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The Functions of Pregnancy-Associated Plasma Protein A in Rheumatoid Arthritis

Gail Greenacre

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Contents

Abstract

Pregnancy associated plasma protein A (PAPP-A) is produced in increasing amounts with advancing pregnancy, a major source being the placenta. It was originally described as a homotetramer with ~ 200,000 M_r subunits. An alternative structure has since been proposed of a disulphide bridged complex consisting of equimolar amounts of the PAPP-A subunit and the proform of eosinophil major basic protein (pro MBP).

The increasing levels of PAPP-A produced during pregnancy and reports of possible proteinase inhibitory activity and immunomodulatory activity suggested that it may be an antiarthritic agent, involved in the amelioration of rheumatoid arthritis (RA) observed during pregnancy.

PAPP-A has been purified from late pregnancy plasma using ammonium sulphate precipitation, gel filtration chromatography, ion exchange chromatography and affinity chromatography. The purity has been confirmed by SDS PAGE and western blot analysis. The purified PAPP-A has been used to investigate functions of PAPP-A which may support or refute a role in the amelioration of the symptoms of RA during pregnancy.

Potential immunomodulatory actions of PAPP-A may be mediated by the inhibition of cytokine production or action. The effects of PAPP-A on the production of proinflammatory cytokines and also on the production of prostaglandin E_2 (PGE_2), an inflammatory mediator, were studied using the THP 1 human monocytic cell line and the MG-63 human osteoblast- like cell line. PGE_2 was measured by radioimmunoassay and the cytokines IL-1, IL-6 and TNF α using commercial ELISA kits from Biosource International. IL-1 α stimulated the production of PGE_2 and IL-6 in the MG-63 cells, whereas PAPP-A had no significant effect on basal or IL-1α stimulated IL-6 and PGE_2 production. In THP 1 cells, LPS, as expected, stimulated the production of PGE_2, IL-1β, IL-6 and TNFα. PAPP-A alone also significantly stimulated their production at higher concentrations, having a much greater effect at higher concentrations than LPS alone. PAPP-A, when incubated with LPS, produced a greater than additive effect on the production of PGE_2, but in combination with LPS had no significant effect on the levels of IL-6 obtained with LPS alone.

Binding of PAPP-A to IL-1α and TGFβ was studied using ^{125}I-cytokines. Formation of PAPP-A cytokine complexes was determined using either native SDS PAGE or gel filtration chromatography. No evidence of cytokine binding was obtained. The binding of PAPP-A to human cartilage was also investigated, since this may facilitate any protective effects of PAPP-A, such as proteinase inhibition, on cartilage. Cartilage binding was studied using immunohistochemical methods. The results indicated that PAPP-A does bind to cartilage, suggesting a possible protective role.

The effect of PAPP-A on retinoic acid stimulated resorption of bovine nasal cartilage was studied and cartilage proteoglycan release measured as glycosaminoglycans using the dimethylemethylene blue assay. PAPP-A appeared to stimulate the resorption of cartilage.

A key issue was whether PAPP-A functions as a proteinase inhibitor thus preventing connective tissue breakdown. Inhibition of elastase, cathepsin B, plasmin and trypsin was studied using colorimetric and fluorescent peptide substrates. PAPP-A was shown to competitively inhibit elastase, possibly due to the electrostatic interactions between the negatively charged PAPP-A and positively charged elastase.. It did not inhibit the other enzymes studied,

A monoclonal antibody to PAPP-A was produced by immunization of mice with the purified PAPP-A. This is currently been evaluated as to its potential use in a screening ELISA assay for monitoring PAPP-A levels.

Overall the effects of PAPP-A on monocytic cell cytokine and PGE_2 production and its stimulation of cartilage proteoglycan breakdown suggest it does not have a major role in the remission of RA during pregnancy. However this work has demonstrated functions of PAPP-A not previously reported and confirmed earlier observations of inhibitory activity towards elastase. Further work is required to determine the key functions of PAPP-A during pregnancy.

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I would like to dedicate this thesis to my mum, dad, sister Nira, husband Carl and my very special nephew Ben.

1.1 Pregnancy in humans

Human pregnancy is a sequence of events that normally includes fertilization, implantation, embryonic and fetal growth and finally terminates in birth.

1.1.1 The early stages

The fertilisation of the female oocyte by the male spermatozoon is followed by a series of mitotic divisions. Each cell or blastomere undergoes a series of divisions, during which the total size of the conceptus remains the same. At around the 8 -16 cell stage the dividing conceptus changes morphology to a compact form called a morula. At the 32-64 cell stage the blastocyst is formed which comprises of 2 cell types, the inner cell mass and the trophectoderm cells. The trophectoderm cells give rise to part of an accessory fetal membrane, the trophoblast of the chorion, which is involved in the nutrition and support of the fetus (Johnson and Everitt, 1995).

The conceptus, throughout its development from the fertilization stage to the blastocyst, is enclosed within the zona pellucida. The zona has two functions, firstly it prevents the blastomeres falling apart during cleavage and secondly it prevents two genetically distinct conceptuses from sticking together to make a single chimaeric conceptus composed of two sets of cells each of distinct genotype (Johnson and Everitt,1995).

1.1.2 The implantation stage

The free living blastocyst can grow and survive for a short time in the uterine secretions from which it draws oxygen and metabolic substrates. However a stage is reached when the blastocyst cannot obtain adequate support. Then a few trophectodermal cells of the conceptus release enzymes that digest and liquify the endometrial cells and contact is made between the maternal epithelium and the conceptus. Within a few hours an increased vascular permeability in the area of stromal tissue underlying the conceptus is seen. The primary decidualization reaction then occurs which encompasses changes in intercellular matrix composition, stromal cell morphology and a progressive sprouting and in growth of capillaries. Some trophectodermal cells fuse together and form syncytiotrophoblast cells while others retain their cellularity as cytotrophoblast cells. A larger secondary decidua forms after 2-3 days as the major endometrium component of the placenta is prepared. The surface epithelium under the conceptus and some localized decidual tissue is destroyed and this functions as the "yolk reservoir" and

releases large quantities of primary metabolic substrates, lipids, carbohydrates, nucleic acids and protein, which are taken up by the growing embryo (Johnson and Everitt, 1995). Implantation is complete at this stage, with the formation and invasion of the decidua, physical clamping of the conceptus to the uterus, establishment of a nutritional source (decidual "yolk") and the inauguration of placental development.

The implantation of the conceptus can only take place when the endometrial epithelial surface in the uterus is receptive. The endometrium prior to this time has a high surface charge, long microvilli and a thick glycocalyx coat and is referred to as being in the pre-receptive stage. A loss of charge, shortening of the microvilli and thinning of the mucin coat results in the endometrium becoming responsive to the conceptus. These changes in the endometrium are controlled by hormones. Both oestrogen and progesterone are critical for effective implantation of the blastocyst in the uterus. Oestrogen stimulates the growth of the endometrium during the proliferative phase while progesterone stimulates the release of glandular secretions which include specific macromolecules and growth factors, which in turn stimulate the activation of the blastocyst. The epithelial cells are stimulated by steroids to make them responsive to a signal from the blastocyst to transmit signals to the underlying stromal cells to initiate decidualization. (Johnson and Everitt, 1995).

1.1.3 Factors involved in the signalling between the conceptus and uterus

The signalling between the conceptus and uterus involves both cytokines and prostaglandins, see table 1.1. The exact sequence of interactions between the various factors, however is yet to be established.

Table 1.1 A summary of the factors involved in the signalling processes between the conceptus and the uterus (adapted from Johnson and Everitt, 1995)

Factor/s	Role in the signalling Process
Oestrogen / Leukaemia inhibitory factor (LIF)	Induction of endometrium to produce Heparin binding EGF- like growth factor (HB - EGF)
HB-EGF	Initiates the conceptus to shed its zona, attach to the endometrium and invade
Proteolytic Enzymes	Digestion of epithelial layer, basement membrane and the stromal extracellular matrix to assist the invasion of the blastocyst
Granulocyte/ macrophage colony stimulating factor (GM-CSF)	Assists in attachment of the blastocyst to the endometrium
Histamine and Prostaglandins	Involved in the stromal response
Transforming Growth Factor β (TGF β)	Promotes the implantation process, vascular permeability and growth of new capillaries

1.1.4 Embryonic development

Following implantation, the inner cell mass of the conceptus begins to differentiate into three primary germ layers, ectoderm, endoderm and mesoderm. They are the embryonic tissues from which all the tissues and organs of the body develop. As the embryo

develops the endoderm becomes the epithelial lining of the digestive tract, respiratory tract and a number of other organs. The mesoderm forms the peritoneum, muscle, bone and other connective tissue and the ectoderm develops into the skin and nervous system.

During the early stages of growth of the implanted conceptus, nutrients and waste products are in the surrounding endometrial fluids. These materials diffuse through the thin "shell" of trophoblast cavities and tissues of the conceptus itself. Further development leads to the formation of embryonic membranes that lie outside the conceptus and protect and nourish it, these membranes are the yolk sac, amnion, chorion and allantois. The yolk sac is never functional in humans, whereas in some animals it functions all through pregnancy and in others it functions until the chorio-allantoic placenta take over. In humans it becomes a nonfunctional part of the umbilical cord.

1.1.5 The placenta

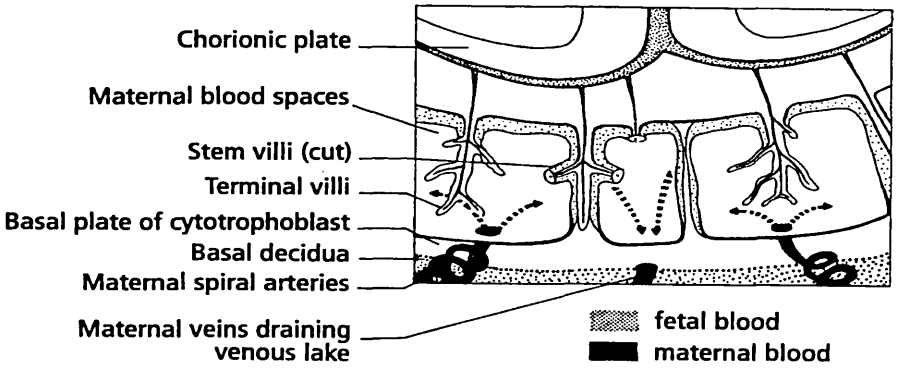
The placenta provides buoyancy and freedom of movement and growth for the fetus, as well as acting as an anchoring device for the fetus in the uterus, but most important, it allows for exchange of nutrients and respiratory gases between mother and fetus.
The chorio-allantoic placenta is formed as a result of the outgrowth of an endodermal diverticulum (the *allantois*) from the hindgut region of the developing embryo. Fingerlike projections of the chorion, called chorionic villi grow into the decidua basilis of the endometrium. These will contain fetal blood vessels of the allantois which will serve as connections in the placenta between mother (chorio-allantoic placenta) and fetus (embryo), and this connection is the umbilical cord (Johnson and Everitt, 1995).

The placenta is generally functional by 3-4 weeks of pregnancy, however a full mature maternal blood flow may not develop until 10-12 weeks thus for the first few weeks of pregnancy the embryo has low oxygen levels in comparison to the later weeks. The placenta increases in size slowly and steadily until birth and then the fetus has a rapid growth phase just prior to birth resulting in a significant lowering of the ratio of placental weight to fetal weight. As the placenta starts to function it gradually takes over responsibility for production of a range of hormones, including progesterone and oestrogen.

The placenta is an organ grown especially to nourish the fetus and to excrete waste products. Maternal and fetal blood streams are in very close proximity but do not mix. The maternal blood circulates across the fine terminal villi which also contain the fetal capillaries, see figure 1.1a. At these sites only a very thin layer of chorionic syncytiotrophoblasts separates fetal vessels from the maternal vessels, see figure 1.1b. Chemical substances diffuse from one bloodstream to the other, nourishment is taken from the maternal blood in the same way and waste products pass back through the placenta into the maternal blood stream to be excreted by the maternal kidneys. Infective agents such as viruses and bacteria, plasma proteins and complex molecules such as cholesterol will not gain access to the fetal circulation unless special transport mechanisms exist, as for example, in the transport of immunoglobulin G (IgG) antibodies across to the fetus.

Figure 1.1a and 1.1b Schematic view of the human placental interface. (Adapted from Johnson and Everitt, 1995).

Figure 1.1a



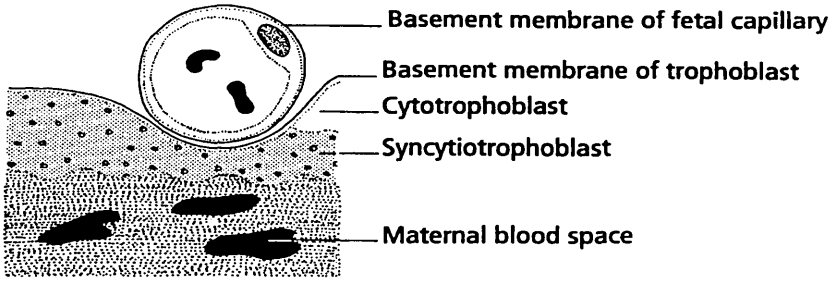


Figure 1.1b

1.2 Pregnancy associated plasma proteins

1.2.1 Pregnancy associated plasma proteins - A, B, C and D

It has been known for many years that during pregnancy several proteins appear in the plasma that are not present in the non pregnant state. Lin et al. (1974a) investigated these proteins in the early 1960s. Hyperimmune antiserum was obtained from rabbits by repeated immunizations with plasma from the third trimester of pregnancy and then absorbing the antisera with non-pregnant female and/or male plasma. Gel diffusion tests were carried out using antisera to pregnancy plasma and four pregnancy proteins were detected. These were designated pregnancy associated plasma proteins (PAPP), -A, -B, -C, and -D (Gall and Halbert, 1972 ; Lin et al., 1973,1974b, 1974c). These proteins were not detected in cord sera, non pregnancy plasma or in male plasma using crossed immunoelectrophoresis, which at that time was the most satisfactory detection method. PAPP-A and PAPP-B did not show any relationship to other plasma proteins that are known to be increased in concentration during gestation, whereas PAPP-C was identical to the pregnancy specific β-glycoprotein (PSβG or SP-1) that had been characterised by Bohn (1971), and PAPP-D was the same as the hormone, human placental lactogen (hPL). Using monospecific antisera or by comparison with purified proteins, PAPP-A and PAPP-B were shown to be unrelated to many other pregnancy associated proteins including human chorionic gonadotrophin (hCG), α-fetoprotein, placental proteins (PP1, PP2, PP3, PP4, PP5) and pregnancy zone protein (PZP).

Heating pregnancy plasma to 60^0C for 30 minutes did not inactivate any of the 4 pregnancy associated plasma proteins. PAPP-A however was destroyed at 70^0C, PAPP-C at 85^0C and PAPP-D at 100^0C after 30 minutes incubation (Lin et al., 1974b). It was also found that all of the proteins were stable between pH 4-10 at 0^0C for two hours, whereas at pH 2 and 12 PAPP-A was completely destroyed. PAPP-C was also destroyed at pH 2 but not pH 12 and PAPP-D was stable at all the extremes. It was also reported that alternate freezing (-70^0C) and thawing (room temperature) for at least 10 cycles did not have any obvious effect on the reactivities of these antigens (Lin et al., 1974b).

The isoelectric points of the pregnancy associated plasma proteins were determined by polyacrylamide disc electrofocusing in a pH 3 to 10 gradient using whole plasma, see table 1.2. The four proteins were easily separated from each other by gel filtration on Sephadex G-200.

Table 1.2 The isoelectric points and M_r of the pregnancy associated plasma proteins (Lin et al., 1974b).

PREGNANCY ASSOCIATED PROTEIN	PI VALUE	M_R
PAPP-A	4.4 +/- 0.1	750,000
PAPP-B	*n.s	1,000,000
PAPP-C	3.8 +/- 0.2	110,000
PAPP-D	5.7	20,000

*n.s. not stated

On immunoelectrophoresis PAPP-A and PAPP-D migrated as α_2 and PAPP-B and PAPP-C as β2 globulins (Lin et al., 1974b).

On comparison of PAPPs with other pregnancy proteins with respect to electrophoretic mobilities and the pI values, it was suggested that PAPP-D or PAPP-A could represent the β_2 pregnancy associated protein. However it was difficult to confirm that the PAPPs were pregnancy specific because of the poor sensitivity of methods available for their detection. (Lin et al., 1974b). There is still some uncertainty as to whether these proteins are present in the non pregnant state and their exact role in pregnancy has not been fully elucidated.

1.2.2 Other pregnancy proteins

Other pregnancy associated proteins have been detected in gestational sera (Horne and Nisbet, 1979). Some of these are described in more detail, table 1.3.

Table 1.3 Pregnancy proteins their sources and the levels detected during pregnancy (Adapted from Persellin, 1981).

Protein	**M_r**	**Source**	**Levels (maternal serum)**
α Fetoprotein	64,000	initially, the yolk sac and then the fetal liver	300ng/ml at term
α_2 Pregnancy associated globulin (α PAG)	364,000	possibly leucocytes although also may be the liver	> 40mg/dl at term
Pregnancy specific glycoprotein (SP 1) (possibly PAPP-C)	90,000	syncytiotrophoblasts	0.1-0.3mg/ml throughout

1.3 Pregnancy Associated Plasma Protein A (PAPP-A)

1.3.1 Levels of PAPP-A detected in pregnancy samples

PAPP-A levels in maternal serum have been shown to rise steadily through the second trimester and then more rapidly during the third trimester (Lin et al., 1974c; Lin et al., 1978). The levels of PAPP-A in maternal serum/plasma have been reported at term by several workers, see table 1.4.and appear to vary between these workers. This may reflect the methods used, and/or the source of PAPP-A.

Table 1.4 Levels of PAPP-A present in serum/plasma samples using a variety of techniques and antibodies.

	TECHNIQUE USED IN THE DETERMINATION	ANTIBODY USED	CONCENTRATION OF PAPP-A AT TERM
Davey et al (1983)	Radioimmunoassay (Serum)	Polyclonal (DAKO)	109.6 +/- 80.1 mg/l
Sinosich et al (1984)	Radioimmunoassay (serum)	Polyclonal (DAKO)	48.7 mg/l
Duberg et al (1982)	Radioimmunoassay (plasma)	Polyclonal (Bischof)	238 +/- 75.6 mg/l
Oxvig et al (1994)	Immunoelectrophoresis (Plasma)	Polyclonal (DAKO)	15mg/l

It was also reported that the concentration of PAPP-A, detected in plasma, may depend to some extent on which anticoagulant is used, as shown in table 1.5 (Bischof et al., 1983; Davey et al., 1983).

Table 1.5 The effect of different anti-coagulants on the apparent PAPP-A concentration measured by radioimmunoassay in the maternal blood collected in the third trimester (Davey et al., 1983).

Anticoagulant	PAPP-A mg/l +/- SD
Heparin	122.6 +/- 67.0
Citrate	119.1 +/- 62.5
Oxalate	118.6 +/- 95.5
Acid Citrate Dextrose	88.8 +/- 43.7
EDTA	136.9 +/- 66.2

Though PAPP-A concentrations tend to be expressed as mg/l, many workers have used the World Health Organisation (WHO) standard to quantify PAPP-A levels in samples. The WHO78/610 standard is derived from pooled late term pregnancy plasma which has been assigned an arbitrary value of 100IU/l (100mIU/ml).

Early studies on PAPP-A showed that the concentration rose steadily in the maternal circulation up to 36/37 weeks and then more rapidly to term. In early pregnancy (6 weeks) PAPP-A levels are significantly higher than in the non pregnant state which may indicate that the production or metabolism of PAPP-A is hormone dependent and that within the first few days after implantation some event, possibly hormonal, triggers the production of PAPP-A. Bischof et al.(1984) has proposed that progesterone may trigger PAPP-A production by the endometrium. More recent reports indicate that a PAPP-A stimulatory factor can be precipitated by ammonium sulphate, suggesting that it is a protein (Bischof, 1986) and that progesterone is only indirectly linked to the production of PAPP-A.

1.3.2 PAPP-A production and localisation in pregnant females.

PAPP-A levels were found to be three orders of magnitude higher in maternal than the fetal blood, 86,000 mIU/l and 22.5 mIU/l respectively, as determined by radioimmunoassay (Grundzinskas et al., 1985). These results strongly suggest that PAPP-A is produced by the mother and is present in the maternal circulation and not in the fetal circulation.

A study was carried out to compare the distribution of PAPP-A with other proteins of known origin, including human placental lactogen (hPL), AFP and Prolactin (PRL) using fetal and maternal tissues (Duberg et al., 1982). The results obtained for PAPP-A suggested that the decidua could be a possible source as the concentration found (57.0 +/- 2.0ug/g)(15%) was more that three times higher than that in the trophoblast (16.9 +/- 5.4ug/g)(4.5%). The highest concentration was found in maternal plasma at term (238.8 +/- 75.6ug/ml)(62.8%), see table 1.6 for results expressed as percentages.

Table 1.6 The percentage distribution of PAPP-A in maternal and fetal compartments as determined by radioimmunoassay (adapted from Duberg et al., 1982).

COMPARTMENTS	PAPP-A (%)
Maternal Tissues	
Maternal blood	62.8
Decidua	15.0
Fetal Tissues	
Chorion	9.8
Amnion	7.4
Amniotic fluid	0.5
Cord blood	0.0(3)
Trophoblast	4.5

PAPP-A would appear to be predominantly maternally derived. However investigations on the localisation of PAPP-A using immunohistochemical techniques, see table 1.7 detected PAPP-A in maternal and fetal cells.

Table 1.7 A summary of the localisation of PAPP-A (refer to figure 1.1).

Cytotrophoblast	Imaizumi 1983 Dobashi et al 1984
Syncytiotrophoblast (as an apical rim)	Lin and Halbert 1976 Wahlstrom et al 1981
Syncytiotrophoblast (within cytoplasm)	Imaizumi 1983 Tornehave et al 1986
Corpus Luteum	Sjoberg et al 1984
Endometrium (Stroma)	Bischof et al 1984 Wahlstrom et al 1985 Sjoberg et al 1984

Doubt was expressed about some of the localisation (Chemnitz et al., 1986) as it was demonstrated immunoelectrophoretically that the PAPP-A antiserum produced by Bischof et al. (1979), which had been used by other workers (Duberg et al.,1982; Bischof 1984; Schindler et al., 1984), was oligospecific and may therefore be detecting other proteins in addition to PAPP-A. The antiserum of Bischof et al. (1979) was then purified by absorbing with serum from non pregnant women followed by absorption with minced fetal connective tissue and precipitation with ammonium sulphate and ion exchange chromatography. The purified antibody produced was found to be monospecific. This monospecific antibody was then used in immunohistochemical experiments and PAPP-A was found to be exclusively in the cytoplasm of the syncytiotrophoblast (Chemnitz et al., 1986). More recently workers have confirmed the synthesis of PAPP- A by syncytiotrophoblasts by in situ hybridisation (Bonno et al., 1994). It would appear that PAPP-A is produced by fetal cells such as syncytiotrophoblasts and maternal decidua during pregnancy.

1.3.3 PAPP-A levels and distribution in non pregnant subjects

In some studies measurable levels of PAPP-A have been detected in the blood of non pregnant women (Bischof et al., 1981; Bersinger and Klopper, 1984), whereas other studies have failed to do so (Anthony et al., 1983; Sinosich, 1984). One possible explanation may be whether measurements were made in serum or plasma, as it was observed that coagulation of the blood changed the immunological properties of PAPP-A (Davey et al., 1983). It was found that the levels of PAPP-A, as determined by RIA, in a given sample were significantly different depending on whether the PAPP-A tracer or standard was derived from plasma or serum, see table1.8. (Bischof and Meisser, 1988).

PAPP-A has also been detected at low concentrations in seminal plasma (Martin-Du-Pan et al., 1983; Bolton et al., 1986). One could speculate that PAPP-A may have a role in the maintenance of sperm motility during transport to the site of fertilisation

Table 1.8 The effects of immunological heterogeneity of PAPP-A on the measured levels of PAPP-A in non pregnant females (adapted from Bischof and Meisser, 1988).

Origin of tracer	Type of standard	Antibody	PAPP-A levels ng/ml +/- SD	
			serum	plasma
Plasma	Plasma	Dako	45.4 +/- 7.6	46.0 +/- 16.4
Plasma	Serum	Dako	24.3 +/- 5.7	19.7 +/- 6.7
Plasma	Plasma	In house Bischof and Meisser, (1988)	60.8 +/- 14.7	48.6 +/- 3.9
Serum	Plasma	In house Bischof and Meisser, (1988)	42.9 +/- 8.3	3.7 +/- 5.5

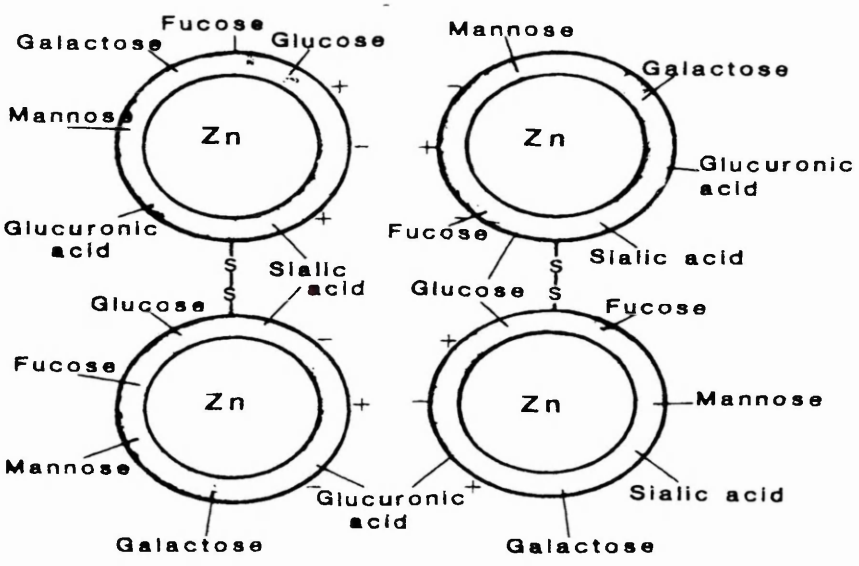
1.3.4 Structure of PAPP-A

Various reports of PAPP-A separation, isolation and characterisation have lead to confusing and conflicting reports on it structure. The following section reviews the literature avaliable.

Bischof et al.(1980) isolated PAPP-A and concluded that the protein was a homotetramer glycoprotein of M_r between 750,000-820,00. Using PAGE gels, at neutral pH, in the presence of 4M urea PAPP-A had an M_r of 450,000, whereas under reducing conditions an M_r of 218,000 was obtained. These results supported the hypothesis that PAPP-A is a dimer with the monomers being held together by electrostatic van der Waals forces and the monomer itself comprising of two chains linked together by disulphide bonds (Bischof et al., 1980).

PAPP-A was found to be deglycosylated by incubation with chondroitinase AC, neuraminidase, α- glucosidase and β- glucuronidase but not β- glucosidase (Sinosich , 1988). This data confirmed the presence of glucuronic acid, as chondroitin sulphate moieties, in the carbohydrate component of PAPP-A, and suggested that PAPP-A is a proteoglycan (Sinosich, 1988). Chondroitin sulphate moieties are highly negatively charged glycans that, like heparin have many potent biological actions including protease inhibition, binding of metal ions and binding to components of the extracellular matrix (Sinosich, 1988). A model was proposed by Sinosich (1988), shown in figure 1.2 with two relatively stable disulphide linked dimeric complexes which ultimately link to form the complete PAPP-A molecule.

Figure 1.2 Schematic representation of PAPP-A molecular conformation obtained from pooled pregnancy serum (Sinosich, 1988).



Elucidation of the structure of this complex protein has been further complicated by the co purification of PAPP-A with pro major basic protein (proMBP) subunits. An alternative model described for PAPP-A is based on two heterodimers with proMBP as an intergral part of the 'active' protein (Oxvig et al., 1993), see figure 1.3.

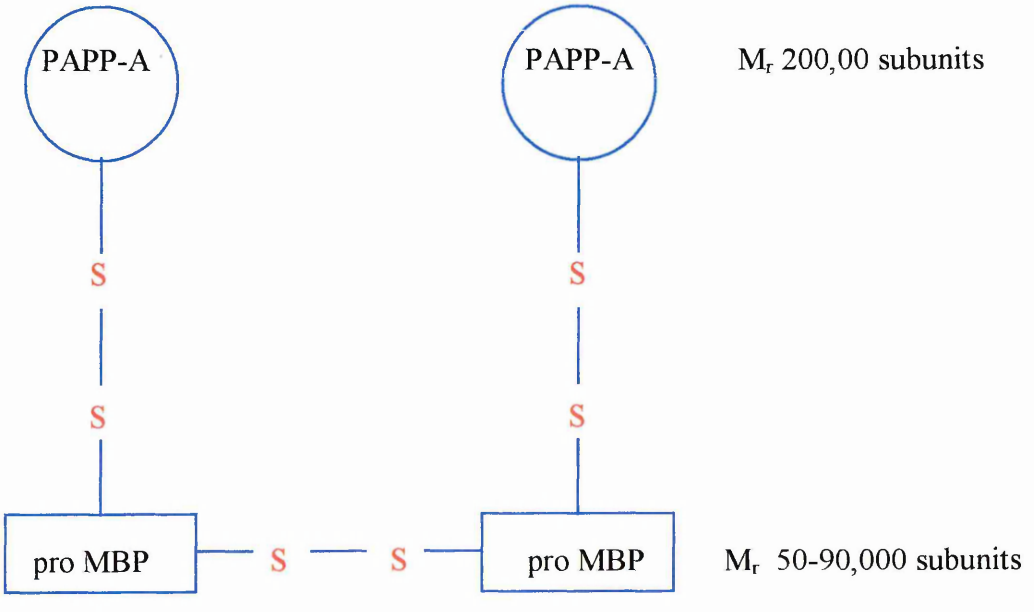


Figure 1.3 Schematic representation of an alternative structure of PAPP-A.

MBP has been characterised from the eosinophil and is derived from the 222 residue prepro MBP (Barker et al., 1988; Wasmoen et al., 1988). MBP isolated from the eosinophil granule is cytotoxic to mammalian cells and has been implicated in tissue damage associated with eosinophil infiltrates (Gleich et al., 1979; Frigas et al., 1980; Hamann et al.,1991).

PAPP-A, isolated from pregnancy serum, and subjected to trypsin and CNBr/trypsin digestion to produce peptides was found to contain several peptides that were derived not from PAPP-A, but from pro MBP. It was suggested that circulating PAPP-A was complexed with pro MBP through disulphide bridges, with one subunit of PAPP-A being bound to one molecule of pro MBP. The pro MBP subunit migrated on SDS-PAGE as a protein of M_r between 50-90,000 rather than the expected 23,400 and it was suggested pro MBP may form very stable oligomers even after denaturation. (Oxvig et al., 1993).

In pregnancy the plasma levels of immunoreactive MBP are greatly increased and it has been located immunohistochemically in the X cells and giant cells of the placenta (Maddox et al., 1983; Maddox et al.,1984; Wasmoen et al., 1987). MBP has been purified from placental tissue and seems to be tightly associated with unknown large proteins (Wasmoen et al.,1989). The actual site(s) of formation of the PAPP-A/proMBP complex is unknown, but probably involves specific interactions between the two proteins. The serum levels of MBP (0.28uM) (Wasmoen et al., 1987) and PAPP-A subunit (0.23uM) (Folkersen et al., 1981; Westergaard et al., 1983a) present in late term suggest that essentially all MBP antigen present is bound to PAPP-A.

The amino acid sequence of PAPP-A derived from pregnancy serum, was determined from partial protein sequencing and from the sequence of cloned cDNA. The PAPP-A monomer was found to contain 1547 amino acid residues, but derived from a larger precursor of placental origin. PAPP-A contains 82 cys residues, which are bridged, 14 putative sites for N- glycosylation and 7 putative sites for attachment of glycosaminoglycan groups.

Results from the sequence analysis show that PAPP-A is not related to α_2m as reported by Sutcliffe et al (1980). The major part of the PAPP-A sequence is unrelated to any known protein sequence to date (Kristensen et al.,1994).

The carbohydrate composition of the isolated reduced and carboxymethylated PAPP-A and the proMBP subunits and of the intact PAPP-A/proMBP were found to be 13.4% (w/w), 38.6% (w/w) and 17.4% (w/w) respectively (Oxvig et al., 1994).
The amino acid compositions of the subunits agreed with those expected from their cDNA sequences (Barker et al., 1988; McGrogan et al., 1988; Kristensen et al., 1994) and also the amino acid composition of the complex is compatible, with PAPP-A and proMBP being present in equimolar amounts (Oxvig et al., 1993).

1.3.5 Similarities between PAPP-A and α_2m

PAPP-A is similar in a number of respects to α_2m, as indicated in table 1.9. Both have similar M_r, identical pIs and similar electrophoretic mobilities and were thought to have similar subunit structures. Because of their similar physicochemical properties they co-elute on gel filtration chromatography and are generally difficult to separate. It was suggested that PAPP-A may be a modified form of α_2m (Sutcliffe et al., 1980), however subsequently this was found not to be the case (Oxvig et al., 1993).

Table 1.9 Comparison of α_2m and PAPP-A (Sinosich, 1988; Sinosich et al., 1983).

	PAPP-A	**α_2m**
M_r	820,000	725,000
Molecular conformation	Homotetrameric	Homotetrameric
Carbohydrate (%)	19.4	8.6
Carbohydrate (M_r)	159,080	62,350
Protide (M_r)	660,920	662,650
pI	4.42	4.42
Zinc atoms per molecule	15.8	4.2

PAPP-A and α_2m differ in their carbohydrate content indicating that α_2m is a glycoprotein and PAPP-A is a proteoglycan containing glucuronic acid in chondroitin sulphate moieties (Sinosich et al., 1982). They also have different amino acid sequences (Sinosich et al., 1990).

1.3.6 Possible roles for PAPP-A

There have been reports of many possible roles for PAPP-A and the following section summarises the current data and literature available to date.

1.3.6.1 The role of PAPP-A as a protease inhibitor

Initial findings indicated that PAPP-A inhibited the caseinolytic and fibrinolytic activity of plasmin (Bischof, 1979). These results prompted workers to suggest that PAPP-A may play a role in the regulation of the fibrinolytic system during pregnancy.

PAPP-A has been shown to inhibit complement induced haemolysis. It specifically inhibits C_3 by binding to this complement component and not by inhibiting C_3 convertase as demonstrated for C_3 inactivator (Bischof et al., 1982; Bischof et al., 1984). It was suggested that heparin contaminating PAPP-A preparations caused this inhibition (Sinosich, 1982).

Thrombin activity was inhibited by PAPP-A in a dose-dependent manner. Its effects on thrombin were similar to those of heparin and it was suggested that it resulted from similarities between the carbohydrate composition of heparin and that of PAPP-A (Meisser et al., 1985). Heparin is an inhibitor of coagulation, of complement, of lymphoblastogenesis, and of elastase (Currie, 1967; Rosenberg and Damus, 1973; Novitskaya et al., 1975; Marossy, 1981; Chang and Boackle, 1986). It was later suggested by Meisser et al. (1988) that the inhibitory effects on thrombin activity originally attributed to PAPP-A (Bischof, 1981) were due to contaminating traces of heparin that had leached from the heparin Sepharose column used in PAPP-A purification and not PAPP-A.

There have been reports that PAPP-A inhibits granulocyte elastase activity and this inhibition was shown to be non competitive (Sinosich et al., 1982). Inhibition of

elastase by PAPP-A (K_i 1.7×10^{-7}M) was approximately ten fold greater than that of α_2m (K_i 1.6×10^{-6} M) (Marossy, 1981; Sinosich et al., 1982). Other workers however (Bischof et al., 1990) suggested that the inhibition of elastase was due to contaminating heparin. More recently Oxvig et al. (1994) have reported competitive inhibition of human leukocyte elastase (HLE) by PAPP-A by a colorimetric assay with synthetic substrates (K_i 5-10 x 10^{-9} M), however significant inhibition was seen only at low ionic strengths. At ionic strengths greater than 150mM inhibition of HLE was negligible.

Fluorometric methods were used to study the effect of PAPP-A on human plasmin and colorimetric methods for bovine pancreatic chymotrypsin, bovine pancreatic trypsin and human leukocyte elastase. The results observed indicated that PAPP-A did not inhibit plasmin, trypsin or chymotrypsin, it did however inhibit elastase as previously described. α_2m was used as a control inhibitor and was shown to inhibit all four proteases (Sinosich et al., 1982). Oxvig et al. (1994) also reported no inhibition of trypsin or chymotrypsin by PAPP-A

It has been well documented that α_2m binds and inhibits trypsin, chymotrypsin, cathepsin B, papain and plasmin (Starkey and Barratt, 1977). PAPP-A samples that contained α_2m as a internal positive binding control were used to study the effect of PAPP-A on trypsin. A sample containing α_2m and PAPP-A was incubated with iodinated trypsin. The majority of the counts in these samples were precipitable with anti α_2m antibody, less than 10% were precipitable with anti-PAPP-A antibody suggesting that α_2m -trypsin complexes are formed but not PAPP-A-trypsin complexes, indicating little or no binding of PAPP-A to trypsin. The experiment was repeated using iodinated plasmin, and as previously described, there was no evidence to suggest that PAPP-A could bind to plasmin (Gore and Sutcliffe, 1984).

Subsequent studies however, have suggested that PAPP-A is capable of binding proteinases with a conformational change that causes the enzyme to be trapped in the same way as in α_2m – proteinase complexes, as described in section 6.1. Colorimetric assays were carried out to measure residual activity of trypsin and plasmin after incubation with PAPP-A. Several substrates of differing molecular weights were used.

The access of the substrates to the enzyme's active site was found to be sterically hindered as inhibition was much more pronounced with large substrates than with smaller ones, although inhibition was still seen with the smaller substrates. The trypsin inhibitory curve was found to be much steeper than plasmin and reached complete inhibition at a molar ratio of 1.0. These observations provide further support that PAPP-A and α_2m are structurally and functionally related proteins despite the different amino acid sequences (Zorin et al., 1995).

The effects of PAPP-A on proteinase inhibition are very conflicting, see table 1.10. Further investigation is required. A key element in this confusion is the differences in purification procedures employed by different workers.

Sinosich et al. (1985) suggested a possible role for PAPP-A as a proteinase inhibitor that may contribute to the follicular protease inhibitor pool which helps maintain proteolytic homeostasis in the pre and post ovulatory phase. It may also contribute to survival of spermatozoa and embryo in the reproductive tract by preventing damage from maternal leukocyte derived proteinases.

1.3.6.2 Modulation of the immune system by PAPP-A

There have been many theories proposed to explain the non rejection of the fetus. One is the suppression of the maternal cell mediated immune response, possibly by inhibition of lymphoblastogenesis of T lymphocytes by plasma proteins. PAPP-A was shown to inhibit lymphocyte responsiveness to phytohemagglutinin stimulation in vitro (Lin et al., 1974b; Bischof et al., 1982), other workers however refute this (McIntyre et al., 1981). Since it is well documented that heparin inhibits lymphocyte transformation (Currie, 1967; Novitskaya et al., 1975) it was suggested that heparin contaminating the PAPP-A preparation used, was responsible for this effect (Meisser et al., 1988).

Table 1.10 A summary of reports of PAPP-A's inhibitory activities

	ELASTASE	PLASMIN	TRYPSIN	CHYMOTRYPSIN
Bischof et al. (1990)	No inhibition *(MeSuc-L-ala-L-ala-L-pro-L-val-ρNA) (AAPV-ρNA)*	*n.s.	*n.s.	*n.s.
Zorin et al. (1995)	*n.s	Inhibition *(Remazol hide powder/ D-val-leu-lys-ρNA)*	Inhibition *(Remazol hide powder/ NαBz-D-L-arg-ρNA(BAPNA))*	*n.s
Sinosich et al. (1982)	Non competitive Inhibition *(N-suc-L-anl-L-ala-L-val-ρNA) (SAAVN)*	No inhibition *(Boc-L-glu-L-lys-L-lys-MCA)*	No Inhibition *(N-tert-but-car-L-ala-ρNA) (BAN)*	No inhibition *(N-benz-L-tyr-ρNA) (BTN)*
Oxvig et al. (1994)	Competitive Inhibition *(MeSuc-L-ala-L-ala-L-pro-L-val-ρNA) (AAPVρNA)*	*n.s	No Inhibition ([1]n.s)	No Inhibition ([1]n.s)

* n.s. Not studied by the investigator, no data available. [1]n.s not stated. *Substrates used.*

1.3.6.3 PAPP-A as a carrier for placental steroids

The binding of steroids to purified PAPP-A was investigated to determine whether PAPP-A could be a carrier protein for placental steroids. No binding was seen between PAPP-A and ^{3}H-progesterone, ^{3}H-oestradiol, ^{3}H-oestrone, ^{3}H-oestriol, ^{3}H- testosterone or ^{3}H-corticosterone (Bischof , 1979).

1.4 Studies into the potential use of PAPP-A as a screening and diagnostic tool during pregnancy.

In studies measuring PAPP-A levels by Laurell immunodiffusion it was found that PAPP-A levels increased as pregnancy advanced, as mentioned earlier in section 1.3.1. The maximal concentration of PAPP-A at term was given as 137+/- 74 arbitrary units (giving a value of 100 units to a late term pregnancy pool) (Smith et al., 1979).

As mentioned previously (1.3.1) in a normal pregnancy PAPP-A levels increase as pregnancy advances (Lin and Halbert, 1978). However a number of studies reported low first trimester levels of PAPP-A in pregnancies with an abnormal karyotype (Muller et al., 1993; Wald et al., 1992; Brambati et al., 1994). This suggested that the PAPP-A measurements may have a clinical value in screening for chromosomal abnormalities, in particular, Downs syndrome (DS) in the first trimester. DS is a congenital disorder with mental retardation and physical abnormalities, with an incidence at birth of ~ 1.3 in 1000. Individuals with this disorder usually have 47 chromosomes instead of the normal 46 (an extra chromosome 21).

Maternal serum human chorionic gonadotrophic (hCG) and α fetoprotein (AFP), with or without unconjugated estriol (UE_3), double or triple test, are routinely measured in the second trimester of pregnancy as a screen for DS as these levels differ for a abnormal fetus compared to a normal fetus (Wald et al., 1994). First trimester screening and early diagnosis would be beneficial with respect to early termination of the pregnancy.

Brambati et al. (1994) reported for the first time that a combination of serum PAPP-A, maternal age and free β-hCG measurements in the first trimester could lead to enhanced detection rates for pregnancies affected by DS and other fetal aneuploidies.

Krantz et al. (1995) also carried out a study using PAPP-A levels with maternal age and free β-hCG. An efficiency of 68% at a 5% false positive rate was achieved. This level of first trimester detection equals or exceeds results reported in the second trimester with either the two or three marker protocol. Wald et al. (1996) concluded that screening using maternal age and serum PAPP-A at 10 weeks of pregnancy was better than the double test (α fetoprotein and hCG with maternal age) and similar to the triple test (α fetoprotein, unconjugated oestriol and hCG with maternal age) at 15-22 weeks.

Sinosich et al., 1982 also suggested that biochemical screening in the first trimester for maternal serum PAPP-A was a useful tool for the detection of fetal chromosomal abnormalities since the serum PAPP-A levels in pregnancies with a DS fetus at 10 weeks were 0.15 multiples of mean (MoM) and at 11 weeks, 0.29MoM. Thus detection of PAPP-A levels of less than 0.3 MOM in singelton pregnancies at 10 weeks followed by confirmatory tests, such as Chorionic villus sampling (CVS), would be useful for DS detection and offer termination possibilities early. It was estimated that all of affected fetuses would be detected at 10 weeks in this way and 50% at 11 weeks (Hurley et al., 1993).

PAPP-A levels were measured using radioimmunoassay (Sinosich et al., 1980) at 9-14 weeks gestation and the study confirmed and expanded on the existing data by confirming the association between depressed levels of PAPP-A in early pregnancy serum and DS (Muller et al., 1993). Further confirmation was provided by Bersinger et al. (1994) who showed that at 10-11 weeks 40% of pregnancies with trisomy 21 have maternal serum PAPP-A levels below the 5^{th} centile of the normal range. This is also the case for 70% of pregnancies with trisomies 18 and 13. The sensitivity of detection using PAPP-A measurement may provide an earlier diagnosis which has some advantages.

In addition to the differences in PAPP-A levels in the serum of mothers of DS babies, levels are altered in other obstetric disorders (Lin et al., 1976).
The development of a radioimmunoassay (Bischof et al., 1981) has made the measurement of maternal circulating PAPP-A during the early stages of pregnancy possible and indicated it to be of potential use as a marker of fetal well being. Maternal

PAPP-A measurements were taken from 51 pregnant women and the results compared favorably with other biochemical tests of placental function.
PAPP-A measurements may be a valuable tool in the management of threatened abortion, particularly in patients who appear normal in ultrasonic examination (Westergaard et al., 1983).

Sjoberg et al.(1987) measuring PAPP-A by radioimmunoassay, (Sjoberg et al. 1984) reported subnormal levels in many cases of ectopic pregnancy and intrauterine abortion, however no distinction could be made between the two disorders.

1.5 Pregnancy and rheumatoid arthritis

Hench, (1938) was quoted as saying that rheumatoid arthritis (RA) in the presence of pregnancy "finds it difficult to progress or indeed to do otherwise than beat a rather precipitous retreat" since he found a marked or complete remission of the symptoms of 'chronic arthritis' during 30 of 34 pregnancies in 20 of 22 patients. The majority of pregnant patients experience symptomatic improvement of RA, however there are approximately 25% who do not improve and in actual fact pregnancy may aggravate the disease (Neely and Persellin, 1977).

During pregnancy an improvement in RA may occur at any time but it is most commonly seen near the end of the first trimester. Amelioration of symptoms in the 1st trimester can be detected by slightly over 50% of patients, with an additional 14% at the end of the second trimester and a further 6% in the third trimester, see figure 1.11. In the vast majority of cases the improvement will continue throughout the whole of the pregnancy.

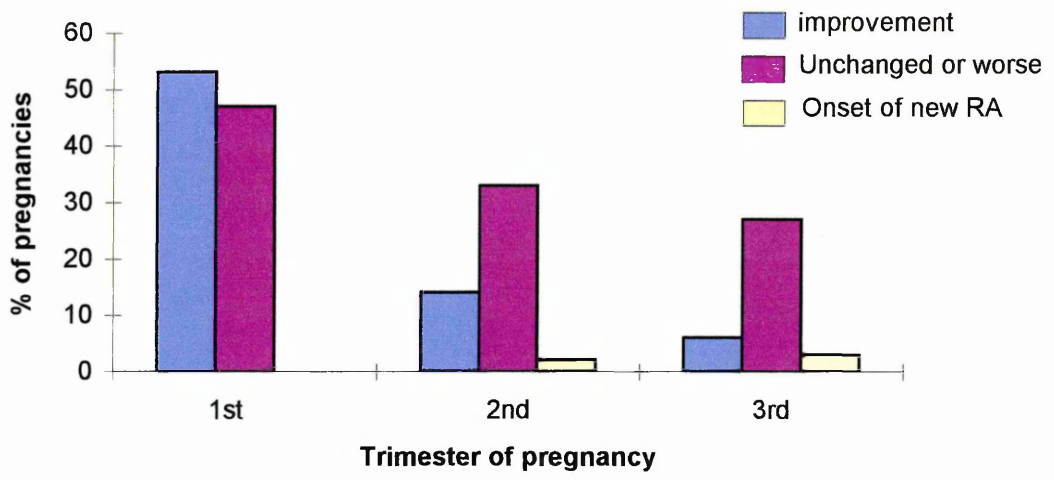


Figure 1.11 The effect of pregnancy on rheumatoid arthritis disease activity : analysis of 308 pregnancies. Improvement, once experienced, usually persisted throughout the course of gestation (adapted from Cecere and Persellin, 1981).

The amelioration of the symptoms can be easily monitored by changes in the patients stiffness, pain and swelling of joints and their general well being. There seems to be no relationship between the age of patient, the duration of disease activity, or the stage of RA, with the degree of improvement of RA during pregnancy. Some patients may have complete amelioration whereas others may be subject to minor flare ups. Patients' who have a remission of their symptoms during their first pregnancy will also experience remission of RA in any subsequent pregnancies and those not benefiting from any remission will not benefit in any further pregnancies. This may suggest an inherent regulation of the mechanism responsible for the amelioration. The reoccurrence of the symptoms of disease develops post partum, with the majority of patients relapsing within 6 weeks (Cecere and Persellin, 1981).

Nicholas and Panayi (1988) believed that the improvement of the symptoms of RA may be due to the induction of an immunosuppressive state that may involve many factors. Immunosuppression has been reported with many factors, including, hCG, AFP, progesterone, SP 1, PAPP-A, prostaglandins and sex steroids.

Generally corticosteroid levels increase progressively throughout pregnancy and they were found to exert multiple effects on the inflammatory and immune responses. It was

suggested that these hormones play a role in the amelioration of inflammatory disorders during pregnancy (Rocklin et al., 1979).

The immunosupressive activities of hCG are somewhat controversial. There have been reports of inhibition of phytohaemagglutinin induced T cell proliferation by hCG (Teasdale et al., 1973), but these effects may be due to contaminants (Patillo et al., 1976).

Davies and Browne (1985) discovered two immunosuppressive activities, so called immunosuppressive factor I and II (ISF I and ISF II) on fractionation of pregnancy serum by gel filtration column chromatography. It was reported that these two factors were present in the non pregnant state as inactive proforms and they are activated during pregnancy by Ca^{2+} and Mg^{2+} and a protein called pregnancy depleted immunoregulatory factor (pd IRF) (M_r 100,000). However, Nicholas et al. (1988) concluded that it would be too simplistic a view that one serum factor alone, such as IRF, produced in excess during pregnancy, could account for disease remission of RA. More than likely there is a multifactorial etiology which will be very difficult to elucidate.

1.6 Rheumatoid arthritis

RA is a common systemic autoimmune inflammatory disorder affecting approximately 1% of the population worldwide (Harris, 1990; Scutellari and Orzincolo, 1998). It most commonly affects women (75%) between the ages of 20-60.

The actual cause of RA is unknown, although several different factors are thought to be involved in triggering the disease, including environmental and genetic factors. Current research is focusing on exogenous infectious agents as well as endogenous substances such as connective tissue proteins (e.g. collagen and proteoglycans) and altered immunoglobulins. Rubella virus, mycoplasma and Epstein- Barr virus have all been implicated as possible causative agents of RA along with other infectious agents. Epstein- Barr virus is well established as a polyclonal activator in the overproduction of immunoglobulins including rheumatoid factor (Slaughter et al., 1978; Djavad et al. 1996).

The majority of RA sufferers produce "rheumatoid factor", an autoantibody (IgM) which binds to the Fc portion of human IgG. Rheumatoid factor is thought to promote inflammation and tissue damage by activating the complement cascade (Pope et al., 1974; Brown et al. 1982).

In its least severe form, RA usually only causes mild pain to certain joints resulting in troublesome hindrance more than disability. However in the most severe form of RA it can cause extreme inflammation of the joints, cartilage, muscles, tendons, bone ligaments and/or internal ligaments resulting in permanent severe joint disfigurement and damage and severe pain.

1.6.1 Joint pathology of RA

The synovium or synovial lining is a thin cellular layer which lines the joint. It produces nutrients such as glucose which diffuse to the cartilage via the synovial fluid (Firestein and Zvaifler, 1992). The synovial lining consists of two cell types, one mucosal and the other phagocytic. Normally it acts as a barrier to serum proteins and as a means of disposal of unwanted materials.

In RA the synovium is intimately involved in the disease process. The earliest change in the joint is vasculitis, inflammation of small blood vessels resulting in increased capillary permeability and oedema (swelling) of the synovium and cellular infiltration (Chapel and Haeney, 1995). This is followed by the infiltration by blood-derived cells, chiefly memory T cells, macrophages and plasma cells into the inflamed synovial joints (Feldmann et al., 1996). The macrophages display surface class II major histocompatibility (MHC) antigens, this expression may be important as class II antigens form complexes with peptides for recognition by T helper cells thereby helping in the induction and maintenance of inflammatory lesions (Ridderstad et al., 1991). The presentation of a relevant antigen to $CD4^+$ T helper cells of an immunogenetically susceptible host may trigger the events in RA. Antigen presenting cells (macrophages or dendritic cells in the synovial membrane) may ingest, process and present foreign protein antigens to T-lymphocytes, which initiate a cellular immune response and stimulates the differentiation of B lymphocytes into plasma cells that secrete antibody (Harris, 1990).

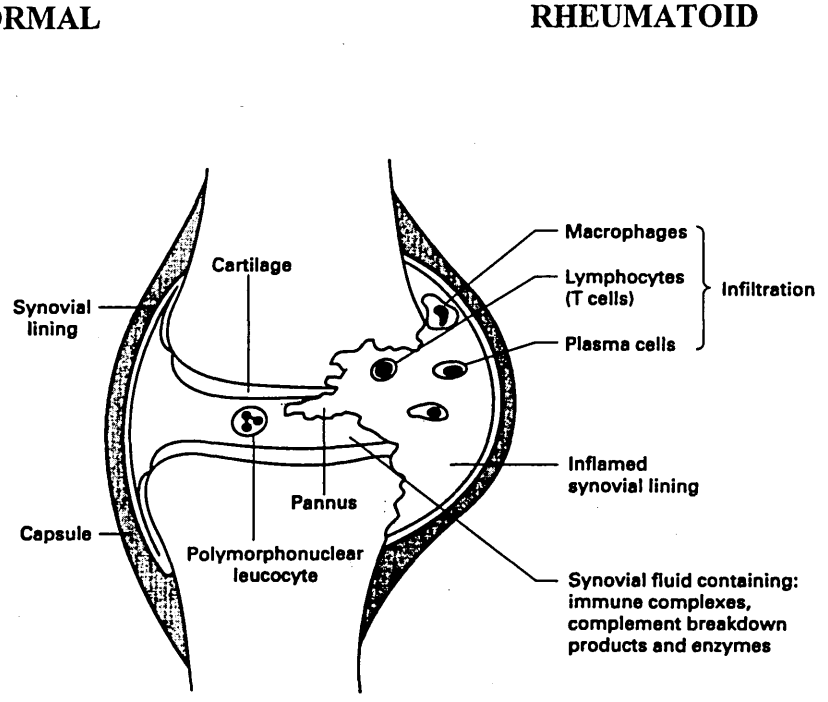
Large numbers of neutrophils are present in the synovial fluid of patients with RA, but very few in the synovial membrane itself. Neutrophils are reported to gain access to the joints by the presence of chemoattractants and cytokines (including IL-1 and TNF), acting directly on endothelial cells to induce the expression of endothelial- leukocyte adhesion molecules that render the endothelial surface hyperadhesive (Bevilacqua et al., 1989). Once inside the joints the neutrophils are rapidly activated by the phagocytosis of cellular debris and aggregates of immune complexes, which then results in degranulation and the release of proteinases (Hibbs et al., 1984; Korchak et al., 1984; Henson et al., 1987). It is estimated that more than one billion neutrophils enter a moderately inflamed knee joint each day (Hollingsworth et al., 1967). The amount of synovial fluid increases with inflammation, causing joints to be swollen, see figure 1.4.

Activation of fibrinolysis via production of plasmin may also lead to plasmin mediated activation of collagenase and stromelysin produced by the rheumatoid synovium (Werb et al., 1977; Okada et al., 1988). Clotting cascades are also activated leading to the production of fibrin that coats the synovial membrane (Van De Platt et al.,1977).

As the disease progresses and becomes established the overgrowth of cellular elements of the synovium causes even more thickening and a marked increase in the surface area, producing an inflammatory granulation tissue called "pannus".
The macrophages in this pannus, which are resident or derived from infiltrating monocytes, and synovial cells, produce destructive proteinases which erode cartilage and bone, starting at the junction between cartilage and subchondrial bone at the joint margin and gradually working inwards until the cartilage is totally destroyed (Dieppe et al., 1989; Bresnihan, 1999). They also produce cytokines which stimulate chondrocytes to self destruct their matrix (Arend and Dayer, 1990).

It is thought that the rheumatoid factor is capable of precipitating out with IgG in superficial layers of cartilage forming complexes that may be attractants for the invasive and destructive pannus (Shiozawa et al., 1980; Jasin, 1985).

Figure 1.4 A normal joint in comparision to a RA joint (adapted from Chapel and Haeney, 1993).



1.6.2 The main cytokines that play a major role in RA

Cytokines are protein mediators that play a part in inflammation, the immune response, cell growth and repair, see table 1.12.

Studies have been carried out to investigate the effects of human TNFα, IL-6 and IL-1β on human articular cartilage chondrocytes in culture. IL-1β and TNFα stimulated the chondrocytes to release IL-6. IL-6 did not affect proteoglycan synthesis or quality. (Malfait et al. 1994). IL-1, IL-6, and TNF α are produced by macrophages and have all been detected in the synovial membranes of patients with RA (Feldmann et al., 1990; Tetta et al., 1990; Firestein and Zvaifler, 1992). These cytokines are involved in the initiation of immune responses and have broad effects on many cells leading to cell proliferation, increased prostaglandin production, matrix degrading protease activity, fever and resorption of bone (Harris, 1990).

IL-1, IL-6 and TNFα have many biological properties which qualify them as potentially important in the pathology of RA such as stimulation of B lymphocytes to produce antibodies, T lymphocytes to produce cytokines, induction of MHC class II expression by macrophages, increased production of PGE_2 and eicosanoids, and stimulation of the production of stromelysin and collagenase by synovial cells and chondrocytes. All are endogenous pyrogens and potent inducers of acute phase proteins (Dayer et al., 1985; Bendzen, 1988; Akira et al.,1990).

These cytokines have been shown to regulate each other, TNFα can stimulate IL-1 which in turn can stimulate the production of TNFα. These two cytokines in concert can stimulate IL-6 expression which via a feedback mechanism can reduce IL-1 and TNFα secretion. Each of the cytokines can stimulate their own production (Akira et al.,1990).

IL- 1 and TNFα production has been shown to be inhibited by PGE_2 production in cell systems (Kunkel and Chensue, 1985; Larrick and Kunkel, 1986) and even though PGE_2 is coproduced with IL-6 its production by chondrocytes is not mediated by IL-6 (Bunning et al., 1990).

Transforming growth factor β (TGF β) appears to counteract many of the inflammatory effects of IL-1, IL-6 and TNFα while acting synergistically to enhance the effects of other cytokines (Edwards et al., 1987). The latent form is activated by the rheumatoid factor (Ridderstad et al., 1991). It is chemotactic for monocytes and induces IL-1 production in these cells (Arend and Dayer, 1990). In cultured chondrocytes the production of type II collagen has been shown, by immunocytochemical techniques, to be enhanced by TGFβ (Frazer et al., 1994).

Table 1.12 A key to the cytokines and their properties

CYTOKINE	M_R	SOURCE	BIOLOGICAL ACTIVITIES
Interleukin 1α Interleukin 1β	17,000	human monocytes/ macrophages	Involved in the activation of T and B cells. Stimulation of non immune cells to produce proteinases (Arend and Dayer, 1990)
Interleukin 6	26,000	monocytes, lymphocytes and fibroblasts	Stimulates the production of acute phase proteins. Key role in stimulating antibody production by B cells (Wong, 1988)
Tumour Necrosis Factor α (TNFα)	17,000	monocytes and macrophages	Shares many biological activities with IL-1 (Arend and Dayer, 1990) osteoclast activating factor, stimulates synovial cells to release PGE_2 and collagenase (Dayer et al., 1985).
Transforming Growth Factor β (TGF β)	25,000	variety of cells including platelets, osteoblasts, synovial fibroblasts and macrophages	Stimulation of matrix synthesis and inhibition of degradation (Arend and Dayer, 1990) Stimulation of production of TIMP and proteinase inhibitors by connective tissue cells (Edwards et al, 1987).

1.6.3 Possible treatments for RA

There is no actual cure for RA to date but there are a number of treatments available which all eleviate the symptoms to varying degrees depending on the severity of the disease.

Non Steroidal Anti-inflammatory Drugs (NSAIDS) can help to control the pain and inflammation in the initial stages of RA. They act by reducing prostaglandin production by the inhibition of the cyclo-oxygenases (COX) enzyme in the RA joint. They can however, lead to complications such as increased gastro-intestinal toxic effects and even death because of the requirement of prostaglandin synthesis in normal physiology (Daoud et al., 1999; Shah et al., 1999).

The use of synthetic inhibitors of matrix metalloproteinases (MMPs) may be useful for the prevention of connective tissue breakdown and it is of great interest whether the blocking of one MMP would be sufficient to halt the progressive and chronic destruction of connective tissue seen in RA (Cawston, 1998). Highly specific MMP inhibitors have been made which remain biologically active after oral ingestion. These have been shown to be active in animal models (Karran et al., 1995).

Therapeutic efforts are now being directed towards disrupting (modifying, blocking, switching off) the cascade of biochemical events considered to be responsible for tissue destruction. Studies have suggested that blocking the TNFα pathway may be of important therapeutic use as results have indicated that blocking TNFα also blocked IL-1 production. This makes TNFα an interesting target for possible treatments of RA (Hauselmann, 1997; Camussi and Lupia, 1998). It has been very recently reported that treatment of arthritic mice with anti IL-12 and anti-TNFα antibodies synergistically suppressed the progression of the collagen induced arthritis (Butler et al., 1999). It has also be reported that treatment with a chimeric monoclonal antibody to TNF α has been shown to suppress inflammation and improve patient well being in RA, the mechanisms of actions however have not been fully explored (Charles et al., 1999).

A new promising drug treatment that has not yet been approved by the FDA is the use of the antibiotic minocycline (a form of tetracycline) as it was found to help reduce

joint inflammation in patients with mild to moderate RA. Researchers do not know exactly why the antibiotic has this effect at present. The antibiotic tetracycline has been shown to be an inhibitor of collagenase either through direct inhibition or by allowing the enzymes to autodegrade (Cawston, 1998).

1.6.4 Cartilage

Human articular cartilage covers the long bones in synovial joints and protects the bone against pressure and acts like a 'shock absorber'. When cartilage is compressed, water is forced out of the system resulting in the proteoglycan being concentrated which in turn increases their osmotic pressure. When the force is released the water flows back into the proteoglycan gel (Lohmander, 1988).

Cartilage is a connective tissue composed of chondrocytes and an extensive complex extracellular matrix of fibrous collagen immersed in a proteoglycan gel which is synthesised by the chondrocytes and can also be degraded by them. The total cell volume is 2% of the total tissue volume, with 75% of the extracellular matrix being water. Of the dry substance, 70% represents collagens (mainly type II), 20% proteoglycans (mainly aggrecan) and the remaining 10% cartilage matrix proteins (Lohmander, 1988; Poole, 1997). Careful regulation of matrix synthesis and degradation is an integral requirement for the maintenance of a healthy functional cartilage.

The metabolism of chondrocytes, particularly proteoglycan synthesis may be modulated by a number of factors, including extracellular macromolecules, mechanical stress and the levels of circulating growth hormones. Insulin like growth factor (IGF) is a known stimulator of proteoglycan synthesis (Handley et al., 1985). Under normal conditions the cartilage does not have its own blood supply and the synovial lining layer is responsible for providing nutrients. Intimal cells produce hyaluronic acid, which along with proteoglycan serve as lubricants for the joint (Firestein and Zvaifler, 1992).

1.6.4.1 Type II Collagen

Collagen is the major protein of connective tissue. Five different types of collagen have been identified in hyaline articular cartilage, (types II,VI, IX, X and XI) with type II

collagen being the most predominant (Van der Rest and Garrone, 1991). This fibre-forming or interstitial collagen is composed of triple helical polypeptide chains, with each chain comprising just over 1000 amino acids. There are three amino acids per turn of the helix and every third amino acid is glycine. The amino acid sequence of the individual collagen chains is Gly-X-Y and there is an abundance of proline and hydroxyproline. The mature type II collagen molecule is incorporated into collagen fibrils and contains non helical telopeptides at its amino and carboxy termini, 18 and 27 amino acids in length respectively. The telopeptides are critical for the formation of stable collagen as they provide some of the cross- links important to the tensile strength of collagen fibrils (Buttle et al., 1994). The structure of the collagen triple helix renders it resistant to the action of most proteases (Van der Rest and Garrone, 1991).

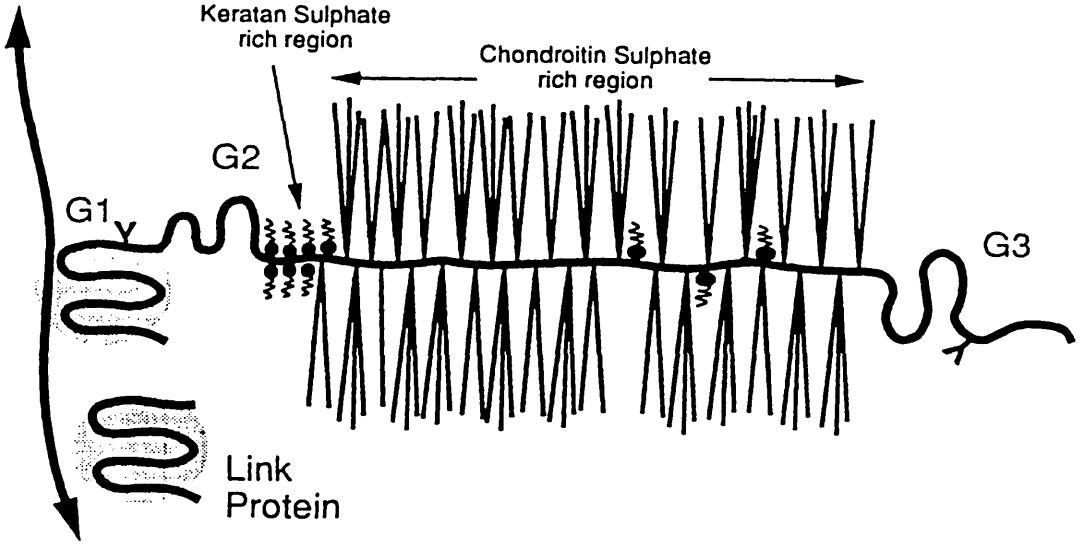
1.6.4.2 Aggrecan

Aggrecan is a large aggregating proteoglycan, the major proteoglycan in cartilage. It binds and retains water in the matrix and plays a fundamental role in the mechanical function of cartilage. Aggrecan is the largest and most complicated of the proteoglycans. It consists of a core protein with three globular domains (G1-G3) and a region of M_r > 200,000 to which are attached 200 chains of different types of glycosaminoglycans and oligosaccharides, see figure 1.5. The total M_r is in the range 1-3 million. The first globular domain (G1) is known to associate with hyaluronic acid and the link protein and this property enables proteoglycan (PG) monomers to form highly charged aggregates in the M_r range 100-200 million via non covalent attachment (Lohmander, 1988; Cawston, 1995). The link protein stabilises the interaction between PG and hyluronate by binding to both a section of the aminoterminal domain of the PG core of the protein and to a hyaluronate decasaccharide sequence (Lohmander, 1988). These highly charged aggregates are immobilised in the matrix and are responsible for pulling water into the tissue creating a swelling pressure and therefore permitting the cartilage to resist compression (Cawston, 1995).

Carbohydrates represent about 90% of the large cartilage PG. The core protein carries two types of GAG chain, chondroitin sulphate and keratan sulphate. Typically there are 100 chondroitin sulphate chains per molecule that are attached through a glycosidic bond between a serine in the peptide and a xylose residue at the reducing end of the GAG chain (Muir, 1958). The content of keratan sulphate increases with age, with a

large proportion located in an intermediate region of the core protein. There are about 40 chains per molecule (Lohmander, 1988).

Figure 1.5 The structure of the major proteoglycan found in cartilage aggrecan (Cawston, 1995).



G1-G3 are globular domains

1.6.5 Degradation of the cartilage components

Degradation of cartilage involves both damage to the collagen fibrillar network and loss of the large proteoglycan aggrecan. Proteinase cascade reactions are the simplest way to interpret data regarding aggrecan and collagen breakdown.

The actual degradation of the cartilage components themselves has been investigated by looking at cartilage breakdown in culture using cartilage explant culture systems (Tyler 1991; Van den Berg, 1998; Uebelhart and Williams, 1999). It was found that a limited cleavage of the core protein occurs to produce monomers with a slightly smaller average hydrodynamic size which are unable to bind to hyaluronic acid and therefore rapidly diffuse out of the matrix. The majority of monomers of proteoglycans are cleaved adjacent to the binding region between G1 and G2 regions of the core proteins,

these fragments diffuse out of the cartilage. Initially most of the link protein and binding region remains as a fragmented complex devoid of glycosaminoglycan within the explant, presumably still bound to hyaluronic acid. With time these components gradually diffuse out into the matrix leading to total breakdown of the proteoglycan structure.

In vitro collagen breakdown requires much longer incubations of cartilage explants and higher levels of IL-1 (150-350pM) than aggrecan (Tyler 1988; Kozaci et al., 1997). In vivo aggrecan is lost before collagen. The only mammalian proteinases known to be capable of type II collagen helical cleavage are the specific collagenases, interstitial collagenase, matrix metalloproteinase 1 (MMP 1), collagenase 3 (MMP 13) and neutrophil collagenase (MMP 8) (see section 1.6.6.4.1), which hydrolyse a single Gly-Leu bond three quarters of the way from the amino terminus of each α chain (Gross et al., 1965; Gross et al., 1980). The larger components are released and readily degraded into smaller pieces by proteinases such as gelatinase A and B (Roughley, 1978; Campbell et al., 1986; Tyler, 1985;). The proteinases involved can be synthesised by chondrocytes under appropriate stimulation or they may be produced by the cells (macrophages, neutrophils) that have infiltrated the inflamed synovium or synovial cells themselves. It is most likely that a series of proteinases act together in a cascade of proteolytic activation (Buttle et al., 1995).

The loss of aggrecan from human cartilage occurs after a specific proteolytic cleavage within the interglobular domain between G1 and G2. Studies on the fragments of aggrecan have shown by N-terminal sequencing that there is only one cleavage site. It was suggested that aggrecan breakdown is catalysed in vivo by a single glutamyl aggrecanase. However, this proteinase may not be the only enzyme directly involved in aggrecan degradation as collagenase cleaves aggrecan at the Asn-Phe and Glu-Ala cleavage sites but with a much higher preference for the Asn-Phe site (Fosang et al., 1994). Two aggrecanases, aggrecanase 1 and 2 have only recently been identified (Abbaszade et al., 1999; Tortorella et al., 1999) (see section 1.6.6.4.2).

1.6.6 Proteinases

Proteinases may be involved in several aspects of joint disease. Their are four main classes of proteinases, aspartic, cysteine, serine, and metalloproteinases which have been classed on the basis of the chemical groups responsible for catalytic activity. The majority of proteolytic enzymes are synthesised as inactive zymogens or proproteinases. Proteolytic activation of the proteinases involved in the breakdown of cartilage by the proteinases themselves is an important factor in cartilage breakdown, which probably occurs through a 'cascade' system (Buttle et al., 1995).

1.6.6.1 Aspartic proteinases

Cathepsin D is the only aspartic proteinase likely to be involved in the pathogenesis of RA. It has been shown to degrade cartilage proteoglycan most rapidly at pH 5.0. Cathepsin D cleaves the hyaluronic binding region from the molecule and splits the polysaccharide attachment region into fragments (Barrett and Saklatvala, 1985). Cathepsin D is secreted by cartilage cultures stimulated with retinoic acid (Ret) (Fell and Dingle, 1963).

1.6.6.2 Cysteine proteinases

Cathepsin B and Cathepsin L both cleave the N- terminal peptides of collagen that contain the cross links within and between collagen molecules. Cathepsin B cleaves the hyaluronic binding region from cartilage proteoglycan and degrades the glycosaminoglycan attachment region to small fragments. (Morrison et al., 1973; Roughley and Barrett, 1977; Roughley, 1977).

1.6.6.3 Serine proteinases

Serine proteinases include many of the proteins of the "cascade" of coagulation, fibrinolysis, complement and kinins. They include enzymes such as thrombin, plasminogen activators, plasmin, Cls, Clr, and elastase.

Plasminogen activators (PAs) catalyse the cleavage of plasminogen to yield the serine proteinase plasmin. The activities of PAs are increased by the addition of IL-1 (Bunning et al., 1987; Leizer et al., 1987), TNFα, TGFβ (Campbell et al., 1990) and Ret (Meats et al., 1985) in chondrocytes. Plasmin has the capacity to degrade cartilage

proteoglycan (Lack and Roger, 1958) but has little effect on collagen although it does activate latent collagenase. Plasmin may activate latent metalloproteinases involved in cartilage breakdown (Buttle et al., 1995).

Leukocyte elastase attacks elastin which is a crossed linked structural protein that is important for the elastic strength of the joint capsule and is highly resistant to proteolytic attack (Barrett and Saklatvala, 1985). It also acts on cartilage proteoglycan to remove the hyaluronic acid binding region and then fragment the glycosaminoglycan attachment region (Keiser et al., 1976; Roughley and Barrett, 1977; Roughley, 1977). Leukocyte elastase also degrades collagen fibers, initially by degrading the N-terminal peptides which breaks the crosslinking that plays a crucial part in the stabilization of collagen fibers. The individual molecules are then degraded to small peptides and amino acids by further proteolytic attack (Davies et al., 1978; Mainardi et al., 1980).

1.6.6.4 Metalloproteinases

1.6.6.4.1 Matrix metalloproteinases (MMPs)

MMPs are a large group of proteinases that are synthesised by mesenchymal cells and are either secreted directly into the extracellular matrix (as inactive proproteinases) or retained at the cell surface (Buttle et al., 1995). MMPs are enzymes that are dependent on metal ions for activity and are divided into four main groups, stromelysins, collagenases, gelatinases and membrane type MMPs (Nagase, 1997), see table 1.13. A large number of studies indicate that MMPs are involved in the pathological destruction of joint tissue (Vincenti et al., 1994) as this large group of proteases are capable of hydrolysing extracellular matrix components (Murphy, 1993). They are secreted as inactive proenzymes that are activated proteolytically by the removal of a 10 kM_r polypetide chain from the protein. This process removes the cysteine residue that previously blocked the active site of the zinc atom (Andrews et al.,1990). MMPs are controlled by various cytokines, growth factors and other agents that stimulate the production of proMMPs (Goldring et al., 1993). Prostromelysin is activated by plasmin to produce the active stromelysin (MMP 3) which has been found, along with collagenase in rheumatoid synovial fluid (Brinckerhoff, 1991).

Table 1.13 Summary of MMPs and proteins degraded (modification from Nagase, 1997).

Enzyme		*Matrix Substance*
Collagenases		
Interstitial collagenase	MMP 1	Collagen types, I, II, III, VII, X, gelatins, entactin, aggrecan, link protein.
Neutrophil collagenase	MMP 8	Collagen types, I, II, III, aggrecan, link protein
Collagenase 3	MMP 13	Collagens I, II, III
Collagenase 4	MMP 18	Collagen I
Gelatinases		
72k M_r gelatinase A	MMP 2	Gelatin types, I, II, III, elastin, collagen types, I, IV, V, VII, X,XI fibronectin, aggrecan, elastin.
92k M_r gelatinase B	MMP 9	Gelatin types I, V, collagen types IV, V, XIV, aggrecan, elastin, entactin.
Stromelysins		
Stromelysin -1	MMP 3	Proteoglycan, fibronectin, laminin, gelatin types I, III, IV, V, collagen types, III, IV, V, IX
Stromelysin-2	MMP10	Gelatin types I, III, IV, V, weak on collagen types, III, IV, V, Fibronectin.
Membrane-Type MMPs		
MT1-MMP	MMP 14	Collagens I, II, III, fibronectin, laminin-1, dermatan sulfate proteoglycan.
MT3-MMP	MMP 15	Not known
MT4-MMP	MMP 16	Activated proMMP-2
MT4-MMP	MMP 17	Not known
Others		
Matrilysin	MMP 7	Gelatin types I, III, IV, V, fibronectin, laminin, collagen IV, aggrecan, entactin, proteoglycan.
Stromelysin 3	MMP 11	Weak activity on fibronectin, laminin, collagen IV, aggrecan, gelatins.
Metalloelastase	MMP 12	Elastin
Unnamed	MMP 19	Not known

The synthesis and secretion of the collagenases and stromelysins is also stimulated by proinflammatory cytokines such as IL-1 and TNF$^{\alpha}$ (Goldring et al, 1993).

Their involvement in the normal turnover of connective tissue matrix is well established (Case et al., 1989). Collagenase is the only proteinase that can cleave the triple helix of type II collagen in cartilage. This leads to an unwinding of the triple helix which is then readily cleaved by the 72 or 92k M_r gelatinases and also by collagenases and stromelysin 1.

1.6.6.4.2 A disintegrin and metalloproteinase (ADAM) family of proteins

The ADAM family of proteinases contains more than twenty members having homology to the snake venom metalloproteinases (Jia et al., 1996; Black and White, 1998). These proteins, as their name suggests, contain a disintegrin and a metalloproteinase domain so may be involved in cellular interactions and/ or proteolysis, though not all ADAMs are known to have a functional metalloproteinase domain. They are mainly membrane bound proteins. Human chondrocytes have been shown to express ADAMs 10,12 and 15 which may be involved in cartilage breakdown (McKie et al., 1997).

More recently aggrecanase, the key enzyme(s) thought to be involved in aggrecan breakdown in osteoarthritis and inflammatory joint disease, has been purified and cloned and shown to be a member of the ADAMTS family of proteins (Abbaszade et al., 1999; Tortorella et al., 1999). ADAMTS proteins have multithrombospondin (TS) motifs and no transmembrane domain as in ADAMs. These proteins are therefore secreted proteins which may bind to extracellular matrix via their TS motifs.

Aggrecanase brings about the cleavage of aggrecan core protein at Glu373-Ala374 which occurs in arthritic disease (Lohmander et al., 1993). Two aggrecanases have been characterised to date, aggrecanase 1 and 2 (ADAMTS 4 and 11 respectively) (Abbaszade et al., 1999; Tortorella et al., 1999). Aggrecanase is ineffective at cleaving several substrates cleaved by MMPs, including extracellular matrix components, type II collagen and gelatin (Tortorella et al., 1999).

1.6.7 Proteinase inhibitors

The activities of proteinases are modulated by naturally occurring inhibitors. In the RA synovial fluid it is thought that these inhibitors become saturated by the proteinases released into the synovial fluid or even destroyed allowing the free enzymes to degrade connective tissue (Harris, 1990).

1.6.7.1 Alpha 2 macroglobulin (α_2m)

α_2m is a tetrameric plasma glycoprotein of M_r 725,000, composed of identical subunits linked in pairs by disulphide bonds containing 8-11% carbohydrate. It is synthesised by the liver and distributed mainly intravascularly, plasma concentrations ~ 2.5mg/ml, although it can be detected in most serous fluids. In early pregnancy α_2m reaches a new steady state level 20% higher than in the non pregnant state (Ganrot and Bjerre 1967). No changes in turnover of α_2m are observed in patients with rheumatoid arthritis (Norberg et al., 1970) and no disease has been recognised where the determination of α_2m is of diagnostic interest.

α_2m has the unique property of binding and inhibiting the great majority of endopeptidases regardless of their specificity or catalytic mechanism (Barrett and Starkey, 1973; Werb et al., 1974; Starkey and Barrett, 1977). On binding proteinases it undergoes a conformational change which can be detected using native, gradient gel electrophoresis, the α_2m-proteinase complex migrating faster in gradient gels than the native protein. This "fast" form was attributed to a more compact form of the complex (Barrett et al.,1979). The irreversible transition from the "slow" or S form into the "fast" or F form is associated with the binding of a proteinase (Barrett et al., 1979). Only the fast form can interact with the α_2m receptor, termed α_2m receptor-low density lipoprotein receptor related protein (LRP) which is present on a variety of cells including macrophages, hepatocytes and fibroblasts (Van Leuven et al., 1979; Dickson et al., 1981; Pizzo and Gonias, 1984; Moessrupt and Giliemann, 1991).
Similar conformational changes of α_2m can also be achieved by treatment with primary amines such as methylamine. The structure of α_2m-methylamine is nearly identical to that of α_2m-proteinase complexes and are recognised equivalently by LRP (Pizzo and Gonias, 1984).

The "trap hypothesis" was introduced (Barrett and Starkey, 1973) to explain how the α_2m rapidly undergoes a conformational change as the proteinase attacks a vulnerable "bait region" in the α_2m molecule which then physically encapsulates the proteinase within it. Access of substrates to the active site of the enzyme becomes sterically hindered causing inhibition that is most pronounced with large substrates. Further studies have supported this theory, however one such study suggested that the reaction of α_2m with proteinases does not involve proteolytic cleavage (Harpel, 1973).

Some endopeptidases do not react with α_2m at an appreciable rate, indicating they are not "trapped". This is probably due to their narrow specificity and/or large M_r (>80,000). Plasma kallikrein and plasmin (M_r 90,000) are the largest proteinases reported to be bound. Trypsin and chymotrypsin linked to Sepharose have been found to have little capacity to be "trapped" by α_2m (or bind in any way) or even cleave the bait region. This suggested that the bait region may be internal, so that any proteinases that cleave it must already be well within the "trap" (Barrett and Starkey,1973).
Proteinase molecules "trapped' by α_2m are usually able to act on low molecular weight substrates with minor changes in Km and Vmax which can be attributable to the new environment of the proteinase molecule. Substrates of M_r above 10,000 will be totally excluded from the complex due to steric hindrance and therefore larger substrates will not be able to enter the complex, resulting in α_2m having an inhibitory effect on the enzyme. The antigenic activity of proteinase molecules trapped by α_2m being blocked is one of the clearest indications that the entire molecule is enclosed, since antigenic determinants are likely to cover the whole surface of the molecule and therefore would not be masked by an enzyme-α_2m interaction that would be restricted to the active site of the proteinase (Geokas et al., 1977; Sayers and Barrett, 1980).
The high molecular weight of α_2m prevents it from escaping into the synovial fluid of the normal joint, in inflammation however it is able to pass through into the synovial fluid and is present in the same concentration as in plasma. α_2m complexes have a greater tendency to accumulate in synovial fluid than in plasma (Barrett and Saklatvala, 1985).

α_2m may also have a transport function for various hormones and metals. About a third of zinc in plasma is bound to α_2m (Iwamato and Abiko, 1970).

It has been proposed that α_2m may play an important multifunctional role at sites of inflammation by scavenging both active peptides and proteases that are released by cells at the site of injury (O'Conner- McCourt et al., 1987).

1.6.7.2 Tissue inhibitors of matrix metalloproteinases (TIMP)

TIMPs play an important role in the control of connective tissue breakdown by blocking the action of activated MMPs and preventing activation of the proenzymes. There are four TIMPs all with approximate M_r of 21,000, that contain two domains held in a rigid conformation by six S-S bonds (Cawston, 1998). TIMPs are synthesised by connective tissue cells and bind tightly to all known active MMPs with a 1:1 stoichiometry (Gomis-Ruth et al., 1997). TIMP 1 and TIMP 2 have been recently shown to prevent cartilage collagen breakdown in culture which suggests that MMPs are involved in the breakdown of cartilage collagen (Ellis et al., 1994).

1.6.8 Prostaglandins

Prostaglandins are chemical mediators which are continually synthesised by most cells from fatty acid chains cleaved from membrane phospholipids. Their production is increased in most cells when they are activated by a change in their environment.

The unsaturated fatty acid, arachidonic acid is the precursor for the synthesis of the major classes of both prostaglandins and leukotrienes (O'Banion. 1999; Dubois et al., 1998). The action of phospholipase A_2 causes the liberation of arachidonic acid and Lyso - platelet activating factor (PAF) from membrane phospholipids. "Free" arachidonic acid can then be converted to prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) by the enzyme cyclooxygenase COX. PGH_2 is subsequently converted to a variety of eiconsanoids that include PGE_2 , PGD_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane (TX) (Dubois et al., 1998; O'Banion. 1999).

Two cyclooxygenase isoforms have been identified and are referred to as COX-1 and COX-2. Evidence provided by animal models of inflammatory arthritis strongly suggests that increased expression of COX-2 is responsible for increased PG production seen in inflamed joints (Anderson et al., 1996). Studies carried out on primary cells derived from human synovial tissue or cell types (e.g., monocytes) have

shown that pro-inflammatory agents, IL-1, TNFα and LPS induce COX-2 expression (Crofford. 1997).

E series prostaglandins are important mediators of inflammation, fever and certain types of pain. PGE_2 is the most abundant cyclooxygenase product in inflammatory lesions and along with PGE_1 can increase vascular permeability. However they are much less effective than other inflammatory mediators, such as histamine and bradykinin but can markedly potentiate the increase of vascular permeability produced by them (Roberts and Newton, 1982.). PGE_2 has effects on the immune system suppressing the functions of T and B lymphocytes, natural killer (NK) cells. PGE_2 also inhibits the proliferation of synovial fibroblast-like cells and is a known stimulant of osteoclast mediated bone resorption (Shiroky, 1989).

1.7 Aims and Objectives

The aims of this study were to obtain the purest sample of active PAPP-A possible and to determine whether PAPP-A was a proteinase inhibitor and/or modulator of cytokine production or activity during pregnancy. In such a role, PAPP-A may act as a protective agent for inflamed rheumatoid joints and thus be involved in the amelioration of the symptoms of RA, observed during pregnancy.

This investigation had the following objectives :

1. to devise a purification protocol for PAPP-A from pregnancy plasma for the production of PAPP-A for functional studies;
2. to study the effects of PAPP-A on the production of cytokines IL-1, TNFα and IL-6, which may be involved in the pathogenesis of RA, by monocytic and connective tissue cell lines;
3. to study the effects of PAPP-A on cytokine action via PAPP-A binding of cytokines;
4. to study the inflammomodulatory effects of PAPP-A via investigation of its effects on PGE production;
5. to study the binding of PAPP-A to cartilage and its effects on Ret-stimulated cartilage proteoglycan breakdown to determine whether it may have a chondroprotective role;
6. to determine whether PAPP-A has proteinase inhibitory activities which may be important in the reduction of cartilage breakdown.

In view of the possible importance of PAPP-A during pregnancy it was important to review the current literature and confirm, or refute some of the findings described by other workers and also to expand the data currently available on the functions of PAPP-A.

These studies on PAPP-A were intended to increase our knowledge of this molecule, the functions of which are not clear cut. By studying the effects of PAPP-A on the

inhibition of proteinases, cytokine production and binding and cytokine stimulated activities we can better evaluate its possible role, if any, in the remission of RA during pregnancy. Agents, which protect connective tissues from breakdown, may in the future provide models for developing therapeutic agents for the treatment of RA.

Chapter Two

Materials and Methods

2.1 Protein purification techniques

2.1.1 Ammonium sulphate precipitation.

2.1.1.1 Principle

Salt fractionation is a technique that can be used in the early stages or in the bulk purification of proteins. Ammonium sulphate, when added to a protein solution will precipitate a number of these proteins by "salting out" and the remainder will stay in the supernatant. This method can be optimised for a particular protein to firstly remove the majority of the unwanted proteins present in the sample and secondly to concentrate the protein of interest.

2.1.1.2 Method

This method is based on a modification of that described by Lin et al. (1974). Plasma taken from pregnant women in the final stages of pregnancy was adjusted to a final concentration of 30 % saturation with solid ammonium sulphate, this solution being gently rotated at 4^0C for 18 hours. The amount of solid ammonium sulphate required was taken from a standard table. The solution was then centrifuged at 6000g for 30 minutes in a Sorvall RC 5 refrigerated centrifuge at 4^0C. The supernatant was then transferred into a separate centrifuge bottle and further ammonium sulphate was added to give a final concentration of 60 % saturation. This sample was left for 18 hours, again with gentle rotation, at 4^0C. The preparation was centrifuged for 30 minutes at 6000g and 4^0C, the supernatant was discarded and the remaining precipitate was resuspended in the smallest volume of buffer (0.05M Tris[hydroxymethyl]aminomethane/hydrochloric acid (Tris/HCl) containing 0.15M sodium chloride (NaCl), pH 7.8 with 0.02 % sodium azide) possible to ensure complete dissolution of the pellet. The solution was then dialysed overnight at 4^0C in 3 changes of 30 volumes of the resuspension buffer used previously.

2.1.2 Gel filtration chromatography

2.1.2.1 Principle

Gel filtration (exclusion) chromatography utilises polymeric gels of cross linked dextran, agar, agarose or polyacrylamide in beads to separate substances of different molecular size. The basic principle is that the sample passes down the column and the separation depends on the different abilities of the sample molecules to enter the pores of the gel matrix. Very large molecules cannot pass through the pores to enter the gel and therefore move through the chromatographic bed faster whereas smaller molecules which can enter the pores move more slowly through the column relative to their size since they spend a proportion of their time within the bead stationary phase. Molecules entering the pores are therefore eluted in order of decreasing molecular size, with the largest molecules being eluted first.

2.1.2.2 Method

The gel matrix employed was Ultrogel AcA 34 which is composed of an homogeneous network of polyacrylamide and agarose in bead form, with a fractionation range of 20,000-350,000 packed into an XK 50/100 column (5 x 100 cm).

The critical stage was the packing of the column as a poorly packed column will give rise to uneven flow, zone broadening and loss of resolution. The homogeneity of the column was checked using a 2mg/ml dextran blue solution (M_r 2,000,000). The sample obtained from the ammonium sulphate stage, or, approximately 80ml, (if the volume after dialysis exceeded 80ml) was loaded onto the column at a flow rate of 40ml/hour. 0.05M Tris/HCl containing 0.15M NaCl, pH 7.8 with 0.02% sodium azide was run through and 9ml fractions were collected. The column was then washed with at least three column volumes of 0.05M Tris/HCl containing 0.15M NaCl, pH 7.8 with 0.02% sodium azide to elute all the other smaller remaining proteins. Ideally the column should be kept flowing at a decreased speed constantly. The absorbance of the fractions was then measured in a Cecil 1020 UV-Visible spectrophotometer at a wavelength of 280nm to determine the presence of protein in each fraction. The fractions shown to contain protein were also analysed for the presence of PAPP-A using rocket immunoelectrophoresis as described in section 2.2.2.2.

The fractions that produced distinct rocket like immunoprecipitates contained PAPP-A and were pooled and dialysed against three changes of 25mM Tris/HCl containing 50mM NaCl pH 7.6 ready for the next stage of purification.

2.1.3 Ion exchange chromatography (IEC)

2.1.3.1 Principle

The basic principle of ion exchange chromatography (IEC) is that separation of proteins is achieved on the basis of the charges carried by the solute molecules. Many biological molecules are polar and so ion exchange is often used as it is capable of separating molecules with very small differences in charge, and therefore is a technique with potentially high resolution. The presence of charged groups covalently bound to the insoluble matrix is a fundamental property of an ion exchanger. Most ion exchange experiments are performed in two stages, the first stage being sample application and adsorption. Unbound substances are washed from the column using column buffer, the bound substances being exchanged with the counter ions due to their greater affinity for the ion exchanger. The second stage is the elution of the bound substances from the column again by an exchange mechanism. The substances bound are exchanged for counter ions in the elution buffer which now possess a greater affinity than the bound substances and therefore elute from the ion exchanger. The affinities can be controlled by varying conditions such as ionic strength and pH.

2.1.3.2 Method

The ion exchange matrix used was DEAE Trisacryl gel, an anion exchanger, packed into a column (1.5cm x 35cm). The gel was initially washed with 8M urea solution, followed by two column volumes of 25mM Tris/HCl pH 7.6. The column was then connected to a Fast Protein Liquid Chromatography System (F.P.L.C.), which works on the principle of two connecting pumps that can mix the different buffers in pump A and in pump B to produce varying salt gradients or pH gradients. A salt gradient was required for this method. Pump A reservoir contained 25mM Tris/HCl pH 7.6 and pump B reservoir contained 25mM Tris/HCl containing 1M NaCl pH 7.6. Prior to the run the F.P.L.C. was programmed to wash the column with three alternate salt concentrations (ABA), each of 40ml. The dialysed sample previously pooled was then loaded onto the column at a flow rate of 1ml/minute. The eluent was monitored, using a

flow cell, at an absorbance of 280nm, for the presence of protein. A large peak was seen when the sample was loaded onto the column indicating that many of the proteins were not binding to the column.

Following the addition of the sample onto the column it was washed with 25mM Tris/HCl pH 7.6 until the base line on the chart recorder measuring absorbance reached zero, indicating that all the remaining proteins were bound to the column. The F.P.L.C. was then programmed to increase the salt concentration to 0.5M, keeping the flow rate at 1ml/minute. A second protein peak was eluted from the column after a short time and this was collected and stored at 4^0C. The salt concentration was then increased to 1M and any remaining proteins eluted were collected and stored at 4^0C. The column was then washed again as described initially to regenerate it ready for a future run .

2.1.4 Negative affinity chromatography

2.1.4.1 Principle

Affinity chromatography is a technique which enables the purification of a biomolecule on the basis of its biological function or individual chemical structure. Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilised on an insoluble support (matrix). Negative affinity however, as the name suggests, adsorbs all the unwanted proteins onto the ligand and the protein of interest is eluted from the column in the wash stage. The bound unwanted proteins are then eluted from the column using an elution buffer. Recovery of purified proteins is very high although there is a dilution of the sample, requiring concentration by, for example, the Centripreps.

Sepharose 4B is the most widely used matrix to which affinity ligands are bound, due to its high binding capacity and low non-specific adsorption. Cyanogen-bromide activated Sepharose 4B (CN-Br act. Sep 4B) is the suggested matrix to couple proteins and nucleic acids under mild conditions. The activated groups react with the primary amino groups of protein ligands and multipoint attachment ensures that proteins do not hydrolyse from the matrix.

2.1.4.2 Preparation of anti-total human serum affinity column

10g of freeze dried CN-Br act. Sep 4B were washed with 2 litres of 1mM hydrochloric acid (HCl) on a sintered glass filter (porosity G3) for at least 15 minutes. The ligand, anti-total human serum (5-10 mg/ ml of gel) was dissolved in coupling buffer, 0.1M sodium bicarbonate containing 0.5M NaCl pH 8.3 and the mixture rotated end over end overnight at 4^0C. The excess ligand was then washed away with coupling buffer and any remaining active sites were blocked using 0.1M Tris/HCl containing 0.5M NaCl pH 8.0 for 2 hours at room temperature.

The gel was then packed into a column (1.5cm x 35cm) and washed using the F.P.L.C. system with three alternate cycles (ABA) using 0.1M Tris/HCl containing 0.5M NaCl pH 8.0 and 0.1M sodium acetate containing 0.5M NaCl pH 4.0 at a flow rate of 1ml/minute. The samples obtained at 0.5M and 1M salt concentrations from the IEC column were pooled, then loaded onto the negative affinity column at a flow rate of 0.25ml/minute. The eluent should contain all the proteins not found normally in the serum i.e. the pregnancy proteins and these were monitored again through the flow cell at 280nm to determine at which point they were being eluted. The column was then regenerated using 0.1M glycine, pH 2.9 and all the proteins that were bound to the column were eluted. The samples collected throughout the run, including those in the regeneration step were analysed on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE).

2.1.5 Anti PAPP-A affinity column

2.1.5.1 Principle

An affinity column which binds PAPP-A follows the same principle as any affinity column in that the antibody attached to the matrix will bind the protein specific to it, in this case PAPP-A. The other proteins will elute from the column leaving PAPP-A bound and then by altering the buffer conditions the bound proteins can be eluted.

2.1.5.2 Method

CN-Br act. Sep 4B was prepared as described in section 2.1.4.2. using rabbit -anti human polyclonal PAPP-A antibody (5-10 mg/ ml of gel).

The gel was then packed into a column (1.5cm x 35cm) and washed using the F.P.L.C. system as described in 2.1.4.2. The ion exchange samples eluted using buffers containing 0.5M and 1M NaCl, previously concentrated, were loaded onto the column at a flow rate of 0.25ml/minute. The column was washed with 50mM Tris/HCl containing 0.3M NaCl pH 7.6 until all the unbound proteins had been eluted, by monitoring the eluent at 280 nm in the flow cell. The column was then washed with 0.1M glycine pH 2.9 to elute any proteins still bound to the column. The samples from all stages were collected and analysed on SDS PAGE.

2.1.6 Anti α_2 macroglobulin (α_2m) affinity column

2.1.6.1 Principle

The anti α_2m affinity column followed the same principle and method as the anti PAPP-A column, the only difference being that rabbit-anti human α_2m antibody was used as opposed to rabbit-anti human PAPP-A antibody as the coupling ligand.

2.1.6.2 Method

The samples obtained from the pooled 0.5M and 1M NaCl elution steps from the IEC were loaded onto the column at a flow rate of 0.25ml/minute. The unbound proteins were eluted from the column and the eluent monitored at an absorbance of 280nm until baseline absorbance was reached. The column was then washed and regenerated as described for the anti- PAPP-A column in section 2.1.5.2 to elute the bound α_2m. Samples were collected from both of stages and analysed on SDS PAGE.

2.1.7 Wheat germ lectin affinity column

2.1.7.1 Principle

Lectins are proteins which possess the ability to react reversibly with specific sugar residues which enables them to bind polysaccharides and glycoproteins. Immobilised lectins are very valuable tools for isolating and separating glycosylated proteins due to their specificities for commonly occurring sugars. The binding that takes place between the lectin and the sugar residue on the protein is analogous to the interaction between an antibody and antigen. Proteins that are bound to the column can be eluted by using

buffers with higher ionic strengths or a competitive binding substance. Wheat germ lectin-Sepharose 6MB consists of the lectin from wheat germ coupled to macrobeads of Sepharose 6B by the cyanogen bromide method.

Wheat germ lectin binds N-acetyl-glycosaminyl residues and wheat germ lectin-Sepharose 6MB is a group specific adsorbent for the purification of glycoproteins and polysaccharides. α_2m is known to bind with weak affinity to this matrix and PAPP-A has been reported to have a stronger affinity (Sinosich et al., 1987). It may therefore be a very useful step in the separation of α_2m from PAPP-A.

2.1.7.2 Method

Samples obtained from the pooled 0.5M and 1M NaCl elution steps from the IEC were loaded onto a wheat germ lectin column at a flow rate of 0.25ml/minute. The eluent was monitored at an absorbance of 280nm through a flow cell and the eluted proteins collected. The column was then washed with at least 10 column volumes of 0.1M Tris/HCl, pH 7.4 to remove any unbound and weakly bound proteins, these fractions were also collected. The bound substances were then eluted using a competitive sugar, 0.1M methyl-α-D-mannoside. Fractions were monitored at 280nm and protein containing fractions collected. Samples collected were analysed by SDS PAGE.

2.1.8. Gel filtration using Trisacryl GF 2000

2.1.8.1 Principle

The principle of gel filtration chromatography has been described in section 2.1.2.1. This matrix was investigated as an alternative to the AcA 34 Ultrogel because of its large pore size and large fractionation range (10,000-15,000,000).

2.1.8.2 Method

The method was essentially the same as that described in section 2.1.2.2.
The column was packed and the homogeneity of the column was checked using a 2mg/ml dextran blue solution (M_r 200,000). The sample obtained from the ammonium sulphate stage, was loaded onto the column at a flow rate of 40ml/hour and then 50mM Tris/HCl containing 0.15M NaCl, pH 7.8 with 0.02% sodium azide was run through

and 9ml fractions were collected. The column was then washed as described in section 2.1.2.2. The absorbance of the fractions was then determined in a Cecil 1020 UV-Visible spectrophotometer at a wavelength of 280nm to determine the presence of protein in each fraction. The fractions shown to contain proteins were also analysed for the presence of PAPP-A using rocket immunoelectrophoresis, as described in section 2.2.2.2.

2.2 Protein detection and determination techniques

2.2.1 Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS PAGE).

2.2.1.1 Principle

The technique of SDS PAGE is a quick method for the determination of protein relative M_r and purity of a sample. The detergent SDS has long been recognised as a powerful protein denaturant and solubilising agent. It was reported by Weber and Osborn (1969) that proteins dissolved in high concentrations of SDS exhibit electrophoretic mobilities in polyacrylamide gels which are a direct function of their sub-unit M_r. When SDS is present in excess in the sample, all the proteins have the same charge density, and hence the same amount of SDS bound per unit weight of polypeptide. SDS binds to hydrophobic regions of proteins, causing them to unfold and gives them a net negative charge. The application of an electric current through the gel will result in the proteins being separated depending on their ratio of charge to mass, the higher the ratio of charge to mass the faster the molecule will migrate. The M_r of a protein is calculated by running a series of standard protein solutions also on the gel and comparing this protein's electrophoretic mobility with the standard proteins. The percentage acrylamide added to the gel will determine the molecular range of proteins to be analysed on the gel, a high percentage gel separating low M_r proteins and a low percentage gel separating high M_r proteins.

2.2.1.2 Reagents

1. Resolving Buffer :	1.5M Tris/HCl pH 8.8
2. Stacking Buffer :	0.5M Tris/HCl pH 6.8
3. Acrylamide/bisacrylamide:	30% (w/v) acrylamide and 0.8% N,N- bis-methylene acrylamide.
4. Ammonium persulphate :	10% (w/v) in distilled water
5. TEMED	N,N,N',N'- Tetramethylethylenediamine
6. Running Buffer (X10):	0.25M Tris/HCl containing 2M glycine and 1% SDS (w/v) pH 8.3
7. SDS :	10% (w/v) in distilled water
8. Reducing sample buffer : (X2)	0.06M Tris/HCl containing 2% (w/v) SDS,10% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 5% mercaptoethanol
9. Non reducing sample buffer : (X2)	As for reducing but without the mercaptoethanol
10. Stain :	0.25% Coomassie brilliant blue R250 made up in destain
11. Destain:	45% methanol and 5% acetic acid in distilled water
12. Protein standards:	Biorad prestained high M_r markers containing Myosin, (200,000) α-Galactosidase (116,200) Phosphorylase b (97,400), Ovotransferrin (77,000) Glutamate dehydrogenase (55,500), Ovalbumin (42,700).

2.2.1.3 Method

This method is a modification of the method employed by Laemmli, (1970). The gel apparatus used was the Biometra mini gel twin G42 apparatus.

The plates were thoroughly cleaned with 70% ethanol to ensure they were grease and dust free to prevent uneven gel formation that would interfere with the movement of the proteins down the gel, and were then assembled with the spacer ready for use. The

resolving gel mixture (5% acrylamide) was then made up as below:

Resolving buffer	3.0ml
Acrylamide/bis	1.3ml
Distilled water	3.5ml
SDS (10%)	80ul
APS (10%)	100ul
TEMED	40ul
Total Volume	**8ml**

The first four solutions were added, mixed gently and then the remaining two solutions were added. The mixture was carefully poured into the gel cast, followed by the addition of a layer of ethanol on top to prevent exposure to the air that would hinder the polymerisation.

Once the resolving gel had set, the ethanol was poured off the top and the surface washed several times with distilled water. The stacking gel (3.6 % acrylamide) was then prepared :

Stacking buffer	1.25ml
Acrylamide/bis	0.6ml
Distilled water	3.0ml
SDS (10%)	20ul
APS (10%)	100ul
TEMED	40ul
Total Volume	**5ml**

As previously the first four solutions were mixed, the remaining two added and the mixture mixed gently again. The mixture was poured onto the top of the resolving gel and the 10 tooth comb carefully inserted, ensuring no air bubbles were present. The stacking gel was left for about thirty minutes to completely polymerise. During this time the samples were prepared, 15ul of reducing sample buffer was added to 15ul of

sample in an Eppendorf tube and they were then incubated at 70^0C for 10 minutes. The non-reduced samples were made up by the same procedure using non- reducing sample buffer and incubated at room temperature for 10 minutes. The clips were then removed from the gel cassette followed by the comb and spacer and the gel was then placed in the gel tank and clipped into place. The tank buffer was diluted 1 in 10 in distilled water and then poured into the upper and lower chambers of the tank. The samples and standard were carefully loaded into the numbered wells, 15ul in each and the lid placed on the tank. The gel was then run at 150V for approximately 45 minutes. Once the gel run had completed, as determined by the bromophenol blue band at the bottom of the gel, the lid was removed and the gel cassette removed. The plates were then disassembled and the gel placed in destain for 15 minutes to remove any SDS and then placed in stain overnight at room temperature. The following day the gel was washed several times in destain until clear protein bands could be seen and the appearance of these recorded photographically.

2.2.2 Rocket immunoelectrophoresis for PAPP-A detection

2.2.2.1 Principle

Rocket immunoelectrophoresis, first described by Laurell (1966) is a quantitative method by which the presence and/or concentration of a known protein can be determined in a simple, quick and reproducible way. Electrophoresis is performed in an agarose gel containing a monospecific antiserum. The sample proteins are pipetted into wells in the gel and when an electrical current is applied the antigen starts to migrate out of the well into the antibody containing gel, at this point the antigen concentration exceeds that of the antibody. The antigen continues to migrate towards the anode forming a "rocket" shaped precipitate at the point when antigen and antibody concentration are equal. The height of the rocket is directly proportional to the concentration of antigen.

2.2.2.2 Method

Agarose gels were prepared using Litex agarose type H.S.A in which the migration of human IgA and IgG was zero at pH 8.6.

A 1% agar solution was prepared by adding 1g of the agar to 100ml of barbitone buffer (0.07M 5-5 diethylbarbituric acid containing 0.15M Tris, 0.35mM calcium lactate and

2mM sodium azide pH 8.6). The mixture was gently boiled for a few minutes, removed from the heat and then boiled again for a few minutes taking great care not to burn the agar. The initial volume was then restored and the agar was placed in a water bath at 56 ^{0}C ready for pouring. The glass plates (10cm x 10cm) were cleaned with distilled water and 95% ethanol to ensure they were "squeaky" clean. 20ml of agar solution was then pipetted into a test tube and 100ul of rabbit anti-human PAPP-A polyclonal antibody (DAKO) was added to the tube (antibody concentration of 1ul/cm^2), the tube was gently agitated and the agar then poured onto the flat even surface of the glass plate. The gels were then covered and allowed to set. Once the gels had set, 3mm wells were made along one side of the plate to allow for sample application. The electrophoresis tank consisted of two troughs on either side of the cooling plate and 0.07M barbitone buffer pH 8.6 was added to each of the troughs and 3 MM filter paper wicks were presoaked in them. The gels were then placed on top of the cooling bed and 6ul samples applied to the wells.

The wicks were then gently placed onto opposite edges of the glass plates, taking care not to cover the wells but ensuring sufficient contact with the plates to allow the current to travel through the gel. The condensation lid was put over the plates and the electrophoresis tank was run overnight, at room temperature, for 16 hours at 300 V.

The power was switched off and the plates removed from the tank. The plates were washed in distilled water, then three times in saline solution (0.6% NaCl), each wash for 15 minutes, followed by three washes in distilled water. The next stage was to dry the gels before staining, this was done in an oven at 90^{0}C for at least one hour. The gels were then stained in nigrosin rapid stain solution (0.002% nigrosin in 20% acetic acid) for 1 hour and then destained in 5% acetic acid until rocket like peaks were visible.

2.2.3 Bicinchoninic acid (BCA) protein assay

2.2.3.1 Principle

This protein assay is a highly sensitive assay for the spectrophotometric determination of protein concentration in dilute solutions. This system combines the well known reaction of protein with Cu^{2+} in an alkaline medium (yielding Cu^{+}) with a highly selective detection reagent for Cu^{+}, BCA. The purple reaction product formed by the

interaction of two molecules of BCA with one cuprous ion (Cu^{+}) is water soluble and absorbs strongly at 570 nm, this being the wavelength employed in this assay.

2.2.3.2 Method

The bovine serum albumin was serially diluted over the range 0ug/ml - 5000ug/ml in diluent buffer (0.1M Tris/HCl pH 7.6) and 20ul pipetted in triplicate into a 96 well plate. Each unknown protein sample was then pipetted in duplicate (20ul) into the 96 well plate, followed by the addition of 200ul of BCA working solution (49ml of Bicinchoninic acid solution with 1ml of 4% copper sulphate) to all the wells. The plate was then incubated at room temperature for 30 minutes and the absorbance read in a multiskan MS plate reader at 570nm. A protein standard curve was constructed, see figure 2.1 and the unknown protein concentrations calculated.

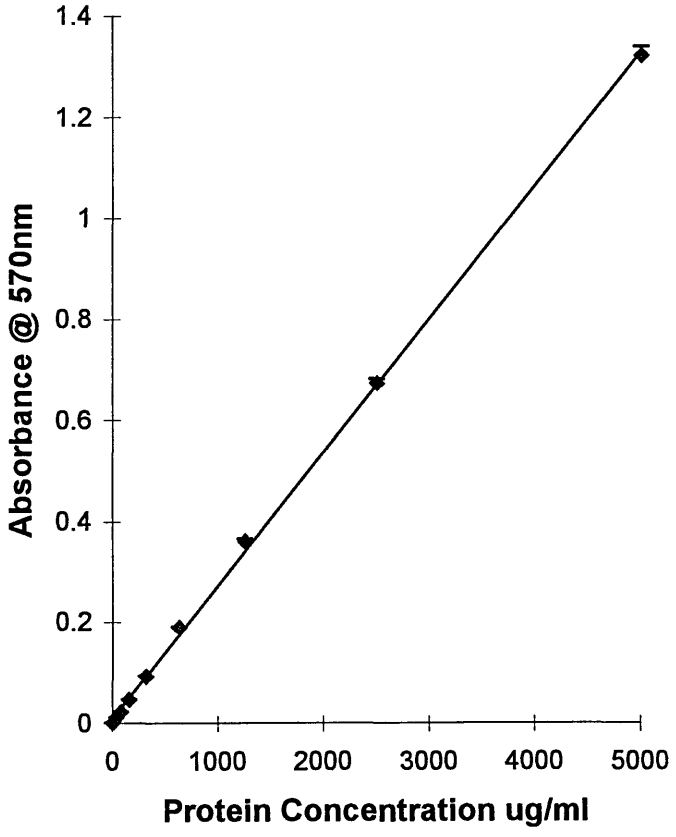


Figure 2.1 A typical protein standard curve, protein concentration is expressed as mean +/- SE.

2.2.4 Western blot analysis for PAPP-A

2.2.4.1 Principle

Western blotting is a powerful technique to identify unknown proteins in samples and also to evaluate the specificity of antibodies.

The antigen or antigens under investigation are first separated by gel SDS PAGE as described in section 2.2.1.3. The gel is then blotted onto a specific membrane to which the antigen (protein) binds. Antibody is then incubated with the membrane and will bind to its specific antigen. An enzyme labelled anti- immunoglobulin will then bind to the antibody and can be readily detected using a colorimetric substrate.

2.2.4.2 Reagents

1. Towbin Transfer buffer :	25mM Tris, 192mM glycine in 20 % methanol and 80 % distilled water
2. Tris buffered saline : (TBS)	20mM Tris, 0.9% NaCl in 2l of distilled water pH 7.5
3. TBS containing Tween 20 : (TBST)	0.05% Tween 20 in 1l of TBS
4. Dilution buffer :	0.2% marvel in 500ml of TBST
5. Blocking solution :	5% marvel in 100ml of TBS
6. Primary antibody :	Rabbit anti-human PAPP-A 1 in 200 in dilution buffer
7. Secondary antibody :	Goat anti-rabbit IgG horse radish peroxidase (HRP) 1 in 10,000 in dilution buffer
8. Substrate :	18mg of 4-chloronapthol in 6ml of methanol mixed immediately before use with 30ml of TBS containing 300ul of 1M imidazole and 30ul of hydrogen peroxide (H_2O_2) (30%)

2.2.4.3 Method

An SDS polyacrylamide gel was run as described in section 2.2.1.3 and washed in Towbin transfer buffer to eliminate any SDS for 15 minutes. Polyvinylidene difluoride (PVDF) membrane was cut to the same size as the gel and prewet in methanol for 30 seconds and then soaked in Towbin transfer buffer with 3MM filter paper and the gel

for 15 minutes.

A " sandwich" was made from the components as follows :

_____ sponge +VE
_____ filter paper
xxxxxxxxxxxxxxx membrane
_____ gel
_____ filter paper
_____ sponge -VE

This was then placed in the cassette and the cassette inserted into the tank, with the gel nearest the -ve electrode. The tank was filled to the mark with chilled Towbin buffer and the system set up for 1 hour at 150V. The cassette was then carefully disassembled and the gel put into Coomassie R250 blue stain to detect any proteins present that had not transferred. The membrane was washed three times in TBST for 10 minutes each wash. The membrane was then incubated in blocking solution overnight at 4^0C with gentle rocking.

The following day the membrane was washed three times for 10 minutes each wash in TBST and then incubated with primary antibody for 2 hours at room temperature with gentle rocking. The membrane was washed as previously and then incubated with the secondary antibody for 1 hour with gentle rocking. The wash procedure was repeated followed by an additional three washes in TBS for 10 minutes. The substrate was then added to the membrane and after about 10 minutes purple bands could be seen, denoting the presence of PAPP-A on the membrane. The membrane was removed from the substrate, washed in distilled water and air dried. The results were recorded photographically.

2.2.5 Native PAGE

2.2.5.1 Principle

Native, or non denaturing PAGE follows the same principle as the SDS PAGE apart from the fact that the proteins can be detected in their native state, i.e. as they are found in nature and not in their denatured monomer state. Native gels are particularly useful for antibody blotting or detection of biological activity, because the native conformation of proteins is maintained.

2.2.5.2 Method

The actual running method is essentially the same as that described for SDS PAGE, section 2.2.1.3 with some alterations to the actual composition of the gels.
The resolving and separating gel mixtures were the same as for SDS PAGE except for the substitution of distilled water for the SDS solution. The non- reducing sample buffer was the same as the reducing sample buffer without SDS or mercaptoethanol. The samples that were run on the gel were incubated with non- reducing sample buffer for 5 minutes at room temperature and NOT heated at 70^0C as for SDS PAGE gels. The running buffer also contained no SDS.

2.2.6 Paragon serum protein electrophoresis

2.2.6.1 Principle

The Paragon Serum Protein electrophoresis (SPE) kit is intended for the electrophoretic separation of proteins into five generally distinct, well resolved zones, which are composed of many individual proteins. The five distinct fractions are, albumin, alpha 1, alpha 2, beta, gamma globulins. The principle of electrophoresis is that when proteins are placed in an electrical field they will migrate toward one of the electrical poles. The Paragon kit was used to determine the purity of the monoclonal antibodies produced, as described in section 2.16.4.2.

2.2.6.2 Reagents

1. SPE Gel	As supplied
2. Barbital Buffer	0.05M Barbital buffer, pH 8.6
3. Acid-Alcohol Solution	20% Acetic acid- 30% Methanol
4. Acetic Acid Solution	5% Acetic acid
5. Paragon Blue Stain	0.5% 8-Amino-7-(3-nitrophenylazo)-2-(phenylazo)-1-napthol-3,6-disulfonic acid disodium salt in 5 % Acetic acid
6. Serum Sample	Undiluted normal serum obtained from BTS.
7. Monoclonal Antibody	Undiluted

2.2.6.3 Method

Each compartment of the Paragon electrophoresis cell was filled with 45ml of barbital buffer. The containers of the wet processor system were filled with 300ml of the following solutions, in sequence :

Acid-Alcohol solution
Paragon Blue Stain solution
5% Acetic Acid solution
Acid-Alcohol solution
5% Acetic Acid solution

The SPE gel was removed from the foil package and blotted gently with the gel blotter. The template was then carefully aligned on the gel with position "A" dots located on the edges of the gel and then placed on the gel surface ensuring it was sealed. The serum samples and pure monoclonal antibody samples were loaded into each of the slots, 5ul into each slot and allowed to diffuse into the gel for 5 minutes. The template was gently blotted and then discarded to ensure no sample smearing on the gel. The gel was placed onto the gel bridge assembly, aligning positive and negative sides of the gel with corresponding positions marked on the gel bridge and placed into the paragon electrophoresis cell and the lid secured. The gel was allowed to run at 100 volts for 25 minutes.

Upon completion of the electrophoresis the gel was removed from the cell and placed in the gel frame. The gel was placed in Acid Alcohol solution I for 3 minutes and then dried in the Paragon Dryer. The dried gel was dipped in the following :

Paragon Blue Stain solution	3 minutes
5% Acetic Acid solution	2 minutes
Acid-Alcohol solution	2 minutes
5% Acetic Acid solution	2 minutes

The gel was then removed from the gel frame and blotted dry with the gel blotters or tissue until completely dry. The gel was then visually evaluated for the presence of proteins.

2.3 Cell culture techniques

2.3.1 Introduction

Cell culture is the growth of cells in vitro by providing them with nutrients to support growth. There are a wide range of cell culture media and tissue culture grade plasticware available, along with an ever expanding collection of cell lines available from the European Collection of Animal Cell Culture (ECACC) and also from the American Tissue Culture Collection (ACTCC).

2.3.2 Preparation of media

Foetal calf serum (FCS) was heat inactivated by placing it in a water bath at 56^0C for 30 minutes before use. Complete media were prepared by adding 55ml of FCS (final concentration 10%) and 5.5ml of L-glutamine (final concentration 2mM) to a 500ml bottle of Dulbecco's Modified Eagle's Medium- with Glutamax (DMEM) or to a 500ml bottle of Roswell Park Memorial Institute (RPMI)1640 media.

2.3.3 Subculturing of cell lines growing in suspension

Cell suspensions were allowed to grow to maximal density. The suspension was then poured into a 50ml centrifuge tube and centrifuged at 200g for 5 minutes. The supernatant was gently poured off and the pellet resuspended in 10ml of complete media. The resuspension was split into a number (obtained from data sheet supplied with the cell line) of tissue culture flasks (75 cm^2) and complete media was added to all of the flasks to give a total volume of approximately 30ml in each flask. They were then incubated at 37^0C in a humidified atmosphere of 95% air / 5%CO_2 until they reached maximal density and required further subculturing.

2.3.4 Subculturing of cells growing in monolayer

Cells growing in monolayer are physically attached to their substratum and have to be detached before being subcultured. The cells in a 75 cm^2 flask were washed three times with 30ml of phosphate buffered saline (PBS) followed by the addition of 5ml of a trypsin/EDTA (0.05% trypsin, 0.02% EDTA) solution to dislodge the cells from the flask. Cells were incubated at 37^0C until they detached from the flask, the time varying with the cell line. The reaction of the trypsin was stopped by the addition of complete media (DMEM) as the FCS present in the medium inhibits trypsin. The suspension was

then centrifuged for 5 minutes at 200g. The supernatant was discarded and the pellet resuspended in 5 ml of complete media and the cells counted using a haemocytometer. The cell suspension was then split into tissue culture flasks (75 cm^2) according to the split ratios (details supplied with cell line) and then incubated at 37^0C in a humidified atmosphere of 95% air / 5%CO_2 until they reached maximal density and required further subculturing.

2.3.5 Trypan blue exclusion method

This method is used for viable cell counting as live cells do not take up the trypan blue dye whereas dead cells do. Trypan blue solution (0.4% in PBS) was mixed 1:1, with a cell suspension, the cells were then counted using a haemocytometer and the number of viable and non-viable cells /ml were calculated.

2.3.6 Cryopreservation of the cell lines

Suspension and monolayer cells were subcultured, the pellets were resuspended in heat inactivated FCS and the cells counted. The cell suspension was placed on ice and adjusted to a final concentration of 1-10 X 10^6 cells per ml with FCS. The precooled "Mr Frosty" was then filled with the appropriate number of cryovials. Dimethyl sulfoxide (DMSO) was then added dropwise, with swirling, to the cell suspension to obtain a final concentration of 10%. The mixture was aliquoted into the precooled cryovials, 1ml in each and the "Mr Frosty" placed in the -70^0C freezer overnight. The cryovials were then placed in liquid nitrogen vessels for long term storage.

2.4. Culture of the MG-63 human osteosarcoma cell line

MG-63 cells were grown in 75cm^2 flasks with DMEM containing 10% heat inactivated FCS and 2mM glutamine at 37^0C in a humidified atmosphere of 95% air / 5%CO_2. The cells were allowed to grow to confluency and were then passaged into either 24 well plates (7 x 10^4 cells per well) for experimental purposes or into 75cm^2 flasks (1 x 10^6 cells per flask) to maintain the culture. The MG-63 cells were passaged as described for cells growing in monolayer.

2.5 Culture of the human monocytic THP 1 cell line

THP-1 cells were grown in 75cm^2 flasks with RPMI 1640 containing 10% heat inactivated FCS and 2mM glutamine at 37^0C in a humidified atmosphere of 95% air /

5%CO_2. The cells grow in suspension and were maintained at 2-9 x 10^5 cells per ml. For experimental purposes the cells were plated out into 96 well plates ($6x10^5$ cells per well) as described in the subculture of cells growing in suspension.

2.6 Cartilage resorption assay

2.6.1 Culturing of bovine cartilage explants

2.6.1.1 Principle

This method was described by Dingle et al. (1975).

2.6.1.2 Method

Nasal septa were dissected from bovine noses and mucus membranes removed aseptically in a flow hood, followed by washing thoroughly with three changes of PBS to remove the blood and debris. The cartilage was then cut into strips of uniform thickness (2mm) using a sterile scalpel and discs of cartilage were then punched out of the strips using a sterile leather punch, discarding any damaged cartilage. The explants in a petri dish were then washed a further three times in PBS and incubated for 24 hours in a humidified atmosphere at 37^0C in an atmosphere of 95% air / 5%CO_2 , in culture medium (DMEM) with 2mM glutamine and 100U/ml penicillin and 100ug/ml streptomycin. The following day the explants were plated out into 96 well plates, 1 disc per well for the cartilage resorption assay. The outer wells of the plate contained PBS only to prevent any "edge" effect.

Retinoic acid was used as the resorbing agent (Meats et al., 1985). A stock solution of retinoic acid was made up in DMSO to a concentration of 10^{-2} M, this was further diluted in ethanol to give a concentration of 10^{-3} M. The solution was further diluted in DMEM to a final working solution of 1 x 10^{-6} M. The cartilage discs were incubated with and without retinoic acid and test substances, 200ul in each well, for a total of 7 days with a change of medium containing test substances at day 3. Media from day 3 and day 7 were stored at -20^{O}C prior to dimethylmethylene blue assay. The cartilage discs were stored at -20^{O}C prior to being digested.

2.6.2 Digestion of cartilage explants

2.6.2.1 Reagents

1. 0.1M phosphate buffer	0.1M sodium phosphate buffer, pH 6.5
2. 50mM EDTA:	465mg of ethylenediaminetetraacetic acid disodium salt (EDTA) in 25ml of phosphate buffer
3. 50mM cysteine/HCl :	197mg of cysteine/HCl in 25ml of phosphate buffer
4. 25mg/ml papain :	625mg of papain in 25ml of phosphate buffer

2.6.2.2 Method

Each cartilage piece was put in a labelled LP4 tube followed by the addition of 0.49ml of phosphate buffer and 70ul of each of the other reagents to a total volume of 700ul. The tubes were capped and placed in a water bath at 65^0C for at least two hours or until the pieces had digested.

2.6.3 Dimethylmethylene blue assay for sulphated glycosaminoglycans

2.6.3.1 Principle

The concentration of proteoglycan released, measured as glycosaminoglycan, chondroitin sulphate, was determined using the dye 1,9 dimethylmethylene blue as described by Farndale et al. (1982). The sample is mixed with the dye and the absorbance change due to the binding of the dye with glycosaminoglycans measured.

2.6.3.2 Reagents

1. Formate buffer :	0.1M sodium formate, adjusted to pH 3.5 with formic acid
2. DMB working solution :	16mg of dimethylmethylene blue dissolved in 5ml of ethanol and then made up to 1l with formate buffer
3. Stock chondroitin sulphate : (porcine rib)	4mg/ml chondroitin sulphate in DMEM or digest buffer

2.6.3.3 Method

Two standard curves were prepared, one for the samples in DMEM and the other for the digest samples. The stock chondroitin sulphate solution was further diluted to give a top standard of 40ug/ml either in DMEM or digest buffer and a range of standard concentrations were then made ranging from 0 - 40ug/ml. Aliquots of 40ul of each standard in triplicate were then added to wells in a 96 well plate followed by 40 ul of each sample in duplicate to the other wells. It was necessary to dilute some of the samples 20 fold for day 3 and day 7 and some of the digest samples had to be diluted 10 fold. DMB working solution, 250ul, was then added to all of the wells and after 2 minutes the absorbances were measured in a multiskan MS platereader at 570 nm. The results were expressed as % cumulative glycosaminoglycan release at days 3 and 7. This was determined from proteoglycan release into the media at days 3 and 7 and proteoglycan remaining in the cartilage discs as determined from the cartilage digests.

2.7 Prostaglandin E radioimmunoassay.

2.7.1 Principle

The amount of prostaglandin E_2 (PGE_2) was measured using a modification of the radioimmunoassay (RIA) technique as described by Andrews (1988). The antiserum does not distinguish between PGE_1 and PGE_2, see table 2.1, the amount of PGE_2 is more correctly the amount of PGE present in the samples.

Cross Reactant	% Cross Reactivity
PGE_1	240
PGE_2	100
PGA_1	10
PGA_2	8
PGB_1	0.7
PGB_2	0.9
$PGF_1\alpha$	3.2
$PGF_2\beta$	4.2

Table 2.1 Specificity of PGE_2 antisera

Radioimmunoassays work on the principle that a limiting and constant amount of antibody is incubated with a fixed concentration of labelled antigen to produce a known % bound constant. In RIA, the sample to be assayed is mixed with the antibody (Ab) and a small amount of labelled antigen (Ag*) and the following reactions takes place:

$$Ag + Ab \Leftrightarrow Ag.Ab$$

$$Ag^* + Ab \Leftrightarrow Ag^*.Ab$$

The radiolabelled antigen competes with the antigen in the sample for the available binding sites on the antibody, so the higher the concentration of antigen in the sample the less radioactive antigen will be able to bind to the antibody, resulting in a greater radioactive content in the free antigen (unbound). The reaction is completed after a fixed time period that has been optimised for the individual assay. In a number of systems reaction mixtures are left overnight at 4^0C. The concentration of the unlabelled antigen in the sample can thus be calculated by setting up a standard curve using known amounts of unlabelled antigen. In the PGE_2 RIA, free PGE_2 (antigen) is removed using adsorption techniques with dextran-coated charcoal, leaving the bound PGE_2 antigen/antibody and labelled antigen/antibody complexes in the supernatant. Radioactive counts obtained from the supernatant ("bound counts") can then be used as a measure of unlabelled PGE concentration.

The amounts of labelled antigen and antibody are kept constant, and have been chosen to give optimal conditions i.e. when a maximum of about 50% of labelled antigen is bound to antibody.

2.7.2 Reagents

1. Stock Buffer : 0.1M Tris/HCl containing 1.4M NaCl pH 7.4

2. Working Buffer : 0.5g of gelatin (0.1%) and 0.1 g of sodium azide (0.02%) were dissolved in 100ml of distilled water, 50ml of stock buffer was added and the volume made up to 500ml with distilled water. The pH was checked and adjusted to pH 7.4 with 1M HCl

3. PGE_2 standard : 1mg of PGE_2 was dissolved in 1ml of absolute ethanol
10ul of this solution was then diluted to 1ml with ethanol, a further ten fold dilution was made to give a stock of 1ug/ml. 100ul aliquots were made and stored at $-20^{O}C$ for a maximum of 6 months.

4. Tracer : [5,6,8,11,12,14,15(n)-^{3}H]-prostaglandin E_2 (specific activity 181Ci/mmol, 6.70T Bq/mmol) was diluted 1 in 1500 in working buffer to give an approximate count of ~ 2500cpm/100ul of tracer.

5. Antibody : One vial of rabbit anti-PGE antiserum was made up to 5ml in working buffer and stored in 1.25ml aliquots at $-20^{0}C$. For use an aliquot was made up to 10ml with working buffer.

6. Dextran coated charcoal : 300mg of activated charcoal and 62.5mg of dextran T70 were suspended in 50ml of working buffer and left stirring on ice for at least 30 minutes before use.

2.7.3 Method

The PGE_2 standard was diluted in DMEM to construct a standard curve in the range 0.039 -10ng/ml and the samples were diluted, if necessary in DMEM. All tubes were kept on ice throughout the assay and were set up according to table 2.2, with the standards being assayed in triplicate and the samples in duplicate, antibody being added last.

	NON SPECIFIC BINDING (NSB)	TOTAL COUNTS (TC)	MAXIMUM BINDING	STANDARD	TESTS
Sample					100ul
Standard				100ul	
Buffer	100ul	300ul			
DMEM	100ul	100ul	100ul		
Tracer	100ul	100ul	100ul	100ul	100ul
Antibody			100ul	100ul	100ul

Table 2.2 Protocol for the PGE_2 radioimmunoassay

The tubes were incubated at 4^0C for 24 hours. The following day 200ul of the ice cold dextran coated charcoal was added to all of the tubes except the TC. The tubes were vortexed and then left at 4^0C for exactly 5 minutes, they were then centrifuged at 1400g for 15 minutes in the MSE mistral 2L at 4^0C to separate the free from the bound. Aliquots of 200ul of the supernatant was then carefully removed and pipetted into labelled scintillation vials and 2ml of optiphase Hisafe scintillation fluid was then added to each vial. The vials were then capped and the radioactivity determined in each sample in a Wallac 1212 Rackbeta liquid scintillation counter.

A standard curve was constructed of % bound counts vs PGE_2 concentration, see figure 2.2 and used to determine unknown amounts of PGE_2 in samples of culture media. Results were expressed as ng per ml culture media.

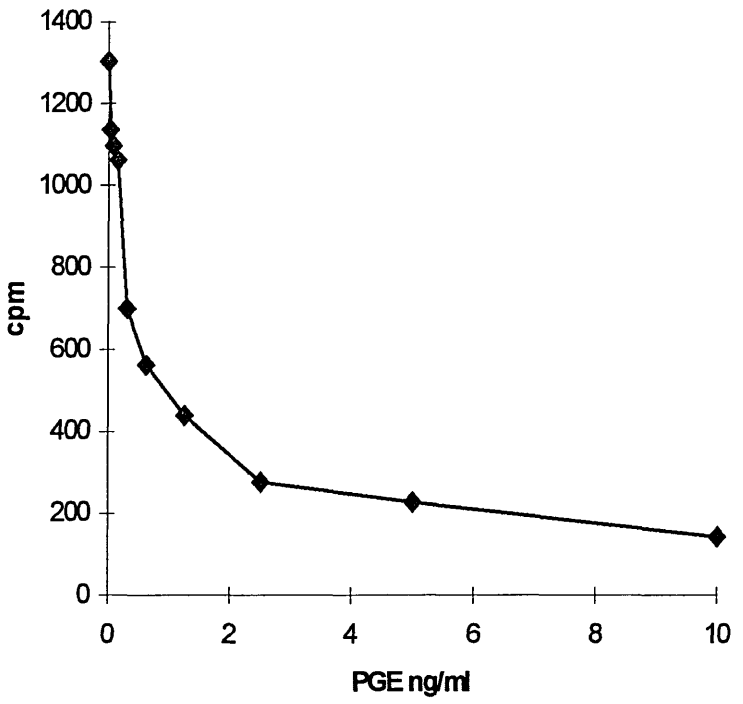


Figure 2.2 Typical standard curve for PGE_2 radioimmunoassay

2.8 Cytokine assays

The cytokine assays carried out were all commercial ELISA kits from Biosource, with the following range sensitivities :

Human IL-1β	0 - 250 pg/ml
Human IL-6	0 - 250 pg/ml
TNF α	0 - 1000pg/ml

2.9 Immunohistochemistry using human cartilage sections

2.9.1 Principle

Immunohistochemistry is a technique for identifying and localising antigens in cells and tissue sections using specific antibodies. PAPP-A binding to cartilage was determined by incubation of PAPP-A with unfixed cryostat sections of human articular cartilage. After washing of the sections, any bound PAPP-A was detected using anti

PAPP-A antibody and a peroxidase labelled secondary antibody. Antibody to type II collagen was used as a positive control in this system and rabbit immunoglobulin fraction as the negative control.

2.9.2 Reagents

1. PBS	1x as supplied
2. Block	1% BSA in PBS
3. PBS/BSA	0.1% BSA in PBS
4. Primary antibody	Rabbit anti human - PAPP-A
	1: 200 dilution in PBS/BSA
5. Positive control	Rabbit anti-human type II collagen antibody
	1: 200 dilution in PBS/BSA
7. Negative control	Rabbit immunoglobulin fraction
	1 :1200 dilution in PBS/BSA
8. Secondary antibody	Goat anti-rabbit HRP labelled
	1: 3000 dilution in PBS/BSA
9. DAB	1mg/ml 3,3'- Diaminobenzidine tetrahydrochloride
10. PBS/Glycerol 1:1	10ml of PBS+ 10ml of glycerol
11. PAPP-A	500ug/ml

2.9.3 Method

Cryostat sections from macroscopically normal femoral head cartilage were incubated overnight at 4^0C in a moisture chamber with either PAPP-A (500ug/ml) or PBS under the same conditions. All of the slides were then washed three times in PBS for 3 minutes each wash. The primary antibody, positive and negative control, were then added to the slides (100ul to each) and the slides incubated at room temperature for 1 hour in a moisture chamber. The slides were then washed in PBS as previously described and secondary antibody or PBS were added. The slides were incubated for a further hour followed by the wash step to remove any traces of the unbound antibody. The substrate was then incubated with the slides for about ten minutes for colour to develop and then rinsed in distilled water. The sections were mounted in PBS/glycerol and examined under the Olympus light microscope and photographs taken.

2.10 Elastase assay.

2.10.1 Principle

The assay used was a colorimetric assay and was a modification of that described by Sinosich et al. (1982) and Oxvig et al. (1994). Elastase enzyme cleaves the ρ-nitroanilide (ρNA) substrate releasing the chromphore ρ- nitroaniline which can be monitored by the change in absorbance at 405nm.

2.10.2 Reagents

1. Assay buffer	50mM Tris-HCl containing 50mM NaCl pH 8.0
2. Substrate	10mg of MeO-Suc-Ala-Ala-Pro- Val-ρNA in 1ml of DMSO and then further diluted in assay buffer to give a final working concentration of 1mM
3. Enzyme	Human leukocyte elastase, specific activity 20-22U/mg protein) made to a concentration of 1uM in assay buffer, to give a final concentration in the cuvette of 12.5nM.
4. PAPP-A	PAPP-A was diluted in assay buffer to a concentration of 2.5uM to obtain a final concentration in the cuvette of 62.5nM, which is the normal level at term.

2.10.3 Method

The assay was optimised using a range of substrate and enzyme concentrations and varying amounts of enzyme. To a cuvette, 1.925ml of substrate (0.1-1.0mM) was added followed by the addition of 25ul of the elastase enzyme. At each substrate concentration 50ul of PAPP-A or 50ul of buffer was added to the cuvette and the rate of reaction was monitored over 5 minutes at a wavelength of 405nm in a Cecil 5000 scanning UV- visible spectrophotometer at 25^0C. The effect of PAPP-A at varying concentrations was also determined, 0.625 - 62.5nM using fixed enzyme (12.5nM) and substrate concentrations (0.5mM). Aliquots of PAPP-A, 50ul, were also incubated with 25ul of enzyme for 30 minute at room temperature prior to addition to the enzyme

assay system to determine whether any inhibitory activity was destroyed by enzymic degradation. A control containing 50ul of buffer and 25ul of enzyme was also tested under the same conditions.

2.11 Plasmin assay

2.11.1 Principle

Plasmin was assayed using a colorimetric assay with a peptide ρ- nitroanilide substrate, (ρNA). Plasmin releasing the chromphore ρ- nitroaniline which can be monitored by the change in absorbance at 405nm.

2.11.2 Reagents

1. Assay buffer	50mM Tris-HCl containing 50 mM NaCl pH 8.0
2. Substrate	H-D -Ile-Phe-Lys-ρNA dissolved in DMSO to a final concentration of 100mM
3. Enzyme	Human plasmin (specific activity 7 U/ mg of protein) made up to 20uM in assay buffer, to give a final concentration in the cuvette of 0.4uM
4. PAPP-A	PAPP-A was diluted in assay buffer to a concentration of 2.5uM

2.11.3 Method

The assay conditions were optimised using a range of enzyme and substrate concentrations. To a cuvette containing 930 ul of assay buffer and 25 ul of substrate (0.01-1.0 mM), 25ul of either PAPP-A or buffer was added followed by the addition of 20ul of enzyme. The colorimetric reaction was monitored by the change in absorbance at 405nm in the Cecil 5000 scanning spectrophotometer at 25^0C.

Aliquots of PAPP-A, 25ul, were also incubated with 20ul of enzyme for 2 hours at room temperature prior to addition to the enzyme assay system to determine whether any inhibitory activity was destroyed by enzymic degradation. A control containing 25ul of buffer and 20ul of enzyme was also tested under the same conditions.

2.12 Trypsin assay

2.12.1 Principle

Trypsin was assayed using a colorimetric assay with a peptide nitroanilide substrate, Bz-DL-Arg-ρNA-HCl (DL-BAPNA).

2.12.2 Reagents

1. Assay buffer	100mM Tris-HCl containing 20mM $CaCl_2$ pH 8.0
2. Substrate	DL-BAPNA dissolved in DMSO to a final concentration of 100mM
3. Enzyme	Human trypsin (specific activity 2.5 U/ mg of protein) made up to 45uM in assay buffer, to give a final concentration in the cuvette of 0.45uM
4. PAPP-A	PAPP-A was diluted in assay buffer to a concentration of 2.5uM

2.12.3 Method

The assay conditions were optimised using a range of enzyme and substrate concentrations. To a cuvette containing 965 ul of buffer and 25ul of substrate (0.1-2.5 mM) 10ul of enzyme was added. The colorimetric reaction was monitored by the change in absorbance at 405nm in the cecil 5000 scanning spectrophotometer at 25^0C. At each substrate concentration 25ul of PAPP-A or 25ul of buffer was added to the cuvette and the rate of reaction was monitored over 5 minutes. Aliquots of PAPP-A, 25ul, were also incubated with 10ul of enzyme for 2 hours at room temperature prior to addition to the enzyme assay system to determine whether any inhibitory activity was destroyed by enzymic degradation. A control tube was also set up as described for the previous enzyme assays.

2.13 Cathepsin B assay

2.13.1 Principle

Cathepsin B was assayed using a fluorescent assay with a peptide napthylamide substrate, Z-Arg-Arg-βNA.

2.13.2 Reagents

1. Assay buffer	50mM potassium phosphate buffer pH 6.0
2. Activating Buffer	20.5mg of dithiothreitol (DTT) and 100ul of Brij 35 detergent were made up in 100ml of the 50mM phosphate buffer pH 6.0.
3. Substrate	50mg of the substrate, Z-Arg-Arg-βNA was dissolved in 425ul of distilled water and 425ul of assay buffer was then added to give a concentration of 100mM
4. Enzyme	Human cathepsin B (specific activity 588.7U/ mg of protein) diluted to 98nM in assay buffer, to give a final concentration in the cuvette of 9.8nM
5. PAPP-A	PAPP-A was diluted in assay buffer to a concentration of 2.5uM

2.13.3 Method.

The assay conditions were optimised using a range of enzyme and substrate concentrations. The enzyme was activated for 5 minutes at 40^0C by the addition of 180ul of activating buffer to 20ul of enzyme in a 96 well plate followed by the addition of 5ul of the substrate (0.1-2.5mM). The reaction was monitored in the Wallac fluorescent plate reader at λ_{ex}355- λ_{em}460 nm for 3 minutes. The effect of PAPP-A on the enzyme was determined by the addition of either 4ul of PAPP-A or 4ul of buffer to the wells just before the addition of the substrate.

2.14 Calculations of K_m and K_I

The value for K_m was obtained from the enzfitter programme and the value for K_I was determined by the following equation (Dawes, 1972):

$$K_{m(I)} = K_m \left(1 + \frac{[I]}{K_I} \right)$$

Rearranging :

$$K_I = \frac{[I]K_m}{K_{m(I)} - K_m}$$

K_m is the Michaelis constant, defined as the substrate concentration at half maximum velocity of reaction and is characteristic for a given enzyme.

$K_{m(I)}$ is the substrate concentration at half maximum velocity of reaction in the presence of the inhibitor.

K_I is the dissociation constant of the enzyme inhibitor complex.

[I] is the inhibitor concentration.

2.15 Methods for determining cytokine binding to PAPP-A

The role of PAPP-A as a cytokine binding plasma protein was investigated using a number of techniques. Binding of TGFβ and IL-1β were chosen since it has been previously observed that α_2m, which has similarities with PAPP-A, binds both these cytokines (Borth and Luger, 1989; O'Conner-McCourt and Wakefield, 1987).

2.15.1 Gel filtration Chromatography

2.15.1.1 Principle

The method described is a modification of the method described by O' Conner-McCourt (O'Conner-McCourt and Wakefield, 1987). The cytokines used were radiolabelled IL -1β (^{125}I IL-1β) and TGF β (^{125}I TFGβ). The PAPP-A sample, after mixing with the labelled cytokine, could be separated into PAPP-A - cytokine complexes, free PAPP-A and free cytokine on a pre-equilibrated Sephadex G200 column. The complexes and free cytokine could then be determined by the radioactive counts associated with the fractions collected.

2.15.1.2 Method

Sephadex G200 powder was preswollen overnight in column buffer (0.1M Tris/HCl containing 50mM NaCl, pH 7.4) at 4^0C. The gel slurry was then carefully poured into a 10ml plastic pipette (glass wool at the bottom acted as the filter) and packed under gravity. The column was washed with 3 column volumes of column buffer and the column was calibrated with a 2mg/ml dextran 2000 solution to determine the void volume.

Aliquots of 200ul of PAPP-A (100ug/ml) (1.25uM) were then applied to the column and 1ml fractions were collected. The protein content of each was determined using the BCA method, (section 2.2.3.2) to monitor the exact point of elution of PAPP-A from the column. Various PAPP-A concentrations (62.5ug/ml-1000ug/ml) (0.078uM – 1.25uM) were preincubated with different concentrations of the labelled cytokines (0.05nM-50nM) for different times, 30 minutes to 24 hours. Aliquots of 25ul of the incubation mixtures were then carefully loaded onto the gel surface and allowed to enter the gel. Column buffer was then carefully added, dropwise, to the column

avoiding any disruption on the surface of the gel .

Fractions of 1ml were collected in glass tubes until the PAPP-A and the labelled cytokine had been eluted. Elution of labelled cytokine could be monitored with a Geiger counter. The fractions were then counted for γ radioactivity in a Wallac 1261 multigamma gamma counter and results expressed as counts per minute.

2.15.2. PAGE analysis using autoradiography

2.15.2.1 Principle

The method employed was a modification of that described by Huang et al. (1988). PAPP-A and labelled cytokines ^{125}I IL-1β and ^{125}I TFGβ were incubated and then subjected to native PAGE to determine if they were bound by PAPP-A. Radioactive PAPP-A -cytokine complexes and free labelled cytokines were determined by autoradiography of the gels. The relative position of the radioactive bands seen on the film would determine whether the labelled cytokine was free or bound to the PAPP-A.

2.15.2.2 Reagents

1. PAGE gels:	Ready made 4 % acrylamide gels
2. Tank buffer :	0.25M Tris/HCl containing 2M glycine, pH 8.3
3. Markers :	Prestained high M_r markers, Myosin 205,000, β-Galactosidase 118,000, BSA 85,000 and Ovalbumin 47,000.
4. Diluent buffer :	0.1% BSA in PBS
5. PAPP-A :	62.5ug/ml-1000ug/ml in diluent buffer
6. ^{125}I IL-1β :	0.5nM-50nM in diluent buffer
7. ^{125}I TGFβ :	0.5nM-50nM in diluent buffer
8. Crosslinker :	5mM suberic acid bis (N -hydroxy-succinimide ester) in DMSO
9. Glycine :	2.5M in distilled water
10. Fixer :	1:4 dilution in distilled water of concentrate
11. Developer :	1:5 dilution in distilled water of concentrate
12. Silanising fluid :	Silane A174 (γ methacryloxypropyl- trimethoxysilane)

2.15.2.3 Method

Aliquots of 25ul of PAPP-A at varying concentrations were incubated with 25ul of ^{125}I IL-1β at varying concentrations in small covered glass vials for 2 hours at room temperature. 5ul of glycerol were then added to 25ul of this sample and it was then loaded onto a native PAGE gel. The gel was run at 150V for approximately 3 hours or until the bromophenol blue, present in the markers, ran off the bottom of the gel. The gel was then very carefully taken off the plates and dried onto filter paper. The dried gel was then placed onto developing film in a cassette, the corner marked to determine the orientation and autoradiographed at -70^{0}C for 48 hours. The film was then fixed and developed in the dark room. This procedure was repeated with different incubation times for PAPP-A and cytokines, from 30 minutes to 24 hours and also on extended autoradiography incubation time of 7 days at -70^{0}C.

Crosslinking the samples was also carried out by incubating the incubation mixture with 0.20 volume of 5mM suberic acid bis (N -hydroxy-succinimide ester) for 2 minutes at 4^{0}C and stopping the reaction with 0.05 volume of 2.5M glycine.

PAGE analysis with autoradiography was carried out in the same way for TGFβ, the only difference being that ^{125}I TGFβ, bound very strongly and quickly to plastic and glass, therefore all steps had to be carried out in silanised tubes and transfers with silanised pipettes to prevent any loss of ^{125}I TGFβ. Silanisation was carried out by soaking all glassware and pipettes in distilled water, followed by 100% ethanol, and then finally in silanising fluid before leaving them to dry in an oven before use.

2.15.3 PAGE analysis using gel dissection

A minor modification of the PAGE gel analysis was performed using gel dissection as opposed to autoradiography.

2.15.3.1 Method

The PAGE gel was run as previously described in section 2.15.2.3 and then the gel very carefully removed from the plates and placed onto filter paper that had been premarked with the lane distances. The gel was then carefully sectioned, using a scalpel blade, into lanes and then each lane was cut into 5mm sections. Each section was placed in a

labelled LP4 tube along with the filter paper underneath and then counted in the Wallac 1261 multigamma gamma counter .

2.16 The preparation of a monoclonal antibody to PAPP-A

An overall scheme for the production of monoclonal antibodies is shown in figure 2.3.

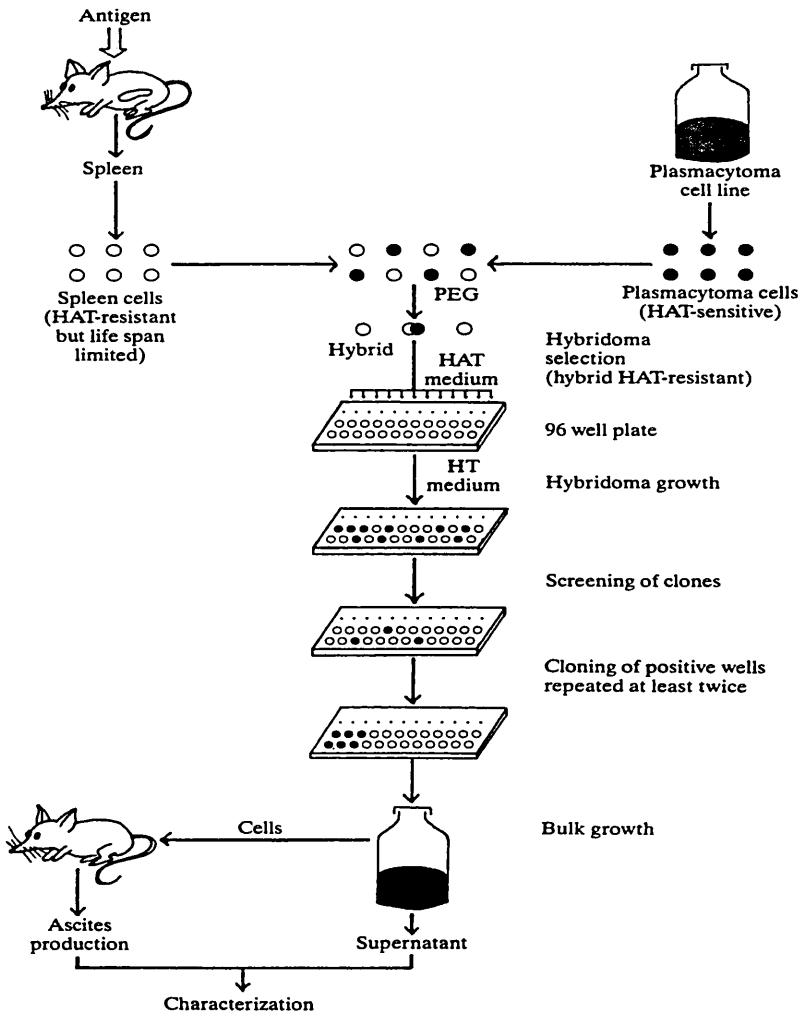


Figure 2.3 A schematic diagram to show the stages in the production of a monoclonal antibody (Newell et al., 1988).

2.16.1 Immunization of the mice

This work was carried out by the Sheffield Hybridoma centre, University of Sheffield.

2.16.1.1 Principle

The primary aim of immunization was to stimulate the production of clones of antigen-committed B lymphocytes, to a point of differentiation which was suitable for fusion. BALB/c mice were used for immunization as they are readily available and easy to handle.

It has been found that antigens are much more immunogenic when presented to the host suspended in an adjuvant. Freund's complete adjuvant is the most commonly used and consists of a mixture of oil (Bayol F) and detergent (mannide monooleate) containing *Mycobacterium tuberculosis*. The timing and the number of immunizing boosts may make a considerable difference to the immune response, a reasonable response may be seen with some antigens after just one injection whereas others may require several injections.

2.16.1.2 Method

A sample of pure PAPP-A (50ug made up in 50 ul of sterile PBS) was mixed with an equal volume of complete Freunds adjuvant. The solution was mixed on a vortex followed by several passes through a syringe and needle until an emulsion formed which did not separate on standing. The mixture was then injected into five 6-10 week old BALB/C mice subcutaneously. After 14 days the injections (intraperitoneal) were repeated using the same amount of antigen but mixing it 1:1 with incomplete Freunds adjuvant. The mice were injected at day 35 as previously. At day 45 tail bleeds were taken from all the mice by excising the tip with sterile scissors and then collecting the blood into a capillary tube. The circulating antibody level was determined using the assay described in section 2.16.3. The best responder, as determined from the assay, was further injected with 100ug of antigen (PAPP-A) in PBS on day 56 to give a final boost and 3 days later the mouse was sacrificed for fusion.

2.16.2 The fusion and hybridoma selection procedure

This work was carried out by the Sheffield Hybridoma centre, University of Sheffield.

2.16.2.1 Principle

Polyethylene glycol (PEG) is used for the fusion of spleen cells with myeloma cells to produce hybridomas. The PEG causes aggregation of the integral proteins in the cell membrane, resulting in areas of lipid bilayer stripped of protein which would merge with stripped membrane from adjacent cells allowing the fusion to occur. On fusion of plasma membranes a multinucleated cell is formed. Subsequently nuclei fuse and contain the chromosomes from each parent cell. The hybridoma is unstable at this stage and tends to lose chromosomes from either parent until stability is attained (Kuby, 1997).

Medium containing aminopterin is used for the hybridoma growth to ensure the main DNA synthesis pathway is blocked, by addition of an inhibitor of the enzyme dihydrofolate reductase, aminopterin. Only the fused hybrid cells that have a functional hypoxanthine- guanine phosphoribosyl transferase (HGPRT) inherited from the parent spleen cells survive and grow as this is required to circumvent the aminopterin block. Unfused myeloma cells do not survive due to the deficiency of this enzyme as they cannot synthesise DNA via the salvage pathway using HGPRT and de novo synthesis is blocked by aminopterin (unfused spleen cells only survive a few days in culture).

2.16.2.2 Method

The myeloma SP2/0 cells were grown in RPMI containing 10% FCS and 24 hours prior to fusion the cells were split to between 3.0-5.0 x 10^5 cells/ml so they were in the logarithmic phase of growth required for optimal fusion efficiency.

The source of B lymphocytes used in the fusion experiments was the spleen of the immunised mouse which was aseptically removed. The cells were perfused out of the spleen by gently pricking and forcing medium through the tissue. This procedure usually removes between 0.5-2.0 x 10^8 lymphocytes per spleen. The fusion ratio of spleen : myeloma was 1:5. The pre-determined number of myeloma cells, were then centrifuged in a universal together with the spleen cells at approximately 400g for 5 minutes at 30^0C. The cell pellet was then resuspended in 10ml of RPMI (containing no FCS) and then centrifuged again for 7 minutes as previously.

The cell pellet was gently warmed in the hand and 1ml of prewarmed (37^0C) PEG M_r 1500 was added with constant swirling to ensure adequate mixing. RPMI, 1ml (prewarmed to 37^0C, no FCS), was then added over 1 minute, followed by a further 9ml over 2 minutes. The cell suspension was then centrifuged for 15 minutes at 300g at 30^0C. The pellet was resuspended in warm RPMI / HAT media (containing Hypoxanthine, Aminopterin, and Thymidine). The cells were then plated at a density of approximately 1.0×10^6 total cells/ml in multiwell plates and incubated in a humidified atmosphere at 37^0C in an atmosphere of 95% air / 5%CO_2. The fused hybridoma cells shared the properties of both cell types. They could survive and divide in culture indefinitely, a property of immortal myeloma cells, and they produced antibody, a property of differentiated B lymphocytes.

The cells were fed every 2-3 days with 100ul of complete HAT medium. There were only a few hybrids in each well. Within 7-10 days each hybrid was showing clonal growth (clumps of > 100 cells appeared and the medium began to turn yellow). The supernatants were tested for antibody using the screening assay described in section 2.15.3. The cells from the wells of positive supernatants were removed and cloned to ensure only strongly growing and antibody producing cells were present. This cloning procedure was repeated several times.

2.16.3 The screening procedure

The screening procedure was carried out "in house".

2.16.3.1 Principle

The screening procedure should preferably be of the same type as that proposed for a diagnostic kit, i.e. it should be rapid and applicable to large numbers of samples. The initial screening may involve several hundred samples and may have to be repeated several times before all the positive clones have been selected. It is also important to note that a monoclonal antibody that is detected using one detection system, for example ELISA, may not function in another, such as western blotting. The ELISA is probably the most useful assay for the screening of hybridoma supernatants and was therefore the method chosen.

2.16.3.2 Reagents

1. PBS	0.1M PBS pH 7.6
2. PAPP-A	1ug/ml of PAPP-A in PBS
3. Blocking Solution	1% Gelatin in PBS
4. Negative Control	RPMI media
5. Positive Control	Mouse Serum (Tail bleed) diluted 1: 1000 in PBS.
6. Secondary Antibody	Rabbit-anti-mouse alkaline phosphatase conjugate diluted 1:15,000 in PBS
7. TBS	0.1M Tris buffered saline (TBS) pH 7.6
8. Substrate	0.0742g of 4-nitrophenyl disodium phosphate in 0.1M Tris/HCl containing 20mM magnesium chloride

2.16.3.3 Method

The assay was optimised in the first instance using different concentrations of PAPP-A and different concentrations of positive and negative controls, see reagents section 2.16.3.2, to ensure the correct antigen concentration was used in the screening procedure. The correct antigen concentration is the lowest dilution which gives the highest antibody titre for the positive serum, without giving high backgrounds with the negative serum.

Specialist "Pro bind" 96 wells plates were coated overnight with PAPP-A diluted in PBS, 100 ul per well and the plate left overnight at 4^0C or 3 hours at 37^0C. The unbound PAPP-A was then removed by inverting the plate and tapping it gently on absorbent paper. The wells were then washed six times with 300ul of PBS. 200ul of blocking solution was added to each well to block any section of the plate that did not have PAPP-A bound to it and hence reduce non-specific binding of the following antibodies. The plates were left for 90 minutes at 37^0C and then washed as previously described.

50 µl of supernatant sample was carefully pipetted into each well, or 50 µl of negative or positive control. The plates were then left at room temperature with shaking for 90 minutes and then transferred to 37^0C for 30 minutes to ensure optimal binding. The wells were washed again as previously, followed by the addition of 100ul of the

secondary antibody. The plates were left for another 2 hours with shaking and then washed six times with TBS (phosphate inhibits the final stage). The final stage was the addition of 100 µl of the substrate. The plates were left to develop for approximately 50 minutes and then read in a multiskan MS platereader at 405 nm.

The wells with an absorbance reading close to that of the positive control were identified and the cells cloned. These cells were then retested to confirm that the antibody was still being produced and then recloned several times to ensure single clone growth. The positive single clones were then grown up in cell culture to enable a sufficient amount of cell culture supernatant to be produced to carry out the next stage which was the antibody purification procedure.

2.16.4. The purification of the monoclonal antibody

2.16.4.1 Principle

After the cloning procedure the cells must be bulk cultured to obtain sufficient antibody containing supernatant. This supernatant can then be used to obtain a purified antibody sample. The hybridoma cells can be stored in 90% FCS and 10% DMSO in liquid nitrogen for further use. A few litres of end culture supernatant is sufficient if less than 100mg of antibody is required. End culture supernatant is that obtained after the cells have reached maximal density.

The main task of the purification procedure was to separate the monoclonal antibody from the other contaminants in the culture media. Several different techniques can be used at this stage to purify the antibody, the one used in this procedure was affinity chromatography using Protein G matrix. The principle behind this technique is that the Fc portion of IgG binds to Protein G (cell surface protein of Group G streptococci that has a type III Fc receptor) over a wide pH range (Guss et al., 1986). To elute the IgG it is necessary to lower the pH to approximately 2.5-3.0 depending on the sample.

2.16.4.2 Method

The monoclonal culture supernatant was centrifuged at 10,000g for 10 minutes to remove cells and debris, the sample was then filtered through a 0.45um sterile filter.

The Hi Trap column (containing pre packed Protein G) was then equilibrated with 20mM sodium phosphate buffer, pH 7.0 (start buffer), and washed with at least 3 times the column volume using a 10ml syringe connected to the top of the column. The sample was then applied through the 10ml syringe and all of the flow through collected into a beaker. The column was then washed with 5 column volumes of start buffer and the eluent collected and monitored at 280nm until no more protein could be detected. This stage was crucial as excessive washing had to be avoided as the interaction between the protein of interest and the ligand may be weak. The bound protein of interest was then eluted from the column by the addition of 3 column volumes of 0.1M glycine/ HCl pH 2.7, as previously, the fractions from the column were monitored at 280nm. The 1ml fractions were collected in tubes containing 100ul of neutralizing buffer, (1.0M Tris/HCl pH 9.0) to bring the pH to around 7.0. The fractions were then analysed for protein content by the BCA method (as described in section 2.2.3.2) and for purity by SDS PAGE gel electrophoresis (as described in section 2.2.1.3). The antibody was stored at -20^{0}C in 100ul aliquots.

2.17. Current ELISAs for PAPP-A

At present there are several ELISAs for PAPP-A, however the question as to their specificity required clarification and the vast majority of published data has been using polyclonal antibodies. The use of polyclonal antibodies may give rise to some polyspecific binding of other proteins. Also the sensitivity of the assay is of the utmost importance if PAPP-A determination is to be used for screening and/or diagnostic purposes in the first trimester of pregnancy. In this project several ELISAs have been used, both commercial and in house using our monoclonal antibody.

2.17.1 ELISA using a commercial polyclonal PAPP-A antibody (DAKO)

2.17.1.1 Principle

Dako have produced a commercial protocol for the detection of PAPP-A in samples using their polyclonal PAPP-A antibody and a polyclonal PAPP-A peroxidase conjugated antibody. This assay has many shortcomings, the main one being the use of the same polyclonal in both stages, differing only in that one is conjugated.

2.17.1.2 Reagents

1. Coating buffer:	0.1M sodium carbonate- bicarbonate buffer, pH 9.6
2. Washing buffer:	0.01M phosphate buffer, 0.5M NaCl, 0.1% Tween 20, pH 7.2
3. Dilution buffer:	as above for wash buffer
4. Standard:	Pregnancy serum ~ 45mg/l PAPP-A
5. Primary antibody:	Rabbit anti-human PAPP-A (polyclonal) Diluted 1:1390 in dilution buffer
5. Secondary antibody:	Rabbit anti-human PAPP-A HRP labelled (polyclonal) Diluted 1: 500 in dilution buffer
6. Substrate buffer:	0.05M phosphate citrate buffer, pH 5.0
7. Substrate:	0.4mg/ml o-phenylenediamine (OPD) dihydrochloride in substrate buffer. Immediately before use 40ul/100ml of 30% H_2O_2 was added
8. Stop solution :	0.5M sulphuric acid

2.17.1.3 Method

A 96 well "pro bind" plate was coated overnight at 4^0C, 100ul of primary antibody being added to each well. The plate was then washed 4 times manually. Test samples and standards were then diluted to appropriate concentrations and 100ul was added per well. The plate was then incubated at room temperature for 2 hours, followed by the wash procedure as previously. Secondary antibody, 100ul, was then added to all the wells and the plate incubated for 1 hour at room temperature. The plate was then washed 6 times to remove unbound secondary antibody. The colorimetric substrate, OPD, was then added to all the wells, 100ul per well and the plate placed in the dark for 15 minutes to develop. When colour development was complete the reaction was stopped with the stop solution, 100ul to each well. The absorbance reading for each well was then determined by using the multiskan MS platereader at 492nm absorbance.

2.17.2 Dr Christiansen's ELISA for PAPP-A

2.17.2.1 Principle

A monoclonal antibody was very generously donated by Dr M Christiansen to enable an ELISA system to be optimised for the measurement of PAPP-A in the different stages of the purification procedure. The assay protocol was also very kindly supplied.

2.17.2.2 Reagents

1. Coating buffer :	0.1M sodium carbonate- bicarbonate buffer, pH .9.6
2. Dilution buffer :	0.01M phosphate buffer containing 0.5M NaCl, 1% Triton X-100 and 1% bovine serum albumin (BSA), pH 7.2
3. Wash buffer :	0.01M phosphate buffered saline, pH 7.2, 0.05% Tween 20.
4. Standard:	WHO IRP 78/610 assuming 100 IU/l (45mg/l) of PAPP-A
5. Primary antibody:	Polyclonal anti-PAPP-A pool diluted 1:1000 in carbonate buffer.
6. Secondary antibody:	Monoclonal antibody 234-3, 75ul in 12ml of dilution buffer.
6. Conjugated antibody:	Rabbit anti mouse HRP labelled (DAKO P260) Diluted 1: 2000 in dilution buffer.
7. Substrate buffer:	0.05M phosphate citrate buffer, pH 5.0
8. Substrate:	0.4mg/ml o-phenylenediamine (OPD) dihydrochloride in substrate buffer. Immediately before use 40ul/100ml of 30%. H_2O_2 was added
8. Stop solution :	0.5M sulphuric acid.

2.17.2.3 Method

A 96 well plate was coated overnight at 4^0C with 100ul of polyclonal antibody and left. The plate was then washed once with wash buffer and 100ul of standard, serum or controls were added to each well. The plate was incubated at room temperature for 2 hours with slow shaking. The plate was then washed 4 times in wash buffer and 100ul of monoclonal antibody was added to each well and the plate incubated for 1 hour at

room temperature with slow shaking. The plate was then washed 6 times in wash buffer followed by the addition of 100ul of conjugated antibody, for 1 hour at room temperature. The plate was washed 6 times as previously described, and then 100ul of OPD substrate was added and the plate left in the dark for 30 minutes to allow the colour to develop. The reaction was stopped after 30 minutes with 150ul of stop solution and the absorbance read on the multiskan MS platereader at 492nm

2.17.3 The in house monoclonal ELISA method

2.17.3.1 Principle

The monoclonal antibody produced in house as described in section 2.16 was initially tested in the ELISA system as described in section 2.16.2.3. The monoclonal antibody was simply substituted for the monoclonal antibody provided by Dr Christiansen.

2.17.3.2 Method

The in house monoclonal antibody was diluted 1:100 in carbonate buffer and substituted into the assay in section 2.17.2.3.

2.18 Statistical analysis

All of the experiments were repeated at least three times to ensure the results were reproducible and for a given experiment, means and standard errors were calculated. Analysis of significance was by analysis of variance (ANOVA) followed by a multiple range analysis test to determine least significant difference (Zar, 1998).

2.18 Reagents and Suppliers

Acetic acid	Fischer
30% Acrylamide/ bisacrylamide	Biorad
4% acrylamide PAGE gels	Biorad
Ammonium persulphate	Sigma
Ammonium sulphate	Sigma
Bichinchoninic acid solution	Sigma
Nα-Benzoyl-DL-arginine-p-nitroanilide (BAPNA)	Bachem
Bovine nasal septa	Abbatoir
Bovine serum albumin	Sigma
Brij 35	Sigma
Bromophenol Blue	Sigma
Calcium chloride	Merck
Calcium lactate	Fischer
Charcoal, activated	Sigma
Chondroitin sulphate (porcine rib)	Sigma
4- Chloronapthol	Sigma
Coomassie brilliant blue R250	Sigma
Copper sulphate	Fischer
Cyanogen bromide activated Sepharose 4B	Pharmacia
Cysteine/HCl	Sigma
Cytokine kits	Biosource
DEAE Trisacryl	Pharmacia
Dextran Blue	Sigma
Dextran T70	Sigma
3,3' Diaminobenzidine tetrahydrochloride	Dako
5,5'- Diethylbarbituric acid (Barbitone)	Fischer
Di- sodium hydrogen phosphate	Merck
1,9 Dimethylmethylene blue	Sigma
Dimethyl sulphoxide	Sigma
Di-potassium hydrogen phosphate	Fischer
Dithiothreitol	Sigma
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco Life Technologies

Ethanol	Fischer
Ethylenediaminetetraacetic acid	Sigma
Femoral head sections	Gift from Dr A Frazer
Foetal calf serum	Gibco Life Technologies
Gelatin	Sigma
Glycerol	Sigma
Glycine	Sigma
Goat anti-rabbit IgG –HRP	Sigma
Rabbit anti-human polyclonal α_2m	Dako
Rabbit anti-human polyclonal PAPP-A	Dako
Goat anti-rabbit-IgG peroxidase	Sigma
Hitrap protein G	Pharmacia
30% Hydrogen peroxide	Sigma
Human cathepsin B	Calbiochem
Human leukocyte elastase	Bachem
Human plasmin	Calbiochem
Human trypsin	Calbiochem
Hydrochloric acid	Fischer
Imidazole	Sigma
^{125} I Interleukin1 (IL-1) β	Amersham
Interleukin 1 α	National Institute for Biological Standards and control (NIBSC)
Kodak film fixer	Sigma
Kodax film developer	Sigma
Late term pregnancy plasma	Blood Tranfusion Service
L-Glutamine	Gibco Life Technologies
Litex Agarose type HSA	Park Scientific
Magnesium chloride	Merck
Mercaptoethanol	Sigma
Methanol	Fischer
γ methacryloxypropyl-trimethoxysilane (Silane A174)	Sigma
Methyl α-D- mannoside	Sigma

MG-63 cell line	ATCC
Molecular weight markers	Merck
N,N,N'N'- Tetramethylethylenediamine	Sigma
Nigrosin	Merck
4-nitrophenyl disodium phosphate	Merck
o-phenylenediamine dihydrochloride	Sigma
Optiphase Hisafe scintillation fluid	Fischer
Papain	Sigma
PAPP-A monoclonal antibody 234-3	Gift from Dr Christiansen
PAPP-A standard- WHO IRP 78/610	Gift from Dr Christiansen
Paragon blue stain	Beckmann
Paragon SPE gels	Beckmann
Penicllin/Streptomycin	Gibco life technologies
PGE Standard	Sigma
Phosphate buffered saline	Gibco life technologies
Potassium dihydrogen phosphate	Fischer
Prestained molecular weight markers	Biorad
[5,6,8,11,12,14,15(n)-^{3}H]- Prostaglandin E_2	Amersham
Rabbit anti PGE antiserum	Sigma
Rabbit anti-human PAPP-A antiserum	Dako
Rabbit anti-human PAPP-A –HRP	Dako
Rabbit anti-human serum	Dako
Rabbit anti-human type II collagen antibody	Monosan
Rabbit anti-mouse phosphatase conjugate	Sigma
Rabbit immunoglobulin fraction	Dako
Retinoic acid (all trans)	Sigma
RPMI 1640 media	Gibco Life Technologies
Sephadex G200	Pharmacia
Sodium acetate	Merck
Sodium azide	Sigma
Sodium bicarbonate	Merck
Sodium chloride	Sigma
Sodium citrate	Fischer
Sodium dihydrogen phosphate	Merck

Sodium dodecyl sulphate	Sigma
Sodium formate	Sigma
Suberic acid bis (N-hydroxy-succinimide ester)	Sigma
Sulphuric acid	Fischer
THP 1 cell line	ECACC
Tris[hydroxymethyl]aminomethane	Sigma
Trisacryl GF 2000	Pharmacia
^{125} I Transforming growth factor (TGF) β	Amersham
Trypan Blue	Gibco Life Technologies
Trypsin EDTA	Gibco Life Technologies
Tween 20	Sigma
Ultrogel AcA 34	IBF Biotechniques
Urea	Merck
Wheat germ lectin Sepharose 6MB	Pharmacia

2.19 Addresses of Suppliers

ATCC
Manassas
United States of America

Amicon Ltd
Stonehouse
Gloucester
GL10 2BJ

Amersham International PLC
Little Chalfont
Bucks
HP7 9NA

Becton Dickinson (UK) Ltd
Cowley
Oxford
OX4 3LY

Bachem
Budendorf
Switzerland

Biometra Ltd
Whatman House
Maidstone
ME16 OLS

Beckmann Coulter Ltd
High Wycombe
Buckinghamshire
HP 12 4JL

Biorad Laboratories Ltd
Bio-Rad House
Hertfordshire
HP2 7TD

Calbiochem- Novabiochem (UK) Ltd
Beeston
Nottingham
NG9 2JR

Cecil Instruments Ltd
Cambridge Road
Milton
CB4 6AZ

DAKO Ltd
High Wycombe
Buckinghamshire
HP13 5RE

ECACC
Porton Down
Salisbury

Fahrenheit Lab Supplies
Rotherham
South. Yorks
S60 1RR

Fischer Chemicals
Loughborough
Leicestershire
LE11 5RG

Gibco Life Technologies
Paisley
Scotland
PA3 4EF

Gelman Sciences Ltd
Northampton
NN4 7EZ

IBF Biotechnics
Villeneuve-La-Garenne
France

Life Sciences International
Basingstoke
Hampshire
RG 21 6YH

LKB/Wallac Instruments
Turku
Finland

Merck Ltd
Lutterworth
Leicestershire
LE17 4XN

Monosan
PO Box 540
Netherlands

NIBSC
PO Box 1193
Hertfordshire
United Kingdom

Olympus Instruments Ltd
Hondurous Street
London
EC1Y 0TX

Park Scientific Ltd
Northampton
England

Pharmacia Ltd
Milton Keynes
Buckinghamshire
MK5 8PH

Sanyo/Gallenkamp PLC
Leicester
LE3 2UZ

Sarstedt Ltd
Beuamont Leys
Leicester
LE4 1AW

Sigma Diagnostics
Poole
Dorset
BH12 4QH

Sorvall/Hereaus Ltd
Brentwood
Essex
CM 15 9TB

2.20 Equipment and Suppliers

Autolude model EV peristaltic pump	Fahrenheit lab supplies
Biometra mini gel twin G42 tank	Biometra Ltd
Cecil 1020 UV/Visible spectrophotometer	Cecil instruments Ltd
Cecil 5000 dual beam scanning spectrophotometer	Cecil instruments Ltd
Fast protein liquid chromatography system	Pharmacia Ltd
General Glassware and equipment	Farhenheit lab supplies
Hera cell incubator	Sorvall/Hereaus Ltd
Hera safe hood	Sorvall/Hereaus Ltd
Hereaus transphor tank	Sorvall/ Hereaus Ltd
Labysystems multiskan MS platereader	Life Sciences International
LKB 2117 multiphor electrophoresis tank	LKB/Wallac instruments
MSE Mistral 2l centrifuge	Sanyo/Gallenkamp PLC
1261 Multigamma counter	LKB/Wallac instruments
Olympus light microscope	Olympus instruments Ltd
Paragon Electrophoresis tank	Beckmann Coulter Ltd
1212 Rackbeta liquid scintillation counter	LKB/Wallac instruments
Sorvall RC 5B Plus centrifuge	Sorvall/Hereaus Ltd
Sorvall super T21 centrifuge	Sorvall/Hereaus Ltd
Sorvall TC 6 centrifuge	Sorvall/Hereaus Ltd
Wallac Victor ^{2} 1420 multilabel counter	LKB/ Wallac instrumemts Ltd

Chapter Three

The development of the purification protocol for PAPP-A

3.1. General Introduction

The purification of PAPP-A has been performed previously by other workers using a variety of different protocols, see table 3.1. There are a number of controversies surrounding these protocols, one being the wide use of a heparin sepharose column to which PAPP-A can bind specifically and reversibly, Sinosich et al. (1981). The development of a purification protocol that will produce the purest possible form of PAPP-A eliminating techniques that have been reported to either contaminate, or alter the structure of PAPP-A is of the utmost importance.

One of the purification protocols using the heparin sepharose column was a simple three step procedure involving gel filtration on AcA 34 matrix, followed by heparin sepharose chromatography and negative affinity chromatography (Davey et al., 1983). However the use of heparin sepharose in these purifications may alter the properties of the purified PAPP-A produced. The interaction between heparin and PAPP-A has been shown to produce an alteration in the electrophoretic mobility of PAPP-A in crossed immunoelectrophoresis studies and a significant increase (22 to 64 % $P<0.02$) in levels of PAPP-A determined by electroimmunoassays (Westergaard et al., 1983b) . Heparin may also contaminate the PAPP-A preparation, affecting its activities (Bischof et al, 1990).

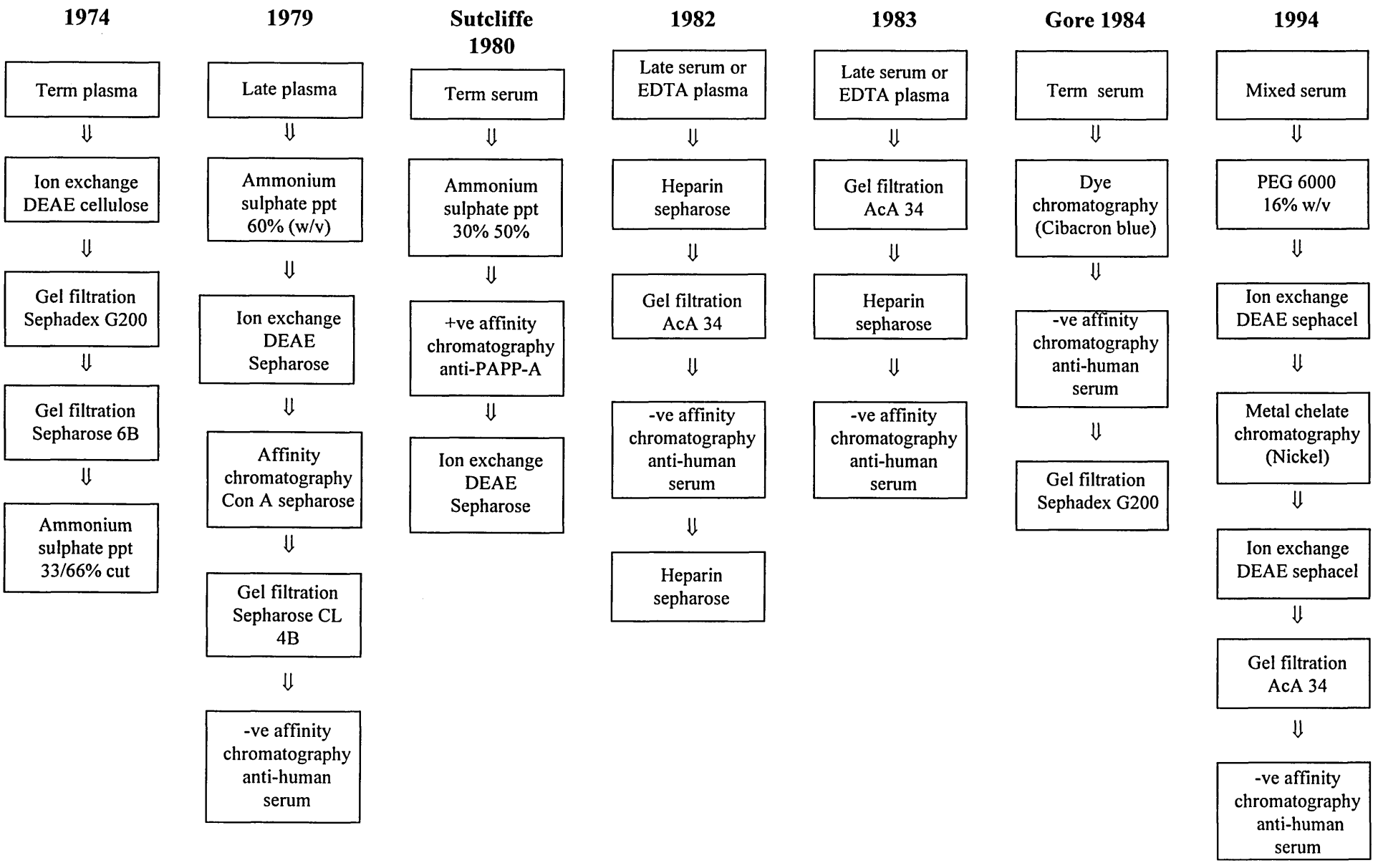
Differing functions of PAPP-A may result from the purification protocols employed. It was suggested by Gore and Sutcliffe (1984) that a purification scheme involving ammonium salts may cause irreversible conformational changes to PAPP-A as it has been reported that ammonium salts can cause these changes in α_2m preventing the binding and inhibition of proteinases (Barrett et al., 1979), this however has yet to be confirmed by other workers. They proposed a purification scheme avoiding high salt concentrations which may disrupt the conformation of the molecule (Gore and Sutcliffe, 1984).

The efficiency of the removal of α_2m, an inhibitor of the four main classes of proteinase (Starkey and Barrett, 1977), from the sample is important since α_2m contamination may have influenced the reported activities of PAPP-A. α_2m shares many similar physical and chemical properties with PAPP-A and therefore has a

tendency to copurify with it (Sutcliffe et al., 1980).

PAPP-A and α_2m have been shown not to be resolved by affinity immunoelectrophoresis with any of the 13 lectins analysed in a study by Sinosich et al. (1987) indicating that the carbohydrate composition of the glycoproteins are qualitatively similar. Most plasma proteins bind to copper chelates but the binding of PAPP-A and α_2m to zinc chelate distinguishes them from the bulk of plasma proteins. Metal chelate chromatography however, has not generally been used in purification procedures reported, as following chromatography on zinc chelate sepharose minor immunoelectrophoretic changes were detected in PAPP-A. Further changes were detected for PAPP-A and α_2m by spectrophotometric scanning. Prior to chromatography, two absorption maxima at 280nm and 214 nm could be detected, however after chromatography on the zinc chelate sepharose the 214nm peak could not be detected.

Following zinc chelate chromatography α_2m loses it ability to inhibit plasmin and chymotrypsin. However both PAPP-A and α_2m retained their inhibitory activity against granulocyte elastase (Sinosich et al., 1982). In conclusion, a chelate chromatography step would not be a good step in the purification as it may lead to changes in the conformational structure of PAPP-A which may then lead to alterations in its activity (Sinosich et al., 1983).


1974
Term plasma
Ion exchange DEAE cellulose
Gel filtration Sephadex G200
Gel filtration Sepharose 6B
Ammonium sulphate ppt 33/66% cut
1979
Late plasma
Ammonium sulphate ppt 60% (w/v)
Ion exchange DEAE Sepharose
Affinity chromatography Con A sepharose
Gel filtration Sepharose CL 4B
-ve affinity chromatography anti-human serum
Sutcliffe 1980
Term serum
Ammonium sulphate ppt 30% 50%
+ve affinity chromatography anti-PAPP-A
Ion exchange DEAE Sepharose
1982
Late serum or EDTA plasma
Heparin sepharose
Gel filtration AcA 34
-ve affinity chromatography anti-human serum
Heparin sepharose
1983
Late serum or EDTA plasma
Gel filtration AcA 34
Heparin sepharose
-ve affinity chromatography anti-human serum
Gore 1984
Term serum
Dye chromatography (Cibacron blue)
-ve affinity chromatography anti-human serum
Gel filtration Sephadex G200
1994
Mixed serum
PEG 6000 16% w/v
Ion exchange DEAE sephacel
Metal chelate chromatography (Nickel)
Ion exchange DEAE sephacel
Gel filtration AcA 34
-ve affinity chromatography anti-human serum

The different protocols used to purify PAPP-A have tended to give poor yields, impure PAPP-A, or both. Table 3.2 summarises the yield and purity achieved by some of these protocols.

The most efficient protocol for the purification is the one described by Oxvig et al. (1994), which was a six stage process and resulted in a 60% yield of pure PAPP-A. The purification did however involve a nickel chelate step which may have caused conformational changes in the structure of PAPP-A and lead to alterations in its activity (Sinosich et al., 1983). The more stages in a purification, the lower the yield of PAPP-A as it is inevitable that there will be slight losses at every stage. Oxvig et al. (1994) have produced a very good yield despite the number of stages in comparison to the five stage process described by Bischof et al. (1979) that only produced a 2.8% yield.

Table 3.2 Summary of the purification yields by other investigators

	PURIFICATION FACTOR	PERCENTAGE YIELD	PURITY
Lin et al., 1974	115	*n.s.	Impure
Bischof et al., 1979	*n.s	2.8	Pure
Sutcliffe et al., 1980	*n.s	16	Impure
[1]Sinosich et al., 1982	700	22	Pure
Oxvig et al., 1994	100	60	Pure

[1] This protocol included a heparin sepharose step

*n.s. None stated

This study describes a protocol for PAPP-A purification that has been developed, avoiding the use of heparin Sepharose chromatography and chelate chromatography. The protocol was based on purification stages used by other workers and involved many developmental steps. The stages used in the final protocol proved to be the most successful in producing the purest PAPP-A with the highest yield from late term pregnancy plasma. The stages that were unsuccessful will not be discussed in any great detail.

3.2 The purification protocol for PAPP-A

A successful purification protocol was established using a number of techniques as described in the methods section, 2.1.

3.2.1 Ammonium sulphate precipitation

This was carried out as described in section 2.1.1.2

To 130ml of late term acid citrate dextrose treated plasma in a 500ml centrifuge bottle, 21.58g of ammonium sulphate was added (30% saturation). The mixture was rotated end over end at 4^0C for 18 hours and then centrifuged at 6000g for 30 minutes. The supernatant was then removed (110ml) and this was poured into another 500ml centrifuge bottle and 25.02g of the ammonium sulphate added (60% saturation). The mixture was again rotated for 18 hours and then centrifuged as previously. The supernatant was kept and the pellet resuspended in column buffer (AcA 34) to a volume of 90ml.

The resuspension was then dialysed overnight in 3 changes 3l of column buffer. The volume obtained after dialysis was about 100ml.

3.2.2 Gel filtration chromatography

This method was carried out as described in section 2.1.2.2.

An 80ml aliquot from the ammonium sulphate precipitation step was loaded onto the AcA 34 column at a flow rate of 40ml/hour, the remaining 20ml from the previous stage was stored at -20^0C. The column was left to run until approximately 650ml had eluted from the column, 9ml fractions were then collected and monitored at 280nm,

see figure 3.1 until the absorbance reached baseline indicating all the proteins had been eluted. The column was then washed with at least three column volumes before being used again, ideally the column should be left running at a slow flow rate constantly to prevent the column getting bacterial contamination and also to ensure that any "sticky" proteins have been eliminated from the column and it is free of all proteins. Initially the first 65 fractions containing protein eluted from the column were collected and the absorbance read at 280nm. This was later optimised to 40 fractions to cover the range before and after the elution of PAPP-A.

Figure 3.1 shows a typical profile for fractions 1 - 48 obtained from the AcA 34 column when monitoring the fractions eluting from the column at 280nm

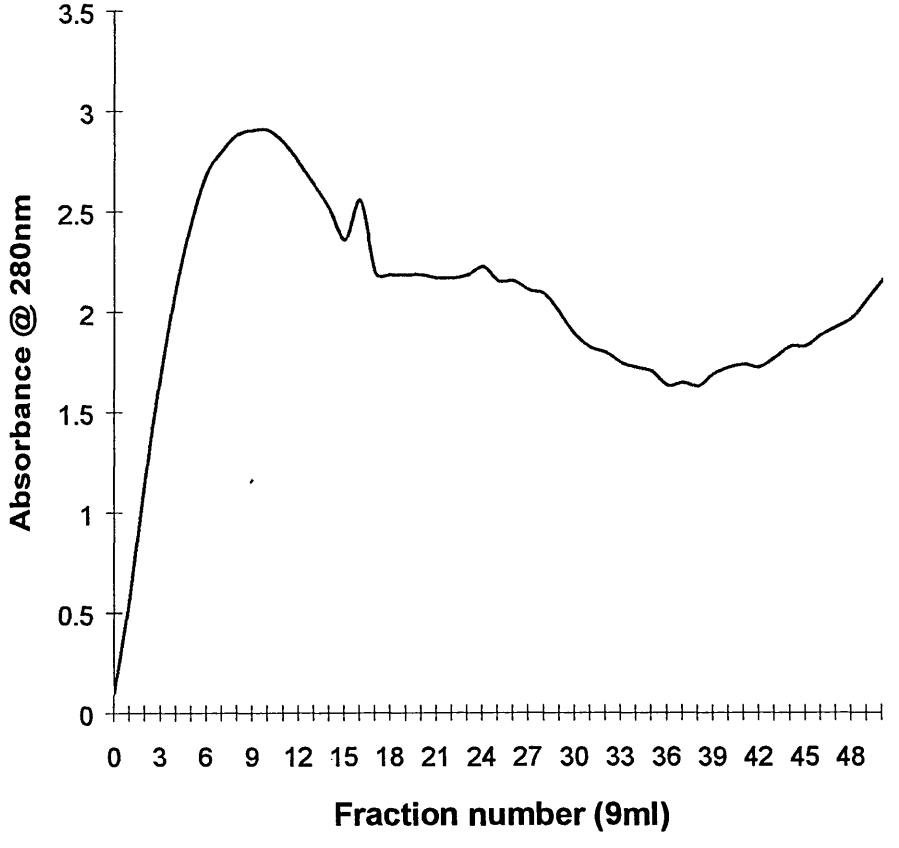


Figure 3.1 AcA 34 column profile of the first 48 fractions containing protein eluted from the column.

The profile indicated that a large amount of protein eluted from the column after fraction 2. PAPP-A eluted in the void volume (see section 2.1.2.1) along with many other proteins and was present in the first large protein peak as determined by rocket immunoelectrophoresis. Initially 65 fractions were tested for the presence of PAPP-A by rocket immunoelectrophoresis, as described in section 2.2.2. Later the testing was restricted to the first 40 fractions. PAPP-A was usually present in fractions 6-28, the highest concentration of PAPP-A being present in fraction 12 indicated by the highest rocket. Figure 3.2 show fractions 1-13 run on rocket immunoelectrophoresis.

Quantitation of PAPP-A using the height of the rockets was initially investigated by assigning pooled term plasma an arbitrary unit value. This proved unreliable as other components present in the plasma, in particular the haptoglobin and lipoproteins masked the rockets and produced a streaking effect, as seen in lane 1 (Bueler and Bersinger, 1989).

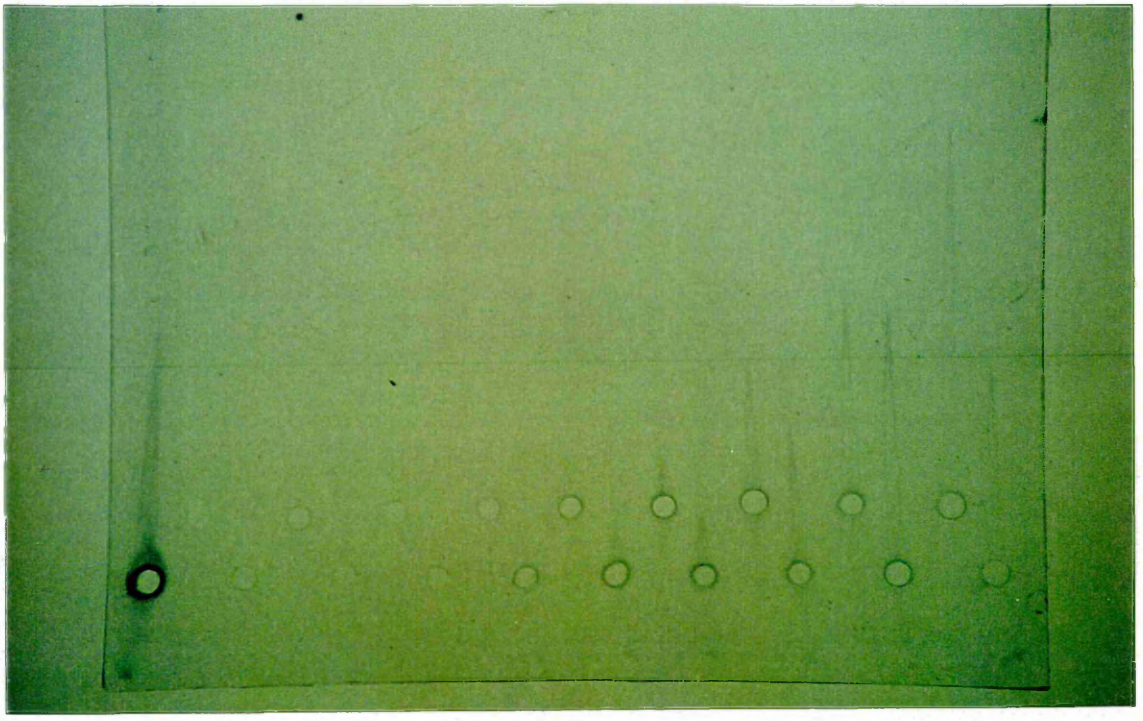


Figure 3.2 One of the plates from the rocket immunoelectrophoresis. Well 1 contains late pregnancy plasma and wells 7 -19 contained fractions 1-13.

More recently the development of the ELISA for PAPP-A has proven a much more reliable and simpler method for screening fractions for PAPP-A, see section 2.17.3.2.

Figure 3.3 shows a typical trace obtained with the fractions monitored at 280nm for protein and in the ELISA (see section 2.17.3.2) for PAPP-A at 492nm.

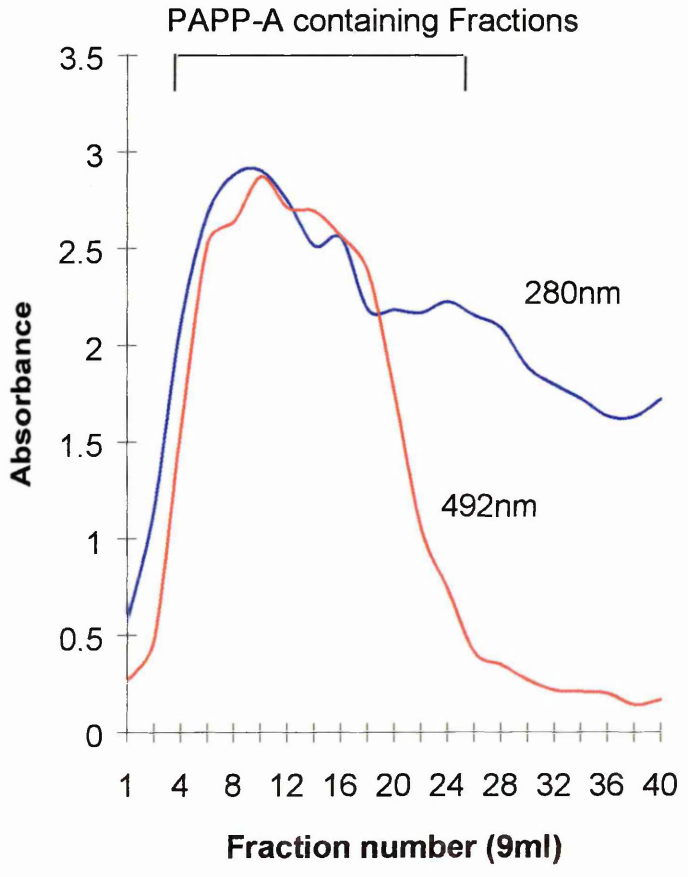


Figure 3.3 A typical profile from the AcA 34 column, absorbance at 280nm monitoring proteins and absorbance at 492nm monitoring the PAPP-A by ELISA.

The fractions containing PAPP-A, determined using the ELISA method, were found to be similar to these containing PAPP-A as measured by rocket immunoelectrophoresis, the only difference being that the ELISA was much more sensitive resulting in a broading of the profile.

The presence of lipoproteins in fractions 2-7, indicated by the appearance of a milky white precipitate in these fractions resulted in only the main peak of PAPP-A being pooled, fractions 8-26. Fractions containing very small amounts of PAPP-A were discarded.

3.2.3 Ion exchange chromatography (IEC)

The method was carried out as described in section 2.1.3.2.

The pooled fractions containing PAPP-A from the gel filtration stage, approximately 180ml, were dialysed against 3l of 25mM Tris/HCl containing 50mM NaCl pH 7.6 with three changes at 4^0C. The dialysed sample pool was then loaded onto the DEAE Trisacryl IEC column, at a flow rate of 1ml per minute and the absorbance at 280nm monitored. After 15 minutes, protein started to elute from the column and these fractions were collected, to be analysed later for the presence of PAPP-A. After the sample had been loaded onto the column it was washed with 25mM Tris/HCl containing 50mM NaCl pH 7.6 until no more proteins could be detected. The collection of the fractions was stopped and the column washed for 30 minutes. The salt concentration of the buffer was then increased to 0.5M and after about 30 minutes a protein peak was eluted and this fraction collected. The salt concentration was then increased to 1M to ensure that the column was completely stripped of any proteins still bound to it and the protein fraction collected. Figure 3.4 shows the elution profile.

The column was then put on a wash programme overnight with alternate high and low salt concentrations to clean it thoroughly. The samples collected from the high salt concentrations, 0.5M and 1M, varied in volume each time the protocol was carried out but they were always concentrated in Centripreps to a total pooled final volume of 5ml for the 0.5M and 1M salt concentrations. Fractions were collected from all the stages of the run, including run through sample load, to be tested on SDS PAGE.

There was no PAPP-A present in the sample load run through, as determined by SDS PAGE. PAPP-A was present in large amounts in the 0.5M salt elution as a very clear and distinct band could be seen at M_r 200,000 on SDS PAGE, under reducing conditions, corresponding to the PAPP-A subunit. A small amount of PAPP-A was also present in the 1M salt elution. At this stage several other bands could be detected on

SDS PAGE, under reducing conditions, one of them being α_2m. Contaminating proteins present at this stage varied from batch to batch, α_2m however was the major contaminant, see figure 3.6, lane 2.

3.2.4 Negative affinity chromatography

The method was carried out as described in section 2.1.4.2.

Non pregnancy proteins will bind to the column and PAPP-A will not. Other pregnancy proteins would not bind to the column also, but these should have been removed in the early stages of the purification.

The 0.5M salt elution and the 1M salt elution from the IEC stage were pooled and loaded onto the column at a flow rate of 0.25ml per minute to ensure maximal binding. The column was then washed in column buffer at the same flow rate until 5ml of buffer had run through, the column flow was then stopped for 18 hours and it was incubated at 4^0C to ensure that the contaminating proteins in the loaded sample had the optimal conditions for binding to the antibodies immobilised onto the Sepharose column. The column was then run again at the same flow rate and the protein peaks were collected in sample tubes on ice. When all the proteins had been eluted from the column, determined by monitoring absorbance at 280nm, see figure 3.5, and 30ml of wash buffer had been eluted without a trace of protein, the column was washed with alternate high/low pH buffers as described in section 2.1.4.2 to elute all the bound proteins. These protein samples were initially saved in the optimisation runs and tested on SDS PAGE to determine which proteins were binding to the column.

The elution profile, figure 3.5 indicated that two proteins were being eluted from the column, these two peaks are in fact both pure PAPP-A. A possible reason why there were two peaks could be that there may be slight differences in the glycosylation of the PAPP-A samples. Both peaks showed an identical band on SDS PAGE.

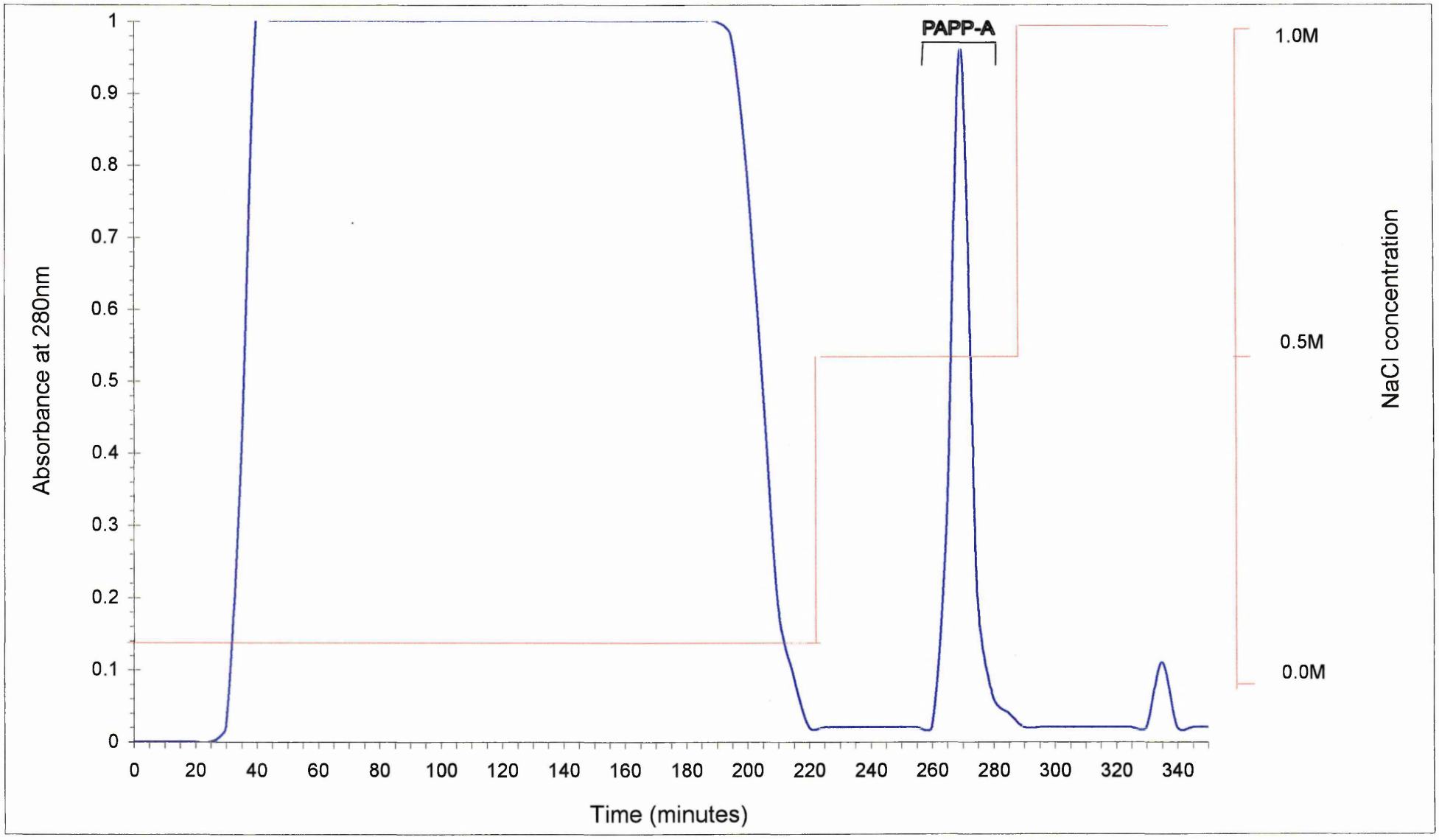


Figure 3.4

A typical profile from the ion exchange chromatography column.

The blue trace indicates elution of protein as determined by A_{280}. The red trace indicates NaCl concentration.

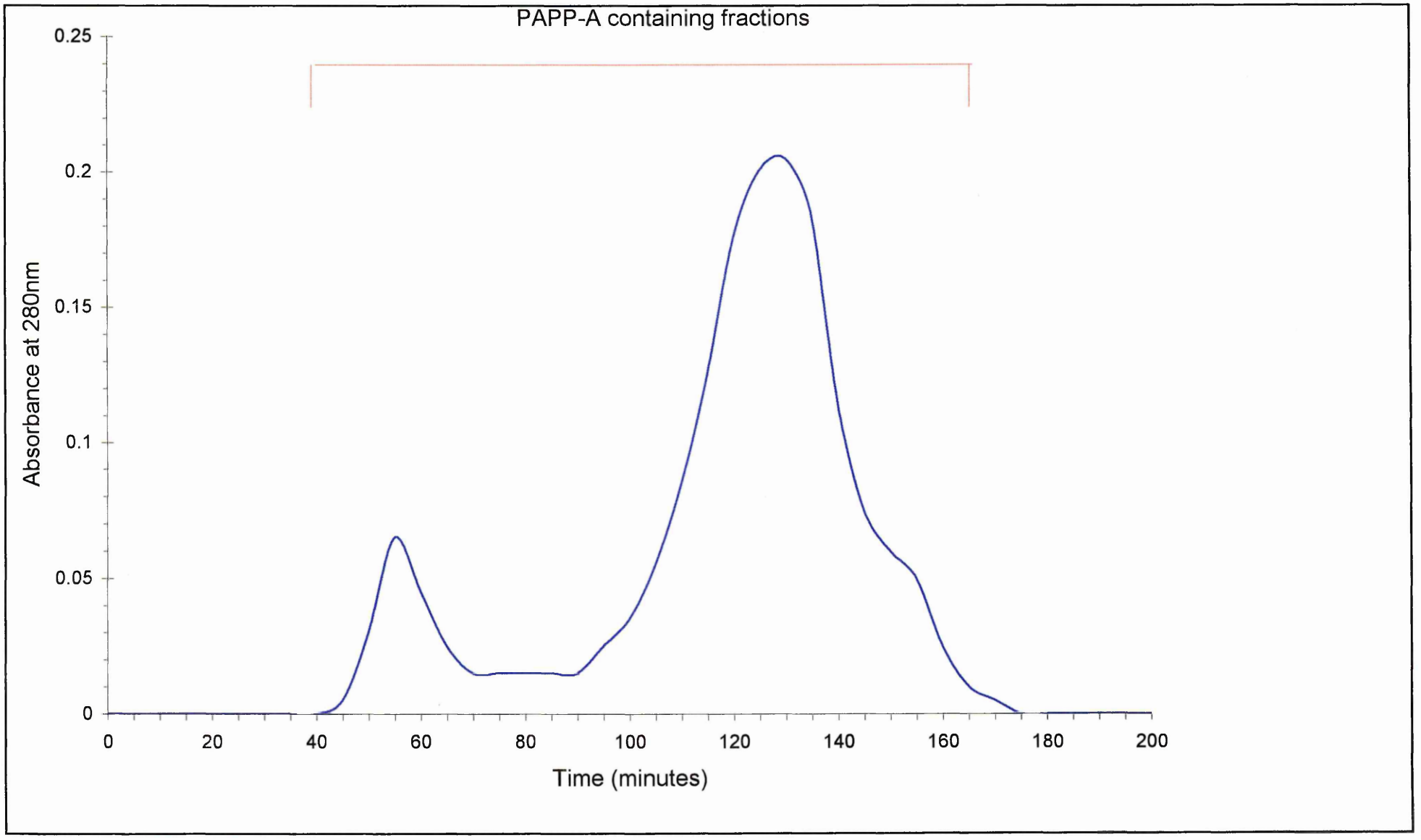


Figure 3.5
A typical profile from the negative affinity chromatography column

The PAPP-A containing samples collected, as previously, varied in volume but they were always concentrated using the Centripreps to 1ml. The pooled sample load was run on SDS PAGE to confirm purity. As expected a single band was seen at M_r 200,000, under reducing conditions, figure 3.6. This band was confirmed to be PAPP-A by western blotting as described in section 2.2.4.3, see figure 3.7.

During the optimisation stages when all the samples were tested on SDS PAGE many bands were detected in the regeneration stages but no band at M_r 200,000 corresponding to PAPP-A could be seen. Due to the nature of the column, over a period of time it lost its binding capacity for human serum proteins as the antibodies were leached from it in the wash stages or were degraded. As the column binding capacity was reduced it was found that not all of the contaminating proteins were eliminated and therefore the column had to be regenerated and the sample loaded for a second time. Reapplying the sample to the negative affinity column was not ideal because at every stage a small amount of PAPP-A was lost.

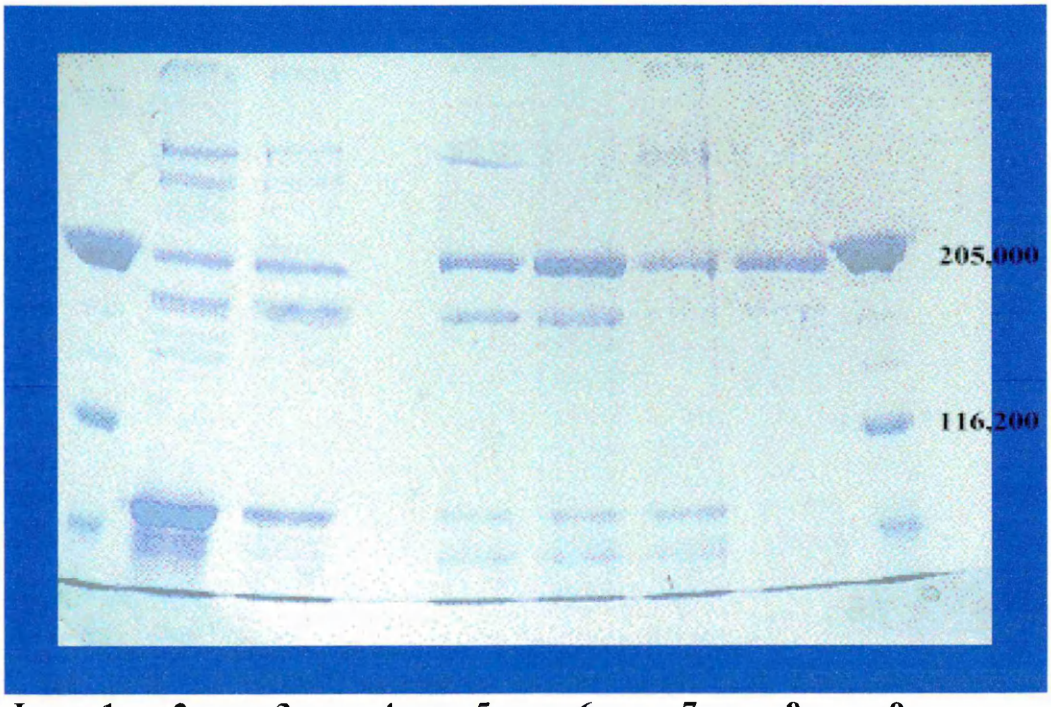
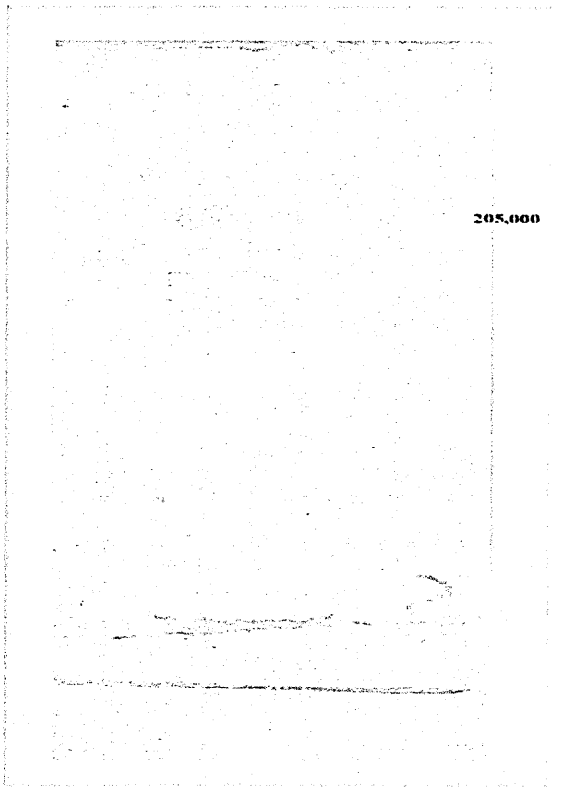


Figure 3.6 A 4% SDS PAGE gel run under reducing conditions. Lanes 1 and 9, high M_r markers, lane 2, 0.5M salt IEC, lanes 3-7 samples from various negative affinity runs and lane 8 pure PAPP-A from the negative affinity step, a single band at M_r 200,000, corresponding to the PAPP-A subunit.



Lane: 1 2 3

Figure 3.7 A western blot using the polyclonal antibody (DAKO) confirming that the single band was PAPP-A. Lanes 1 and 3 contained high M_r markers and lane 2 contained a PAPP-A sample.

The initial plasma pool used for the purification procedure was obtained from up to 100 patients and therefore slight variations in the profile of the products from this stage was inevitable.

3.2.5 The efficiency of the PAPP-A purification

At all the stages in the purification procedure protein content and PAPP-A levels were measured by the BCA method and the ELISA as described in section 2.2.3.2. and 2.17.3.2.

The purification of PAPP-A was a four stage process that yielded a pure sample of PAPP-A, see table 3.3. The yield was 48% which was higher than most yields previously obtained, table 3.1, with the exception of that produced by Oxvig et al. (1993) of 60%.

Table 3.3 PAPP-A purification stages showing PAPP-A concentration and % yield purification at each stage.

PURIFICATION STAGE	VOLUME OF SAMPLE (ML)	PAPP-A CONC^{N} (UG/ML)	FOLD ENRICHMENT %	% YIELD
Term Plasma	130	50		100
Ammonium Sulphate suspension	100 **	62.5	1.25	96
Ion Exchange (50mM salt)	5	800	16	76.8
Negative Affinity	500ul	5,000	100	48

** Only used 80ml from this stage

The values obtained in the table were calculated using the ELISA method described in section 2.17.3.2.

3.2.6 Determination of the N terminal sequence of PAPP-A

The amino acid sequence of PAPP-A has been determined from partial protein sequencing and from sequencing of cloned cDNA. It has a single N-terminal sequence, Glu-Ala-Arg-Gly-Ala-Thr-Glu-Glu-Pro-Ser-Pro (Kristensen et al., 1994). The PAPP-A obtained from the purification procedure described above was analysed by Dr Arthur Moir at the University of Sheffield using the Applied Biosystems 467A protein sequencer. N-terminal sequence shown was found to be consistent with that published previously, see table 3.4 (Kristensen et al., 1994), giving further confirmation that the protein purified was PAPP-A.

However there was a slight problem with salt contamination in the sample causing difficulties in identifying the first two residues though residue two appeared to be Ala (Moir; personal communication).

Table 3.4 A Table of the sequence results obtained from Dr Arthur Moir.

The University of Sheffield

KREBS INSTITUTE
FOR BIOMOLECULAR RESEARCH

Department of Molecular Biology and Biotechnology
University of Sheffield
PO Box 594
Sheffield S10 2UH, U.K.
Tel: (0114) 276 8555
Fax: (0114) 272 8697

PROTEIN SEQUENCE ANALYSIS

RUN No 1201 SAMPLE GAYLE 10µl

RESIDUE	AMINO ACID	PICOMOLES	RESIDUE	AMINO ACID	PICOMOLES
1	X		26		
2	X		27		
3	R	3.08	28		
4	G	2.61	29		
5	A	1.32	30		
6	T	0.70	31		
7	E	1.50	32		
8	E	1.43	33		
9	P	1.49	34		
10	S	0.47	35		
11			36		

3.2.7 Unsuitable Techniques

The techniques that were not suitable in the purification procedure were the gel filtration using the Trisacryl GF 2000, the anti-PAPP-A affinity column, α_2m affinity column and wheat germ lectin column. The anti-PAPP-A and anti α_2m columns were very efficient at removing the respective antigens from the samples but these could not be eluted from the columns, even using harsh conditions such as 0.1 M glycine pH 2.9. The PAPP-A would become inactive if harsh elution conditions were used and

therefore would be useless for activity experiments. The wheat germ lectin column bound α_2m and PAPP-A with the same affinity and therefore they coeluted in the same fractions. The Trisacryl GF 2000 matrix was unsuitable as the fractions collected were very dilute and PAPP-A could not be detected using rocket immunoelectrophoresis.

3.3 Discussion

A purification procedure for PAPP-A has been optimised using a four stage process, ammonium sulphate precipitation, gel filtration chromatography with AcA 34, ion exchange chromatography with DEAE Trisacryl and Negative Affinity chromatography with anti-total human serum. The protocol produced a 48% yield and the purity was confirmed by SDS PAGE and western blotting.

The purified PAPP-A did not produce a second band on SDS PAGE under reducing conditions, as reported by Oxvig et al. (1993). They observed a very large distinct band at M_r 215,000 corresponding to PAPP-A and a "smear" corresponding to a species of approximately M_r 50-90,000. This species was not detected under non reducing conditions and later investigations by western blot analysis led to the species being identified as proMBP. The band observed by Oxvig et al. (1993) was very faint indicating that proMBP may be present in smaller, undetectable (on SDS PAGE), quantities in the sample obtained in the purification process described in this thesis or that proMBP is not present in the sample. Zorin et al. (1995) purified PAPP-A using a three step process involving L-lysine Sepharose chromatography, heparin Sepharose chromatography and negative immunoaffinity chromatography and produced a pure sample of PAPP-A with an approximate yield of 32 %. The PAPP-A, when analysed using SDS PAGE, produced a single band under reducing conditions with an M_r 205,000. A second band corresponding to proMBP was not detected, as in the present study.

There appears to be a discrepancy as to whether proMBP is associated with purified PAPP-A or not. Oxvig et al. (1994) suggested it was an integral part of PAPP-A, however it does not appear to be present in the PAPP-A purified as part of this study. PAPP-A may exist in different forms and different purification protocols may favour different species of PAPP-A. Currently there are no commercially available antibodies

to proMBP which could be used to confirm or refute its presence in PAPP-A.

Despite the apparent lack of proMBP in the purified PAPP-A, its shows similar activities to that purified by Oxvig et al. (1994) as discussed later in the thesis.

Chapter Four

The effect of PAPP-A on the production and action of cytokines and PGE_2.

4.1 General Introduction

It was of relevanceto determine whether PAPP-A had any involvement in the remission of RA in terms of immunomodulatory and inflammomodulatory effects. The effects of PAPP-A on the production and action of cytokines, e.g. IL-1, TNFα and IL-6 and PGE_2, all of which are known to be involved in RA (Firestein and Zvaifler, 1992), may provide evidence as to whether or not PAPP-A may be involved in the remission of RA commonly observed in pregnant women (Hench, 1938).

Initially its effects on cytokines and PGE_2 production were investigated using two different cell lines, a human osteoblastic cell line (MG 63) and a human monocytic cell line (THP 1). Osteoblasts are present in the joint and monocytic cells infiltrate the joint during inflammation. The binding of PAPP-A to cytokines was also investigated to determine whether PAPP-A could act in the same manner as α_2m by binding to the cytokines and inhibiting their actions.

4.2 The effect of PAPP-A on IL-1α stimulated PGE_2 and cytokine production by the MG-63 human osteosarcoma cell line

4.2.1 Introduction

The MG-63 osteoblastic cell line is known to produce PGE_2 and IL-6 when stimulated with IL-1α (Rosenquist et al.,1996; Koka et al.,1998) and was therefore chosen as an example of connective tissue cells to investigate the effects of PAPP-A on these parameters.

4.2.2 Method

The MG-63 cells were plated out into 24 well plates at 6 $x10^4$ cells/well (500ul) and left for 3 days at 37^0C in a humidified atmosphere of 95% air/5% CO_2 to reach confluence.

On day three the media were removed from all of the wells, the cells washed three times in PBS and 500ul of test substances added to quadruplicate wells as follows :

1. Control (DMEM)
2. IL-1α 10U/ml
3. IL-1α 10U/ml + PAPP-A 0.5ug/ml (0.63nM)
4. IL-1α 10U/ml + PAPP-A 5ug/ml (6.25nM)
5. IL-1α 10U/ml + PAPP-A 25ug/ml (31.25nM)
6. IL-1α 10U/ml + PAPP-A 50ug/ml (62.50nM)
7. PAPP-A 0.5ug/ml (0.63nM)
8. PAPP-A 5ug/ml (6.25nM)
9. PAPP-A 25ug/ml (31.25nM)
10. PAPP-A 50ug/ml (62.50nM)

The plates were then incubated for 24 hours at 37^0C in a humidified atmosphere of 95% air/5% CO_2. The supernatants were removed and stored at -70^0C until they were assayed for PGE_2 and cytokine production as described in sections 2.7.3 and 2.8 respectively.

4.2.3 Results

IL-1α stimulated the production of PGE_2, as expected, from previous reports, whereas PAPP-A alone, at all the concentrations tested had no significant effect on the production of PGE_2 by the MG-63 cell line. IL-1α with PAPP-A had no extra stimulatory effects other than those seen with IL-1α alone, figure 4.1. IL-1α stimulated the production of IL-6 as expected, but PAPP-A alone had no effect on the production of IL-6. PAPP-A with IL-1α produced a slight increase in IL-6, compared to IL-1α alone, however this did not reach significance, figure 4.2.

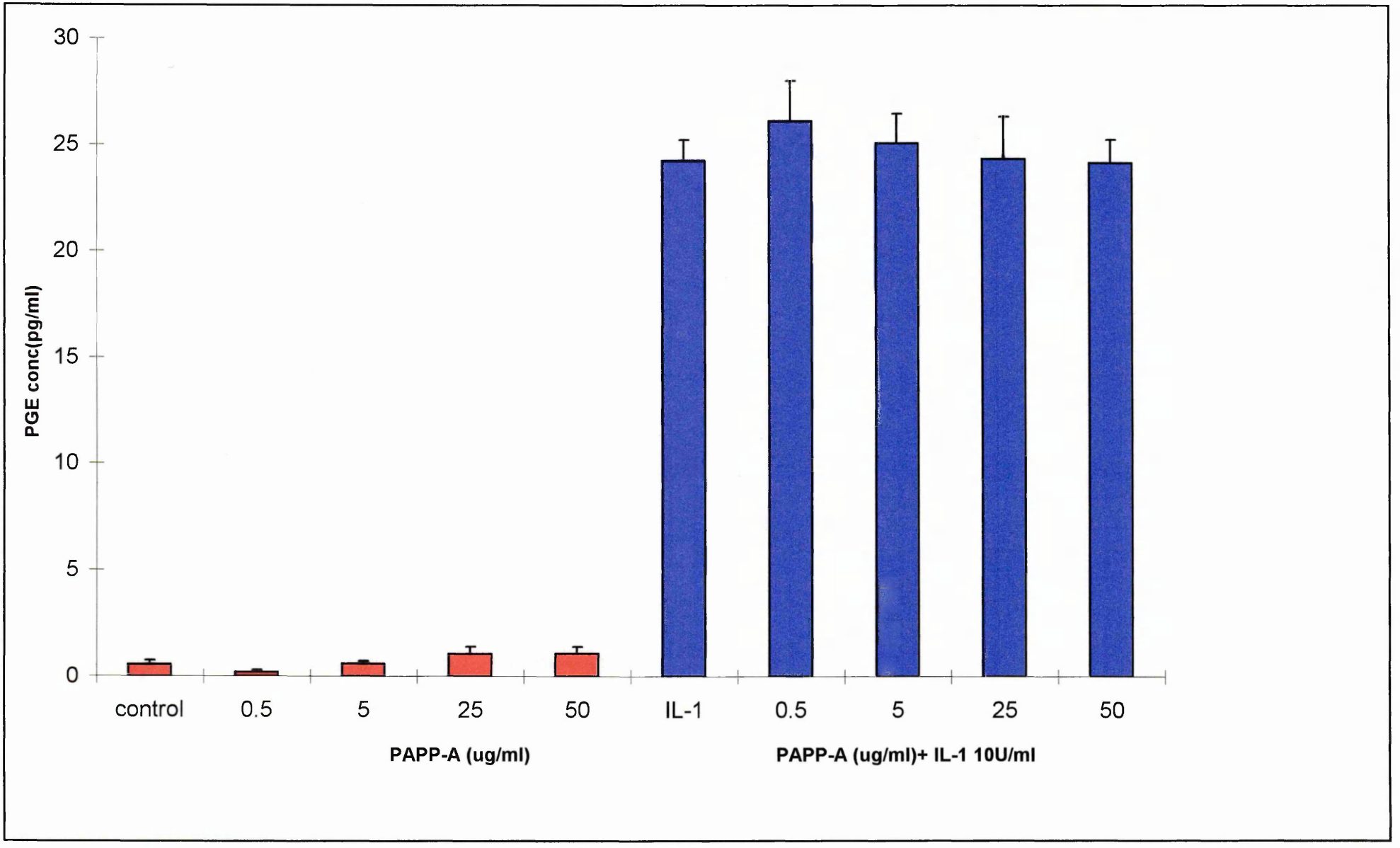


Figure 4.1. The effect of PAPP-A on IL-1 stimulated PGE production by MG-63 cells.
The data is expressed as the mean concentration per 6 wells +/- the standard error.

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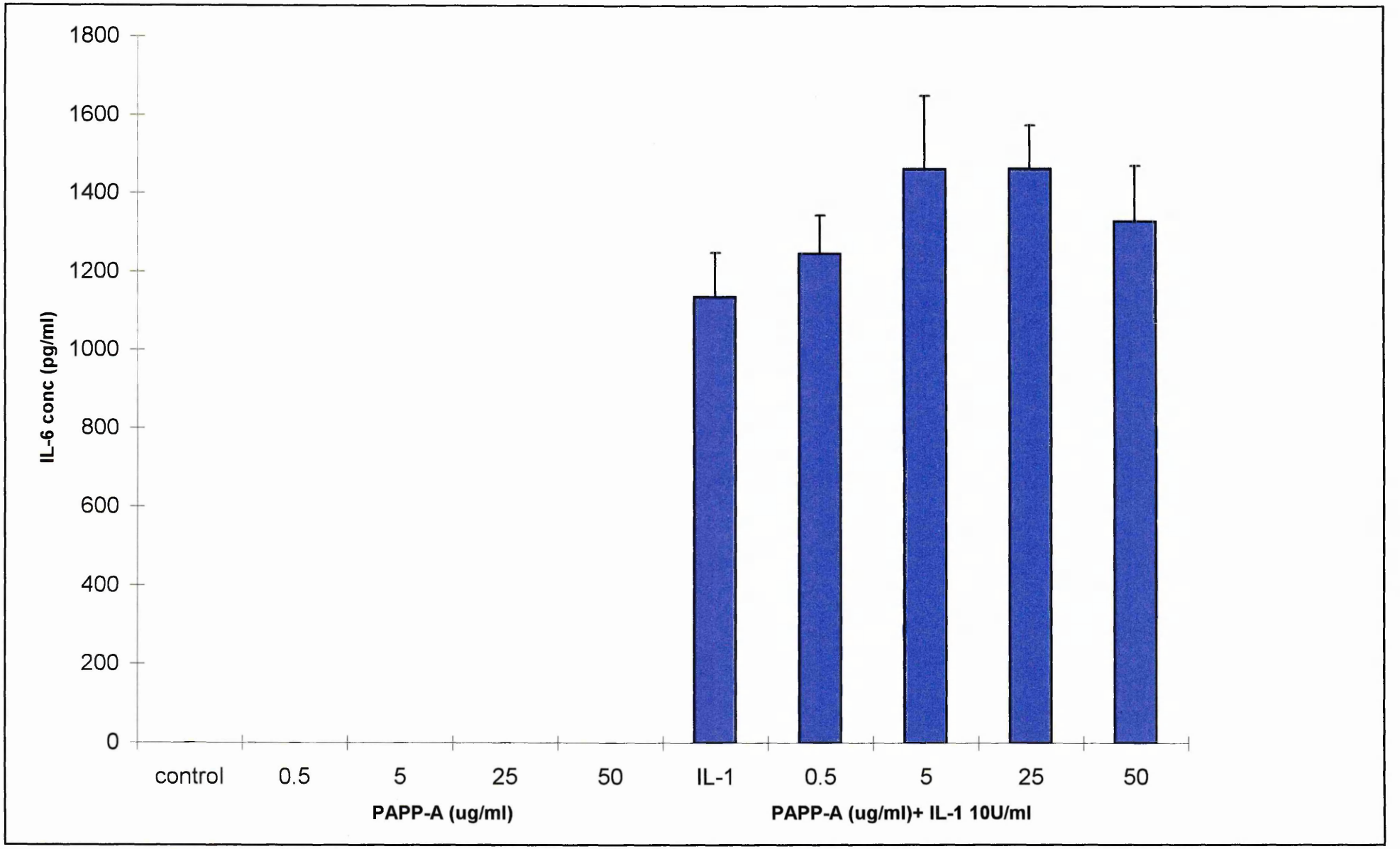


Figure 4.2. The effect of PAPP-A on IL-1 stimulated IL-6 production by MG-63 cells.
The data is expressed as the mean concentration per 4 wells +/- the standard error.

4.2.4 Discussion

It would be expected that PAPP-A would either enhance or inhibit the production of the PGE_2 and or IL-6 because of its similarities with α_2m which is known to modulate cytokine mediated events (Borth, 1989; LaMarre et al., 1991; Crookston et al., 1994).

PGE_2 is a potent inflammatory agent and IL-6 stimulates B cell proliferation and differentiation. Both are present in the synovial fluid in RA (Taylor et al., 1986; Castor et al., 1997) therefore both may have a potential involvement in the pathogenesis of RA. The involvement of PAPP-A, if any, in the remission of the symptoms of RA during pregnancy may be due to its inhibitory activities on one or more of the factors involved in RA. The results however suggested that PAPP-A had no effect on PGE_2 and IL-6 production, suggesting that it is neither anti-inflammatory nor immunosuppressive in this context.

4.3 The effect of PAPP-A on LPS stimulated PGE_2 and cytokine production by the THP 1 human monocytic cell line.

4.3.1 Introduction

The THP 1 monocytic cell line can be differentiated on addition of agents such as dimethyl sulfoxide (DMSO) and phorbol 12-myristate 13-acetate (PMA), into macrophage-like cells which mimic native monocyte-derived macrophages (Auwerx, 1991). It therefore provides a valuable model for studying a wide variety of aspects of macrophage biology, ranging from the role of the macrophage in host defence and as a secretory cell, to involvement in the pathogenesis of atherosclerosis (Auwerx, 1991).

On addition of lipopolysaccharide (LPS) or any other phorbol ester the cell line will stop proliferating and differentiate into macrophage like cells which will then form secretory products. The most extensively documented of these is the secretion of IL-1 (Krakauer, 1985; Matsushima et al., 1986; Fenton et al., 1987, Kovacs et al., 1987; Fenton et al., 1988, Schmidt and Abdulla, 1988, Turner et al., 1988; Kostura et al., 1989, Molina et al., 1989). Stimulated THP 1 cells have also been reported to produce cathepsin B (Li et al., 1997).

Similarly 1,25 -dihydroxyvitamin D3 will promote their differentiation to mature macrophages, increasing surface expression of CD14, the LPS receptor, enhancing their responsiveness to LPS and dramatically increasing the secretion of TNFα , eicosanoids and other proinflammatory mediators (Mazzucco and Warr, 1996).
It was suggested (Tobias et al., 1993) that the receptor for LPS on monocytic cells is likely to be a multimeric receptor consisting of CD14 and as yet unidentified proteins. Studies are presently in progress with the THP 1 cells to identify additional membrane proteins that act together with CD14. Previous studies have documented LPS (1-10ug/ml) induced synthesis of cytokines including IL-1, TNFα and IL-8 by THP 1 cells (Tobias et al., 1989, 1986).

In this study the effect of PAPP-A on LPS stimulated and unstimulated THP 1 cells was studied by investigating its effects on the production of cytokines, IL-1β, IL-6 and TNFα and on PGE_2 production.

4.3.2 Method

THP 1 cells were plated out at 0.6×10^6 cells/ml (900ul) into 24 well plates and 100ul of interferon gamma (IFNγ) was added to a final concentration of 100U/ml. IFNγ was added to the cells, as it has been reported that culturing macrophages in the presence of LPS (1ug/ml) and IFNγ (100U/ml), results in an increased production of PGE_2 as opposed to incubating them with LPS alone (Hoffmann et al., 1988). The plates were then incubated for 3 hours at 37^0C in a humidified atmosphere of 95% air/5% CO_2.

Test substances were then prepared and 10ul added to give final concentrations as follows:

1. Control (RPMI)
2. LPS 5ug/ml
3. LPS 5ug/ml + PAPP-A 1ug/ml (1.25nM)
4. LPS 5ug/ml + PAPP-A 5ug/ml (6.25nM)
5. LPS 5ug/ml + PAPP-A 10ug/ml (12.5nM)
6. LPS 5ug/ml + PAPP-A 50ug/ml (62.5nM)
7. PAPP-A 1ug/ml (1.25nM)
8. PAPP-A 5ug/ml (6.25nM)
9. PAPP-A 10ug/ml (12.5nM)
10. PAPP-A 50ug/ml (62.5nM)
11. Heat treated LPS 5ug/ml
12. Heat treated PAPP-A 50ug/ml (62.5nM)

Heat treated LPS and PAPP-A were tested on the cells to eliminate the possibility that any effects of PAPP-A observed were due to bacterial contamination. Heat treatment consisted of heating samples at 90^0C for 30 minutes. Treatments were set up in quadruplicate and plates were then incubated at 37^0C in a humidified atmosphere of 95% air/5% CO_2 for 24 hours. The contents of the wells were then pipetted into Eppendorf tubes and spun at 6000g in the Sorvall super T21 centrifuge for 15 minutes. Cell supernatants were removed and stored at -70^0C prior to being assayed for PGE_2, IL-1β, IL-6 and TNFα as described in section 2.7.3. and 2.8 respectively.

4.3.3 Results

LPS, as expected, stimulated the production of PGE_2. PAPP-A alone significantly stimulated the production of PGE_2 at concentrations of 5.0ug/ml -50ug/ml, but was not as effective as LPS (5ug/ml) alone. PAPP-A, when incubated with LPS, produced a greater than additive effect on the production of PGE_2, see figure 4.3.

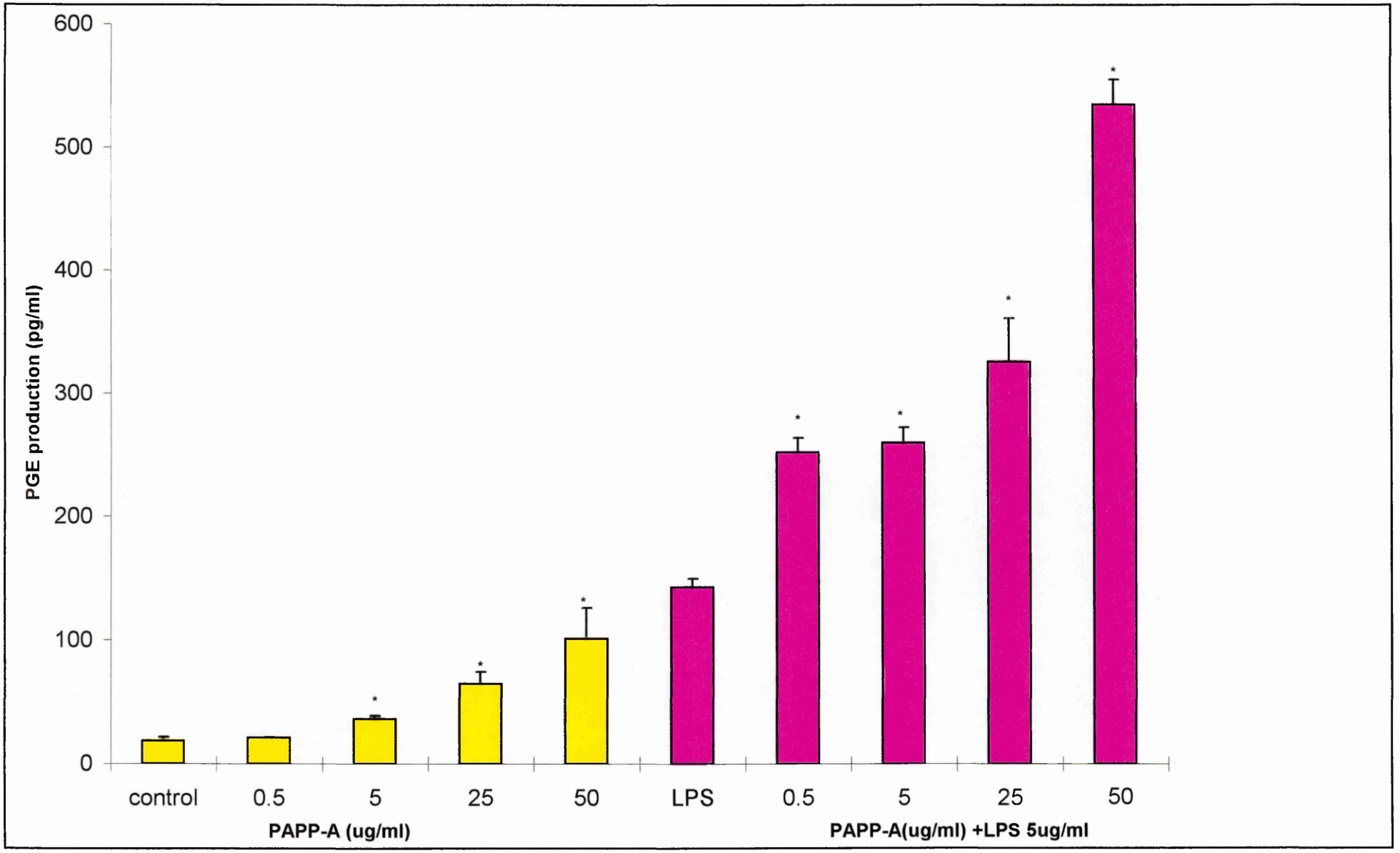


Figure 4.3. The effect of PAPP-A and LPS on PGE production by the THP 1 cell line. The data is expressed as the mean concentration per 6 wells +/- the standard error. Significant differences were determined by ANOVA, * $p<0.05$.

LPS, as expected stimulated the production of IL-1β, IL-6 and TNFα. The effect of PAPP-A on cytokine production was slightly different in these cells compared to the MG-63 cells. PAPP-A alone also stimulated the production of all of these cytokines at concentrations between 5 -50 ug/ml (6.25-62.5nM), having a much greater effect at 50ug/ml (62.5nM) than LPS. PAPP-A, incubated with LPS, would perhaps be expected therefore to have at least an additive effect. This was not the case, IL-1β production did not increase significantly when LPS and PAPP-A between 1-5ug/ml (1.25-6.25nM) were incubated together with the cells compared with the levels produced with LPS alone. Only at PAPP-A concentrations, 10 -50 ug/ml (12.5-62.5nM) was a significant increase in IL-1β seen compared with that with LPS alone, even then it did not reach the level of IL-1β produced with PAPP-A alone, figure 4.4.

The IL-6 production did not significantly increase when PAPP-A was incubated with LPS even at the highest concentration, 50ug/ml (62.5nM) figure 4.5, and levels are approximately half of those obtained when PAPP-A alone was added to the cells.

PAPP-A alone stimulated TNFα production at levels between 1- 50 ug/ml (1.25-62.5nM). PAPP-A incubated with LPS showed an increase in the production of TNFα at 50ug/ml (62.5nM) but this increase was not as great as with PAPP-A alone at 50 ug/ml (62.5nM), figure 4.6. TNFα production showed a similar pattern to that of IL-1β which was not surprising as they have been reported to regulate each other (Ridderstad et al., 1991) and TNFα shares many biological activities with IL-1 (Sherry et al., 1988).

The heat inactivated LPS stimulated the production of IL-1β to give levels of 2,795 +/- 697 pg/ml which were similar to the non heat treated LPS (5ug/ml). The levels obtained for the IL-6 and TNFα also gave similar levels to those obtained for the non heat treated LPS. The heat inactivated PAPP-A at 50ug/ml (62.5nM) did not stimulate the production of IL-1β, levels being undetectable by the ELISA. Similar results were seen for the other cytokines.

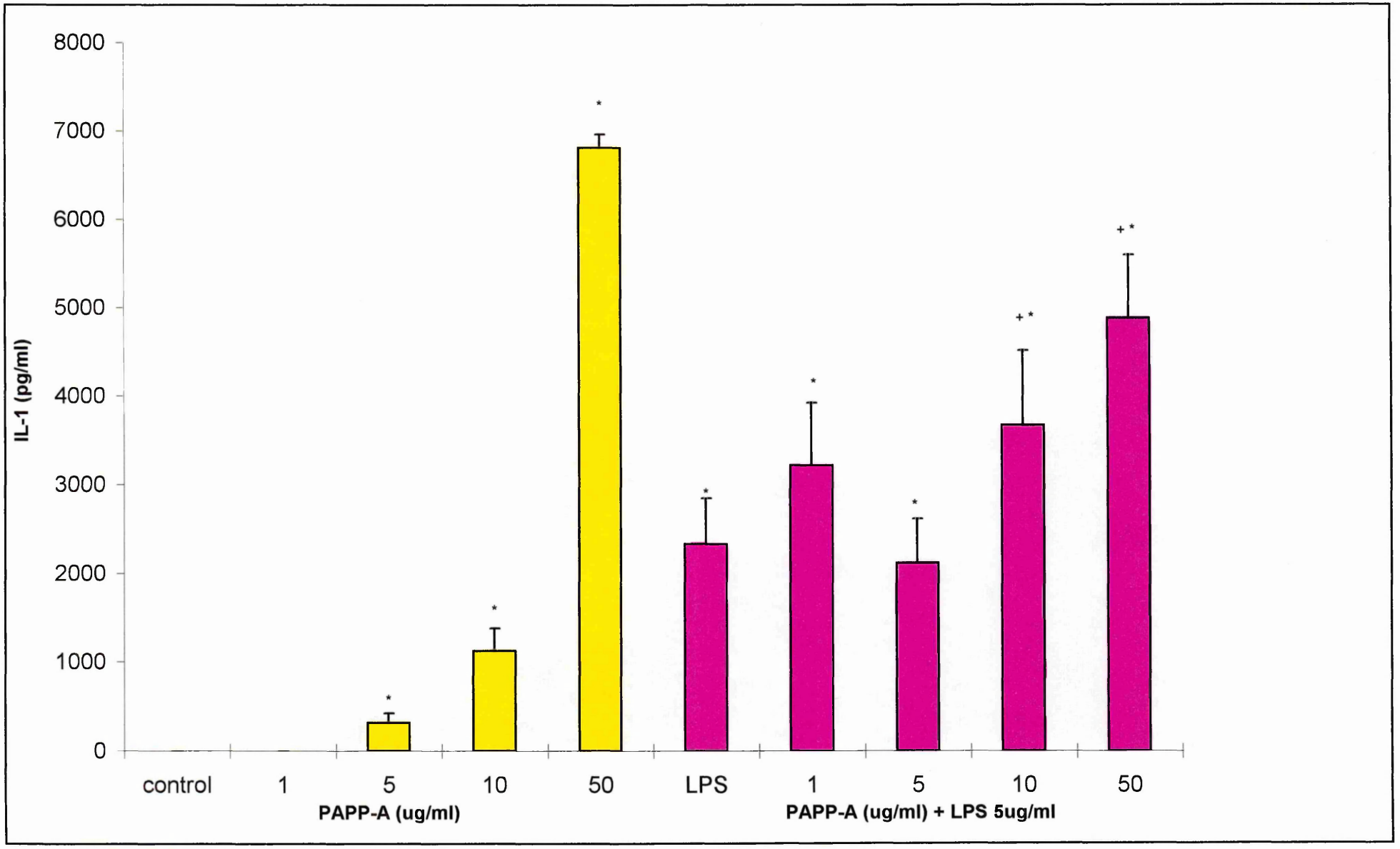


Figure 4.4: The effect of PAPP-A and LPS on IL-1 production on THP 1 cells. Data expressed as the mean conc. per 6 wells +/- the SE. Significant differences determined by ANOVA, * $p < 0.05$, compared with control; +$p<0.05$, compared with LPS stimulated.

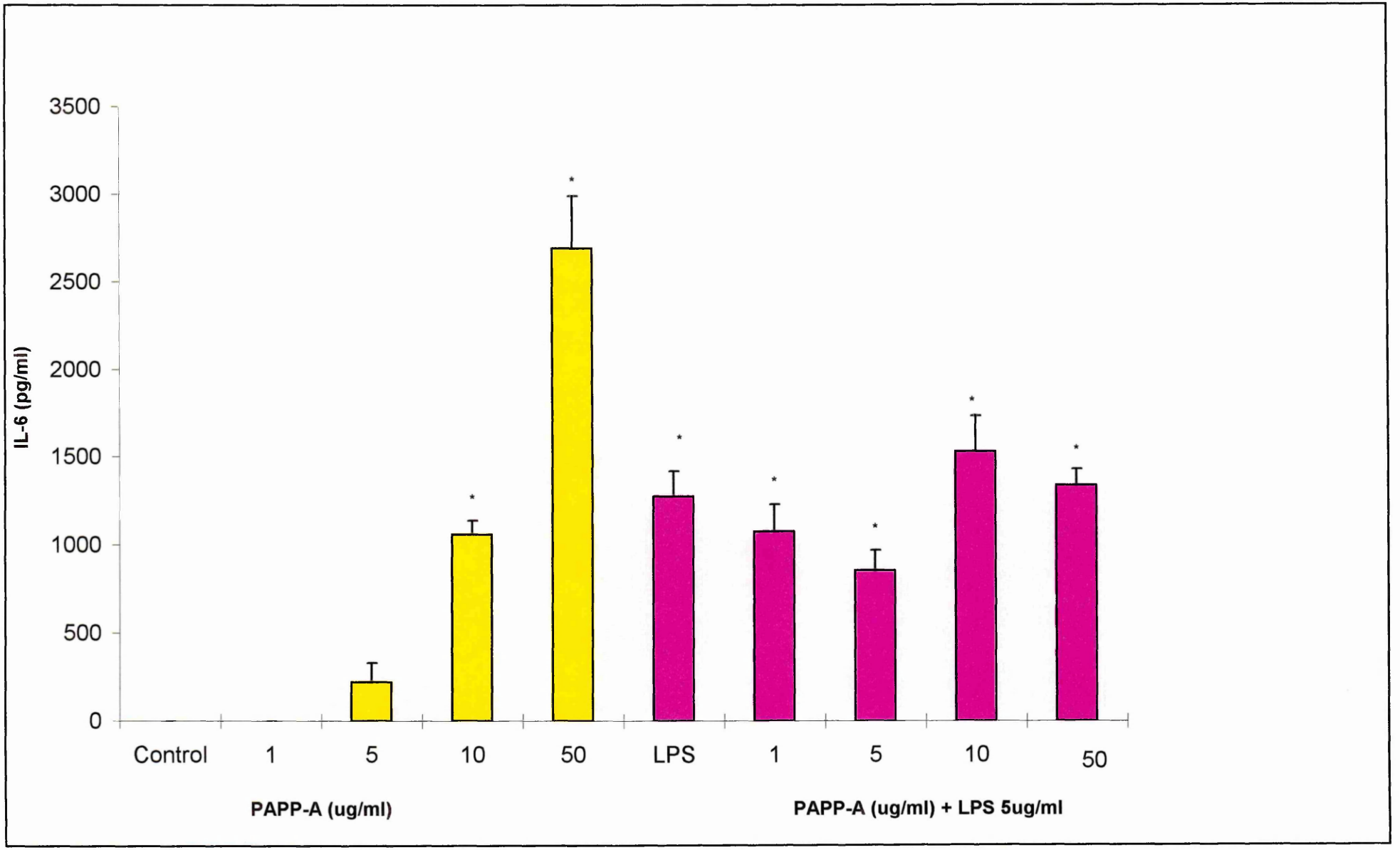


Figure 4.5. The effect of PAPP-A and LPS on IL-6 production by THP 1 cells. Data expressed as the mean conc. per 6 wells+/- the SE. Significant differences determined by ANOVA, * $p < 0.05$, compared to control.

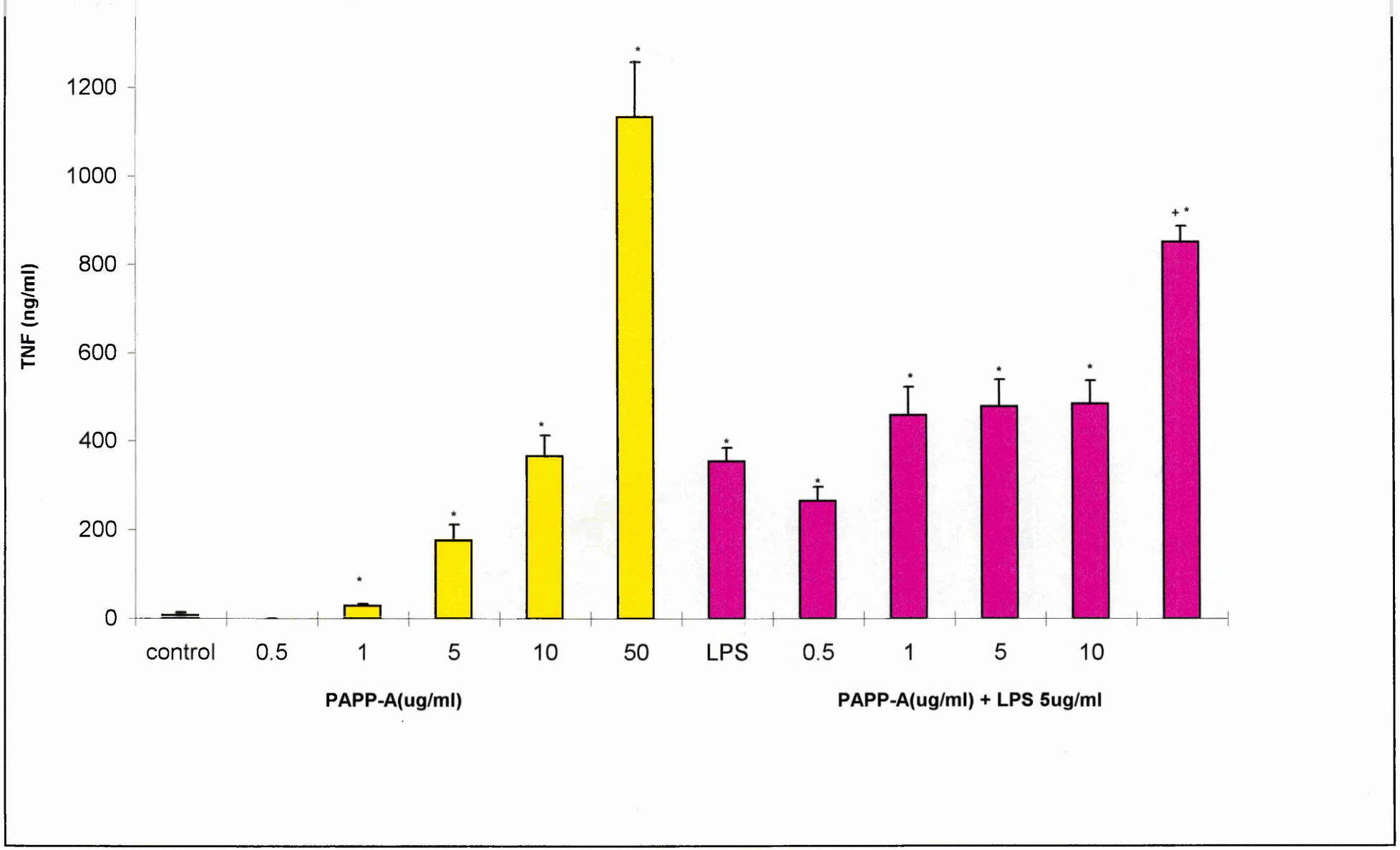


Figure 4.6. The effect of PAPP-A on LPS stimulated TNF production by THP-1 cells. Data expressed as mean conc.per 6 wells +/- the SE. Significant differences determined by ANOVA, * p, 0.05, compared with control; +p<0.05,compared with LPS stimulated.

4.3.4 Discussion

From the results obtained, PAPP-A seems to be acting as a proinflammatory agent in this cell type by stimulating the production of cytokines and PGE_2 which are known to have a role in RA. LPS, as previously documented significantly increased the production of PGE_2 (Hoffman et al., 1988) and all the cytokines tested. PAPP-A had a significant effect on their production also, its effect being greater, at higher concentrations than that of LPS alone. It has been reported that incubation of cultured macrophages with α_2m- trypsin and IFN γ results in an increase in PGE_2 production at 24 hours (Hoffman et al., 1988). It would be expected that PAPP-A plus LPS would have an additive effect on the production of the PGE_2 and cytokines, however this was not seen, PAPP-A alone being a more effective stimulator at high concentrations than in combination with LPS. The results suggest that PAPP-A and LPS may be competing for a similar receptor site(s) on the cells which could be the CD14 receptor. It is also possible that PAPP-A and LPS may bind together and the PAPP-A may become inactive. PAPP-A may be a more effective and less specific stimulator than LPS, acting via one or more receptors resulting in increased stimulation, which may explain why cells incubated with PAPP-A alone had a much higher release of cytokines than with LPS alone.

The results obtained from the heat treated samples confirmed that the effects observed were due to PAPP-A and not some bacterial contamination. There was no stimulation of cytokine production when the PAPP-A was heat treated suggesting the protein had been inactivated whereas the heat treated LPS was not, giving similar results as for the non heat treated LPS samples.

4.4 General Discussion

The results obtained were not consistent with the hypothesis that PAPP-A may have an involvement in the remission of RA. The effect of PAPP-A on IL-1β stimulated production of PGE_2 and IL-6 by the MG-63 cells indicating that it was neither proinflammatory nor immunosuppressive. The effect of PAPP-A on LPS stimulated PGE_2, IL-1, IL-6 and TNFα by the THP 1 cells indicated that PAPP-A was proinflammatory. Such effects would therefore preclude it from being involved in the remission of RA during pregnancy

A possible explanation as to why PAPP-A stimulated the THP 1 cell line to produce PGE_2 and the cytokines and not the MG-63 cell line is that PAPP-A may act by a specific cell surface receptor(s) that is not present on the MG-63 cell line. Future work would be to determine which cell lines PAPP-A has an effect on and possibly determine the nature of the common cell surface receptor. The cells could also be stripped of their surface receptors using trypsin, and PAPP-A incubated with them under the same conditions as previously and the levels of stimulation compared.

4.5 An investigation of binding of PAPP-A to IL-1β or TGF β

4.5.1 Introduction

It has been shown earlier in section 4.2 and 4.3 that PAPP-A does not inhibit the production of cytokines in two cell systems investigated. The aim of this work was to try to establish if PAPP-A has the ability to bind to cytokines and thereby inhibit their actions. It may also be that PAPP-A may bind cytokines and not inhibit their actions but return them to the systemic circulation where they can then be eliminated.

The reason for investigating the binding of cytokines to PAPP-A was that α_2m, which has similarities to PAPP-A, has been shown to be an important cytokine binding protein (Crookston et al., 1994). Several cytokines, including IL-1β, TGFβ and TNFα, bind non covalently to both native and proteinase-activated α_2m with varying affinity. Some covalent binding may also occur with slower kinetics (Crookson et al., 1994). It has been proposed that α_2m serves as a cytokine binding protein modulates cytokine-mediated events or, in the case of protease-activated α_2m, mediates cytokine clearance via the lipoprotein receptor related protein (LRP) (these complexes are targeted to cells expressing LRP) (LaMarre et al., 1991). Cytokine binding is distinct from proteinase binding as it does not involve conformational changes of α_2m (Phillip and O'Conner-McCourt, 1991, Crookston et al., 1993). No peptide bonds are cleaved and there is no disruption of the thiol esters (Crookston et al., 1993).

The major binding protein of TGF β in serum or plasma is α_2m and the complex of TGF β with α_2m has been shown to be a latent form of TGF β (Huang et al., 1988). TGFβ binds to α_2m with a much higher affinity than seen with other growth factors

and is the only growth factor that binds with equivalent affinity to native α_2m and activated α_2m (Danielpour et al., 1990, Crookston et al., 1994).

The ability of PAPP-A to bind cytokines, IL-1β and TGFβ was studied using ^{125}I labelled cytokines. Any complex formation was investigated using native PAGE and gel filtration chromatography after prior incubation of PAPP-A with ^{125}I-IL-1β or ^{125} I-TGFβ.

4.5.2 Native PAGE gels

4.5.2.1 Method

The native PAGE methods were carried out as described in section 2.15.2.3
PAPP-A - cytokine complex formation was studied using a modification of the method described by Legres et al (1994).

PAPP-A (62.5ug/ml -1000ug/ml) (0.078uM - 1.25uM) was incubated with various concentrations of the labelled cytokine, (^{125} IL-1β or ^{125}I TGFβ) (0.5nM -50nM). 50ul of the labelled cytokine was incubated with 50ul of the PAPP-A at 37^{0}C for times ranging from 5 minutes to 24 hours. 30ul of each sample was then loaded onto the Native gels under reducing and non reducing conditions and run for three hours at 150 volts. The gels were then dried on the gel dryer for about 1 hour and autoradiographed at -70^{0}C for up to 14 days. Identical native PAGE gels were also run and stained with Coomassie R250 stain.

The above procedures were also carried out on crosslinked samples as described in section 2.15.2.3 to try to stabilise any complexes that may be produced.

4.5.2.2 Results

The incubated samples when run on the native PAGE gels and subjected to autoradiography, should produce one radioactive visible band at the top of the gel if the labelled cytokine has bound to the high M_r PAPP-A. No bands on autoradiography would indicate that the low M_r labelled cytokine has run off the bottom of the gel into the running buffer and the unlabelled PAPP-A could not be detected

autoradiographically.

The autoradiographs from the native PAGE gels showed no bands were present, even after 2 weeks exposure at -70^{0}C. Only the background was enhanced. Counting an aliquot of the running buffer in the gamma counter indicated that ^{125}I TGFβ and ^{125}I IL-β had run off the bottom of the gel into the running buffer.

It was initially thought that under reducing conditions any complex formation may be broken down by the harsh treatments and therefore it was decided to run the samples under non reducing conditions. The non reduced samples also showed no bands on the autoradiographs indicating that under non reducing conditions the labelled cytokines and PAPP-A were not binding.

Crosslinking the samples and then applying them to native PAGE gels resulted in no bands being detected, indicating that not even weak binding between the PAPP-A and cytokine was occurring.

The native PAGE gels stained using Coomassie stain had very distinct PAPP-A bands present at the very top of the gel, for samples with the PAPP-A alone and PAPP-A incubated with the labelled cytokine. These results indicated that the PAPP-A had not been broken down or undergone any change on incubation with the cytokines.

Gels were also cut up into 5mm sections and then counted. However this gave inconclusive results as the gels expanded slightly on removing them from the plates and even a slight overlap from one lane to the other gave uninterpretable results.

4.5.3 Gel filtration chromatography

The method was carried out as described in section 2.15.1.2 and is a modification of the method described by O'Conner-McCourt and Wakefield, (1987).

4.5.3.1 Method

An initial experiment was carried out loading 50ul of tracer + 50ul of buffer onto the column to determine the elution profile of the tracer. Subsequently buffer was replaced

with PAPP-A. The experiments carried out initially contained either a fixed high concentration of PAPP-A (1000ug/ml) (1.25uM) and variable concentrations of the labelled cytokine (0.5nM -50nM) or with a fixed high concentration of cytokine (50nM) and variable concentrations of PAPP-A (62.5ug/ml-1000ug/ml) (0.078uM - 1.25uM). The aim of these experiments was to try to find an optimal binding ratio. Workers who have looked at the binding of α_2m to cytokines have determined that α_2m must be present in a large molar excess for binding to occur, for example Crookston et al. (1994) incubated 0.5nM of ^{125}I TGFβ with 2.8uM of α_2m . The PAPP-A containing fractions were determined using the BCA protein estimation method (section 2.2.3.2), and the fractions containing labelled cytokine by counting in a Wallac 1261 multi gamma counter.

Crosslinking of the samples was also carried out to try to stabilise any binding that was taking place and prevent any complexes formed from being dissociated when subjected to column chromatography. Crosslinking forms covalent bonds between the weakly formed non covalent bonds of the two components and allows them to remain in their complex form even after severe treatment processes such as gel filtration and PAGE.

4.5.3.2 Results

If PAPP-A binds to either of the two labelled cytokines, i.e. IL-1 or TGFβ the complex formed would be detected in the early stages of the elution profile, as PAPP-A has a high M_r. Whereas if no binding was occurring between the two, the labelled cytokine would be eluted much later in the profile due to its much smaller M_r. If two peaks were seen then this would indicate that the cytokine has bound to PAPP-A and there was also unbound cytokine, suggesting either low affinity binding or the cytokine has saturated the PAPP-A present.

4.5.3.2.1 PAPP-A - ^{125}I IL-1 β Binding

The results obtained, indicated that no binding was occurring between the PAPP-A and ^{125}I- IL-1β. When PAPP-A (1000ug/ml) (1.25um) was incubated with ^{125}I- IL-1β (0.8nm) and loaded onto the column, PAPP-A alone was eluted from the column in either fraction 4 or 5 as determined by the BCA protein estimation method. ^{125}I- IL-1β was eluted from the column in the later fractions as determined by gamma counting,

see figure 4.8. A large radioactive peak was eluted from the column at fraction 12 for all the samples loaded irrespective whether PAPP-A was present or absent suggesting that there was no complex formation between the PAPP-A and ^{125}I- IL-1β.

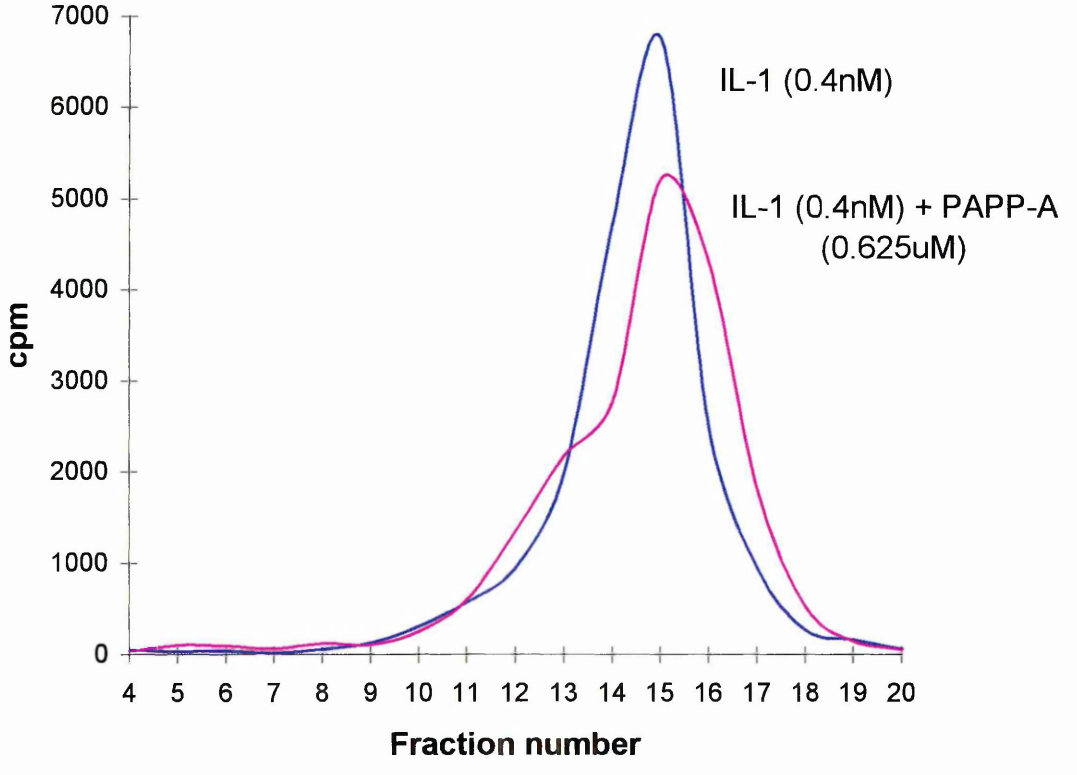


Figure 4.8 Elution of ^{125}I- IL-1β and ^{125}I- IL-1β + PAPP-A on Sephadex G200 column. Results are expressed as cpm.

Results obtained from crosslinking the PAPP-A and ^{125}I- IL-1β showed a major radioactive peak corresponding to ^{125}I- IL-1β and a smaller radioactive peak in the earlier fractions. This peak however, was also detected when the ^{125}I- IL-1β alone was run down the column, figure 4.9, indicating that it was not due to any complex formation between the PAPP-A and ^{125}I- IL-1β but most possibly due to complex formation between the ^{125}I- IL-1β molecules.

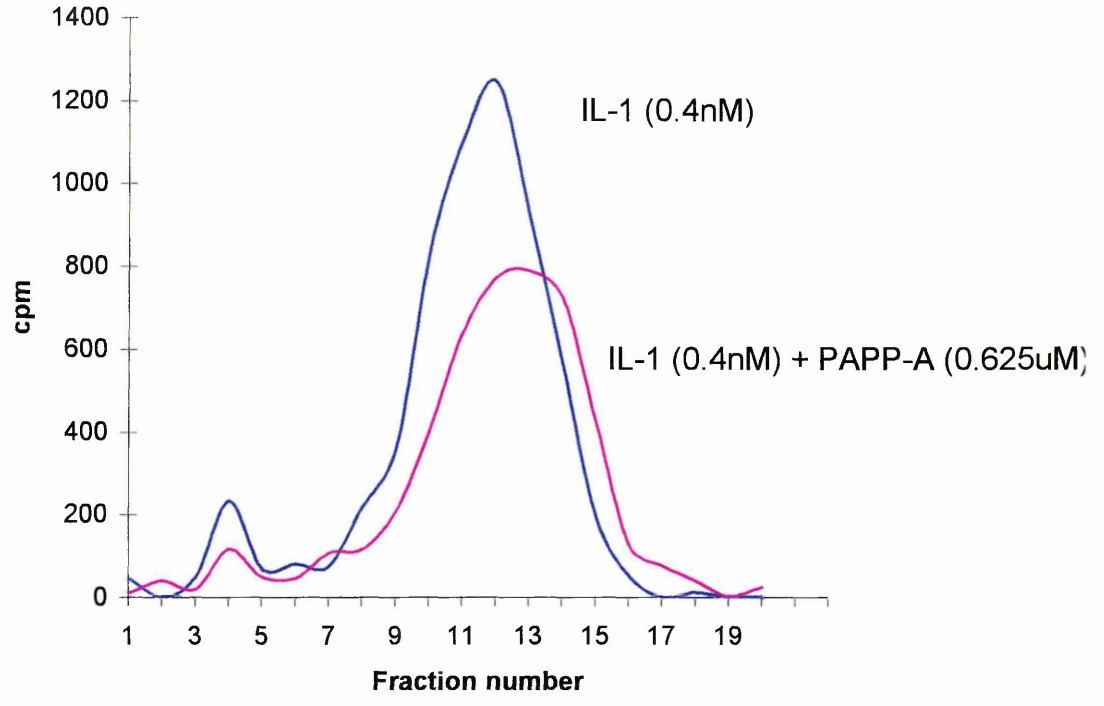


Figure 4.9 Elution of ^{125}I- IL-1β and ^{125}I- IL-1β + PAPP-A on Sephadex G200 column after being subjected to crosslinking procedures. Results are expressed as cpm.

4.5.3.2.2 PAPP-A and ^{125}I TGF β Binding

The elution profile obtained with ^{125}I TGFβ alone was somewhat different from that obtained with the ^{125}I IL-1β. There were three peaks present in the profile, figure 4.10. The results also indicated these three peaks were present when PAPP-A and ^{125}I TGFβ were incubated together, figure 4.10. There was no difference in the number of peaks obtained from ^{125}I TGFβ alone and PAPP-A and ^{125}I TGFβ. The results therefore suggest that there is no binding occurring between PAPP-A and ^{125}I TGFβ.

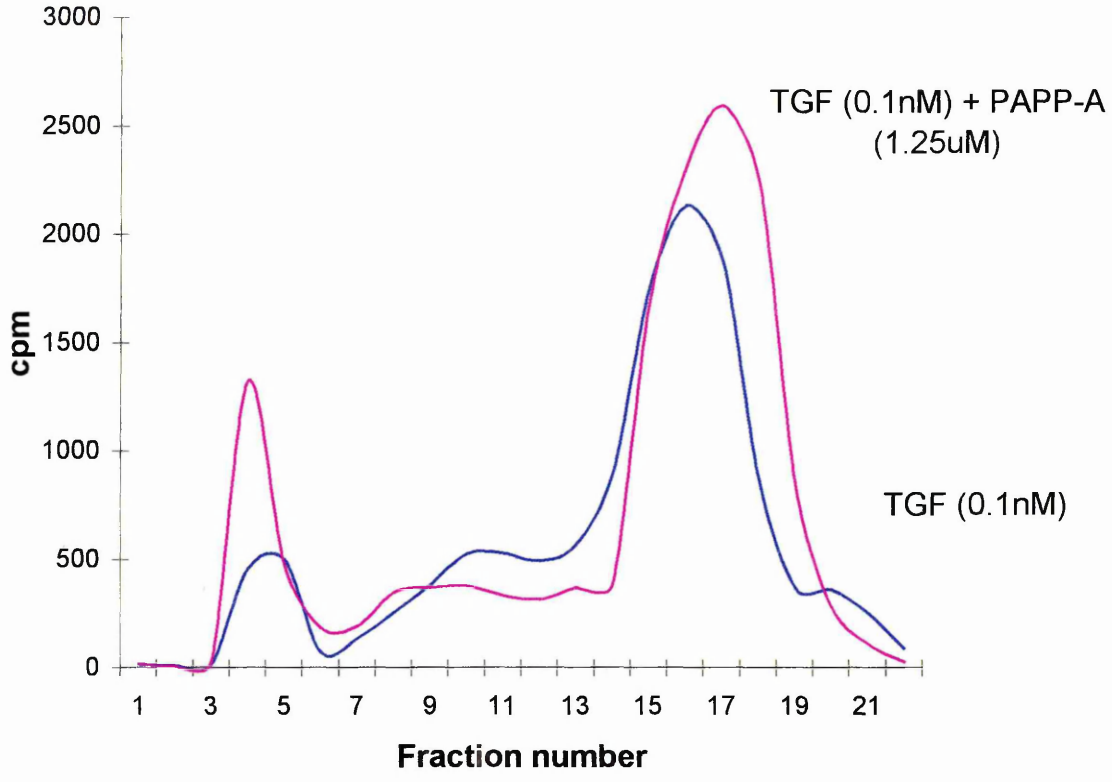


Figure 4.10 Elution of ^{125}I TGFβ and ^{125} I TGFβ + PAPP-A on Sephadex G200 column. Results are expressed as counts per minute.

The fractions containing the "free" ^{125}I TGFβ, that eluted in the later fractions (14-19), were then pooled and reloaded onto the column to determine if the smaller peak (fractions 2-6) was present or that this anomalous peak could only been seen on initial loading. As previously a peak was observed in the early fractions and it was concluded that this peak was possibly due to small amounts of the ^{125}I TGFβ forming a complex with itself.

4.5.4 Discussion

The results obtained from the native gels suggested that no binding was occurring between PAPP-A and the cytokines, despite varying both PAPP-A and cytokine concentrations to try to optimise the binding.

It was initially thought that subjecting the sample to denaturing conditions would break

up any complex formation, particularly if binding was weak, therefore non denaturing, native PAGE was used which would not, break any links between the PAPP-A and labelled cytokine. However it has been shown by Crookston et al. (1994) that TGFβ - α_2m complexes dissociate during non denaturing PAGE electrophoresis and after running a gel for 3 hours 30 minutes the binding was only 32 % and a band could not be detected on native PAGE. This could explain why a band was not detected for PAPP-A. It was also suggested by Crookston et al. (1994) that radioiodination may have affected the structure of some cytokine molecules so that it is possible that the ^{125}I -cytokines would be incapable of binding to PAPP-A but may possibly bind in their native state.

The results from the gel filtration experiments indicated that there was no ^{125}I IL-1β present in the early fractions that contained the PAPP-A also suggesting that PAPP-A does not bind to ^{125}I IL-1β under these conditions. ^{125}I IL-1β and PAPP-A could bind in this system but when they are subjected to the column chromatography they may dissociate and elute from the column separately. However even when the PAPP-A and ^{125}I IL-1 were crosslinked the results obtained suggested that no binding was occurring between the two components.

The results for the^{125}I TGFβ initially indicated, that there was an ^{125}I- labelled peak present where the PAPP-A eluted from the column, which suggested that PAPP-A did bind to ^{125}I TGFβ forming a complex. However the results obtained when ^{125}I TGFβ alone was loaded onto the column refuted this as the early peak was still present suggesting that the effect was not due to complex formation with PAPP-A but to the ^{125}I TGFβ itself.

O'Conner-McCourt and Wakefield (1987) published data indicating that α_2m bound to ^{125}I TGFβ and one of the experiments confirming this, was gel filtration chromatography on Sephadex G200. They noted the presence of three peaks when α_2m and ^{125}I TGFβ were incubated and loaded onto the column. There is no indication in the paper that a control experiment was carried out and it is therefore questionable to whether binding was actually occurring or if it was the ^{125}I TGFβ itself forming the three peaks as seen in the data presented in this study.

Studies have also revealed that many labelled cytokines, including IL-2 and TNFα bind much more effectively to the methylamine converted "fast form" of α_2m than to the native preparation, although some binding to the native form does occur (Cunningham et al., 1990). Similarly PAPP-A may need "activating" in the same way as α_2m in order to bind cytokines.

Chapter Five

The effect of PAPP-A on cartilage.

5.1 General Introduction

A cartilage explant culture system was used to determine whether PAPP-A had any inhibitory effects on cartilage proteoglycan breakdown. The ability of PAPP-A to bind to human cartilage sections was also investigated to determine whether it may form a protective layer.

5.2 The binding of PAPP-A to human articular cartilage

5.2.1 Introduction

The possibility of PAPP-A binding to cartilage was investigated as this may facilitate any protective effects of PAPP-A on cartilage breakdown, such as proteinase inhibition.

5.2.2 Method

The method was carried out as described in section 2.9.3.

Normal cartilage sections were incubated with PAPP-A (500ug/ml) and sections incubated with PBS under the same conditions. After washing, the slides were then incubated, with 100ul of the following :

The PAPP-A incubated sections

1. Primary antibody (rabbit anti-human PAPP-A antibody)
2. Primary antibody (rabbit anti-human PAPP-A antibody)
3. Positive control (rabbit anti-human collagen type II antibody)
4. Negative control (rabbit immunoglobulin fraction)

PBS incubated sections

1. Primary antibody (rabbit anti-human PAPP-A antibody)
2. Positive control (rabbit anti-human type II collagen antibody)

The slides were left at room temperature for 1 hour in a moisture chamber, they were then washed in PBS as previously described. All sections were then incubated with 100ul of goat anti-rabbit IgG, peroxidase labelled antibody, for 1 hour at room

temperature, with the exception of slide number two which was incubated with PBS. All of the sections were then incubated with peroxidase substrate, DAB, and then washed with PBS to remove any excess unbound substrate. The sections were then mounted in PBS : glycerol and examined under the light microscope and the results recorded photographically.

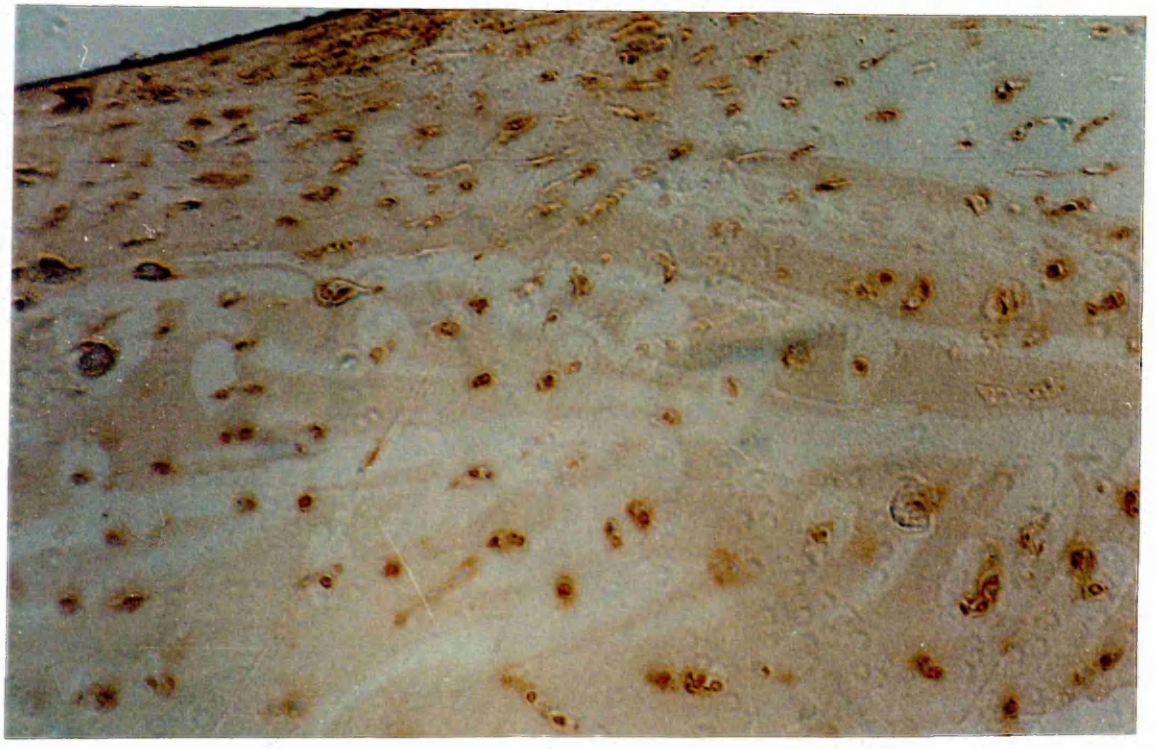
5.2.3 Results

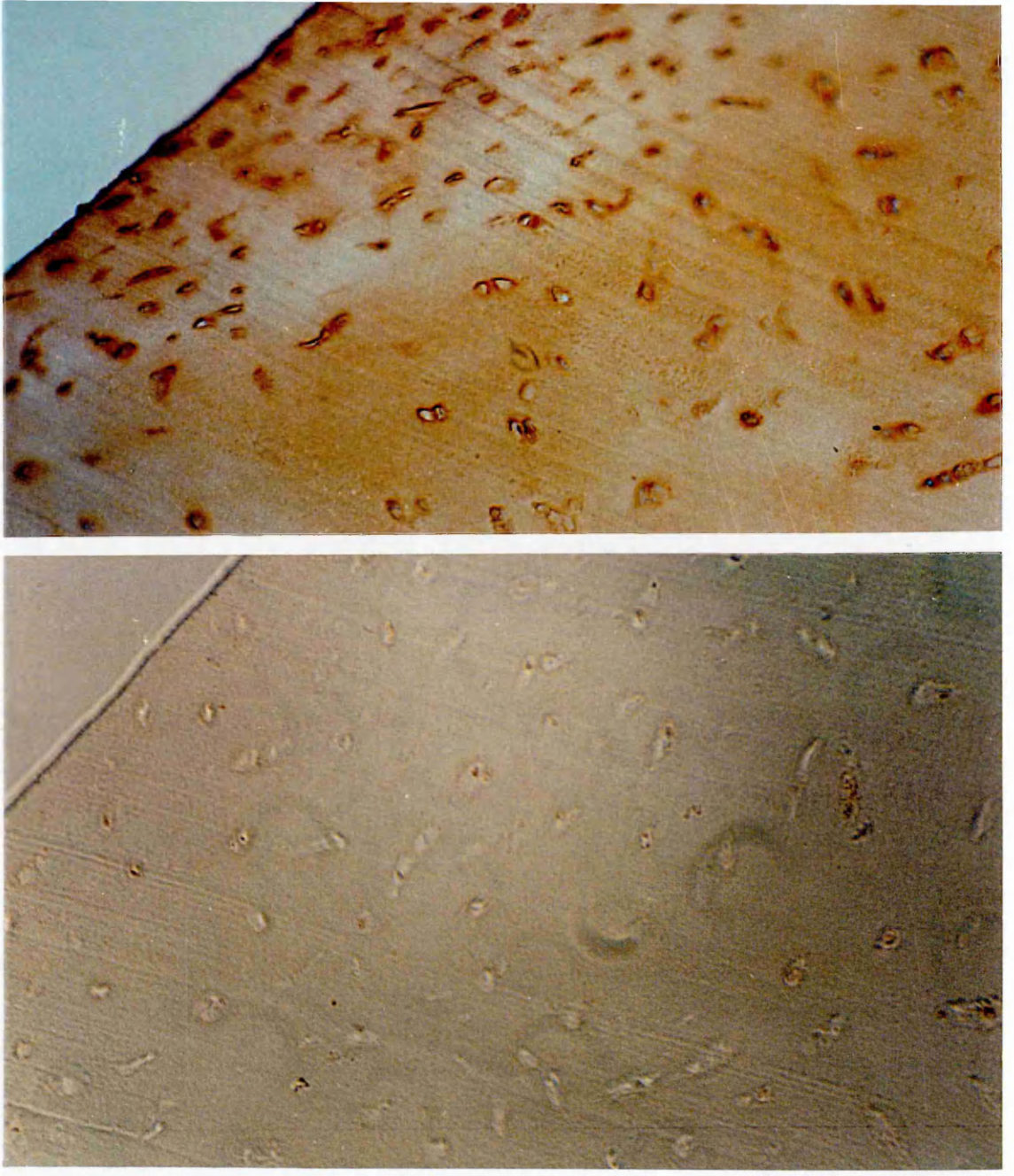
Intense staining was seen with the PAPP-A and PBS incubated sections that had been incubated with the type II collagen antibody, particularly round the edges of the section, see figure 5.1A.

Staining was observed when PAPP-A was incubated with the sections indicative of PAPP-A binding to cartilage. The staining seemed to be mostly around the edges of the cartilage and around the chondrocyte lacunae, see figure 5.1B

No staining was seen when the PAPP-A incubated section was incubated with the rabbit immunoglobulin fraction, instead of primary antibody, see figure 5.1C and no staining was detected in the section that had been incubated with PBS and then the PAPP-A antibody (results not shown)

A





C

Figure 5.1 Immunohistochemical studies of PAPP-A binding to human articular cartilage

A) cryostat sections of human cartilage incubated with PAPP-A. PAPP-A binding is indicated by brown peroxidase staining

B) staining of human articular cartilage for type II collagen (positive control), type II collagen is indicated by brown peroxidase staining

C) cryostat sections of human cartilage incubated with PAPP-A and treated with rabbit immunoglobulin instead of PAPP-A primary antibody (negative control)

5.2.4 Discussion

The results indicate that PAPP-A binds to cartilage. The staining seen was not due to the PAPP-A antibodies binding non specifically to the cartilage, as no staining was detected when the PAPP-A antibody was incubated with the section without PAPP-A. The exact mechanism for binding is not known. The binding of PAPP-A to cartilage may form a "protective" barrier to prevent attack from proteinases via proteinase inhibition or physically preventing them attacking the cartilage, due to the large M_r of PAPP-A causing steric hindrance. PAPP-A is also reported to bind to heparin (Davey et al., 1983). Heparin is an acidic glycosaminoglycan composed of sulphated glucosamine and uronic acid residues (Jacques, 1979; Casu, 1985) and has the ability to interact with basic plasma and cell surface components (Dziarski, 1989). PAPP-A may bind to cartilage proteoglycan by a similar interaction as for that with heparin.

5.3 The effect of PAPP-A on retinoic acid stimulated resorption of bovine nasal cartilage.

5.3.1 Introduction

As an experimental model of cartilage breakdown chondrocytes in explant culture can be induced to degrade their surrounding proteoglycan by application of compounds such as retinoic acid (Ret) (Meats et al.,1985) or proinflammatory cytokines IL-1 (Saklatvala et al., 1984) and TNFα (Saklatvala 1986). The effect of PAPP-A on cartilage proteoglycan breakdown in culture was investigated by incubating PAPP-A alone with cartilage explants and also with Ret.

5.3.2 Method

The method employed was described in section 2.6.

Nasal septa were dissected from bovine noses and the cartilage cut into uniform 2mm diameter discs. The explants were incubated in a humidified atmosphere at 37^0C and 95% air/5% CO_2, for 24 hours. The following day the explants were plated out into 96 well plates, 1 disc per well.

The control and/or test substances were made up in DMEM as below and 200ul was added to each well.

1. Retinoic acid 10^{-6} M
2. Solvent control (DMSO + DMEM)
3. Retinoic acid 10^{-6} M + PAPP-A 0.5ug/ml (0.62nM)
4. Retinoic acid 10^{-6} M + PAPP-A 5.0ug/ml (6.25nM)
5. Retinoic acid 10^{-6} M + PAPP-A 50.0ug/ml (62.5nM)
6. PAPP-A 0.5ug/ml (0.62nM)
7. PAPP-A 5.0ug/ml (6.25nM)
8. PAPP-A 50.0ug/ml (62.5nM)
9. DMEM control

The cartilage discs were incubated for a total of 7 days with a change of medium containing the test substances at day 3. Media from day 3 and day 7 were stored at -20^{O}C prior to dimethylmethylene blue assay as described in section 2.6.3.

5.3.3 Results

Ret (10^{-6}M) stimulated the release of proteoglycans from the cartilage, as expected. PAPP-A at 50ug/ml (62.5nM) also stimulated cartilage proteoglycan breakdown, but this effect was not as great as with Ret alone. PAPP-A incubated with Ret augmented Ret stimulated proteoglycan release. PAPP-A alone at 5ug/ml (6.25nM) had no significant effect on the release of proteoglycan from cartilage but when incubated with the cartilage explants in the presence of Ret there was a significant increase seen compared to Ret alone, see figure 5.2, suggestive of synergy.

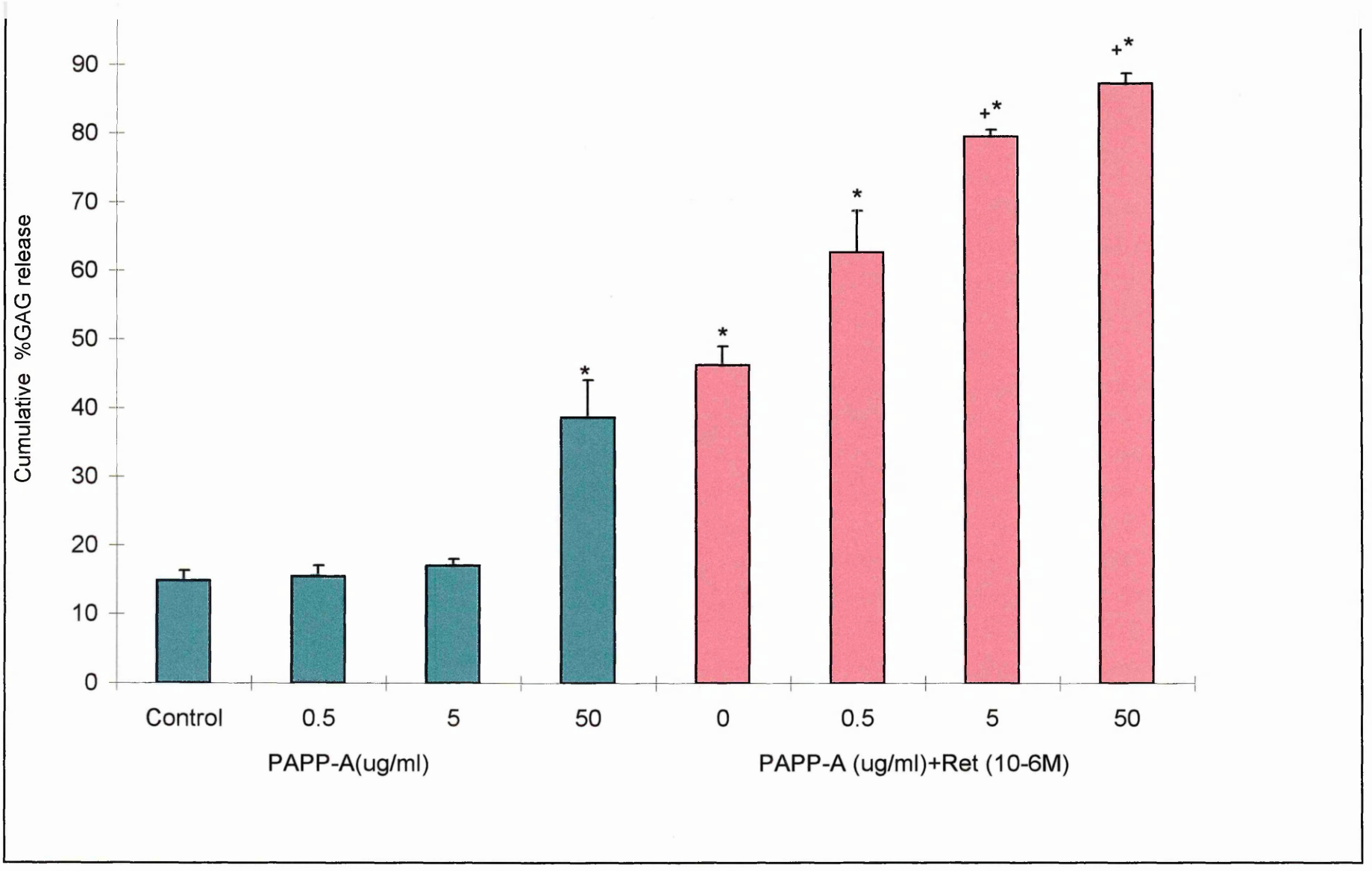


Figure 5.2: The effect of PAPP-A and Ret on % GAG release by bovine cartilage. Data expressed as the mean conc. per 6 wells +/- the SE. Significant differences determined by ANOVA, *p< 0.05, compared to control; +p< 0.05, compared to Ret alone.

5.3.4 Discussion

The results show that, as expected, Ret stimulated the resorption of cartilage. PAPP-A, however, also stimulated the resorption of cartilage at high concentrations, 50ug/ml, and augmented Ret-stimulated resorption. These results suggest that PAPP-A does not prevent cartilage breakdown in rheumatoid arthritis and therefore may not be involved in the remission of RA during pregnancy.

A possible explanation for this stimulation of proteoglycan release could be that PAPP-A as a proteoglycan (Oxvig et al., 1994) could be degraded by factor (s) released from the cartilage discs during the incubation period, releasing proteoglycan which was then detected in the DMB assay, thus giving anomalous results. PAPP-A itself, when incubated in the assay, in the absence of discs, does not release GAGs, detectable in the DMB assay.

Werb et al. (1989) discovered that fragments of other molecules, e.g. fibronectin are capable of binding to chondrocytes in a receptor mediated manner and stimulating matrix degradation. Degraded matrix molecules may therefore have a profound effect on cytokine and proteinase synthesis and release by cells. As described in section 5.2 PAPP-A can bind to cartilage, as may PAPP-A fragments, and therefore could act in a similar way to fibronectin fragments and stimulate cartilage degradation. Furthermore GAGs such as heparin and heparan sulphate have been shown to stimulate plasminogen activation providing additional evidence for such a mechanism (Brunner et al., 1998). PAPP-A has been shown to contain a glycosaminoglycans that may be related to hyaluronic acid or heparan sulphate (Oxvig et al., 1994).

Buttle et al. (1993) investigated the mechanism of cartilage proteoglycan breakdown studying the effects of specific inhibitors of cathepsin B and MMPs on cartilage proteoglycan release. Their results demonstrated that a cathepsin B inhibitor was capable of inhibiting proteoglycan release from explants stimulated with IL-1 α but not from explants stimulated with retinoic acid. The MMP inhibitor however was able to inhibit both IL-1α and retinoic acid stimulated release. This suggested two pathways of cartilage proteoglycan breakdown, one cytokine mediated and the other Ret mediated (Buttle et al., 1993). PAPP-A may activate the cytokine mediated pathway or augment

Chapter Six

The effect of PAPP-A on proteinases.

6.1 General Introduction

It has been shown that α_2m is able to inhibit proteinases from all four major classes and its mechanism of action has been described in detail by Starkey and Barrett (1977). In view of the similar properties of PAPP-A and α_2m (Sinosich, 1988) it was of great interest to investigate whether PAPP-A shared proteinase inhibitory activities.

Past reports of proteinase inhibitory activities of PAPP-A have been very controversial. Sinosich et al (1982, 1990) studying the inhibitory activity of PAPP-A, derived from serum, on elastase, using the synthetic substrate methoxysuccinyl-L-ala-L-ala-pro-L-val-ρNA found it to be an uncompetitive inhibitor whereas Bischof et al. (1990) using the same substrate found no inhibition of elastase by plasma derived PAPP-A. It was initially thought that the source of PAPP-A, whether from serum, plasma and/or the type of anticoagulant used were reasons for the differing results (Bischof and Meisser, 1988).

Sinosich et al.(1982) presented evidence for a trapping mechanism for PAPP-A's inhibition of elastase, as seen with α_2m, but later studies (Sinosich et al.,1990) indicated this was not the case as covalent protease entrapment by α_2m is mediated by limited proteolysis of internal thioester groups which were not present in PAPP-A. Furthermore Oxvig et al. (1994) suggested that PAPP-A, derived from serum, was a competitive inhibitor of elastase at low ionic strengths, and that the inhibition was not due to the "trapping mechanism", as proposed for α_2m, but to the electrostatic interactions between the negatively charged PAPP-A and elastase, which is a basic protein.

Subsequently other workers have presented evidence that proteinase inhibition by PAPP-A is via a trapping mechanism. Zorin et al. (1995) reported the inhibition of trypsin and plasmin by PAPP-A, derived from plasma, using the large M_r substrate remazol brilliant blue hide powder. Inhibition was seen to a much lesser extent using smaller M_r substrates, Bz-DL-Arg-ρNA-HCl (DL-BAPNA) for trypsin and D-val-leu-lys-ρNA for plasmin. It was suggested that the mechanism of interaction was similar to that of α_2m and the access of the substrates to the active site of the enzymes "encapsulated" within the PAPP-A molecule becomes sterically hindered, causing

inhibition that is more marked for large M_r substrate molecules. The enzymes were also found to be protected from inhibition by soya bean trypsin inhibitor and pancreatic trypsin inhibitor, due to their large size but both enzymes could be inactivated by PMSF. Zorin et al. (1995) also demonstrated PAPP-A trypsin complexes on native PAGE. In addition when the mixtures were subjected to reducing SDS PAGE, PAPP-A was specifically cleaved by trypsin and plasmin to produce a major fragment of M_r 85,000 and many minor fragments in the range M_r 45,000-200,000. The major 85,000 fragment generated was similar to that produced on cleavage of α_2m at the bait region. (Zorin et al., 1995).

It has been suggested that PAPP-A may undergo slight modifications due to the purification procedure used and the action of endogenous proteinases present during the purification. This could partially explain the conflicting reports of the activities of PAPP-A. Gore and Sutcliffe (1984) developed a purification protocol for PAPP-A under mild conditions, to minimise any conformational changes, as previously mentioned in section 1.1 and then studied the effects of the PAPP-A obtained, on trypsin, plasmin and complement activities. In contrast to other studies, there was no evidence that PAPP-A had any inhibitory activities and it was suggested that the proteinase-inhibitory activity of PAPP-A, observed by some workers, was due to contaminating α_2m (Gore and Sutcliffe, 1984). Leached heparin, from the heparin sepharose column during the purification has also been reported to be responsible for the inhibition of elastase, previously attributed to PAPP-A (Bischof et al., 1990).

The aim of this study was to determine the proteinase inhibitory activities of the pure PAPP-A preparation obtained using the purification procedure described in section 3.1.

6.2 Plasmin inhibition

6.2.1 Introduction

Plasmin is a broad spectrum serine proteinase consisting of two polypeptide chains held together by disulphide bonds. The light chain contains the active site and has amino acid sequence homologies with other serine proteases such as trypsin, chymotrypsin and elastase (Robbins et al., 1978). Plasmin is generated from its zymogen plasminogen, by the catalytic action of plasminogen activators, urokinase type plasminogen activator (u-PA) and tissue type plasminogen activator (t-PA). Plasmin preferentially cleaves lysyl bonds and seems to have evolved for the function of breaking down fibrin clots. Not only is plasmin itself very active in degrading fibrin, but plasminogen has a high affinity for fibrin so when a clot forms it has a built in mechanism for its own dissolution. Plasmin has been shown to degrade proteoglycan aggregates and monomers isolated from cartilage (Mochan et al., 1984), but has little action on collagen although it does activate latent collagenase and stromelysin (Werb, 1977). Plasmin has been shown to activate MMP-2 indirectly via other plasmin-activated MMPs (Nagase et al., 1997). A more recent report suggests that plasmin degrades MMP-2 (Mazzieri et al., 1997) but when bound to cells MMP-2 is protected from degradation by plasmin. The major plasma inhibitors of plasmin are the α_2 plasmin inhibitor and α_2 m (Harpel, 1977).

6.2.2 Method

Plasmin was assayed using a colorimetric substrate H-D -Ile-Phe-Lys-ρNA as described in section 2.11.3

6.2.3 Results

The results were obtained as scanning traces and the rate of reaction was calculated in absorbance change per minute, see table 6.1. The rate of formation of ρ-nitroaniline was calculated using the extinction coefficient at 405nm of 10,400 mol/L/cm, see table 6.1.

Table 6.1 The effect of PAPP-A (62.5nM) on plasmin activity (0.4uM) at varying substrate concentrations expressed as the amount of ρ- nitroaniline produced in pmole/ minute +/- SE

Substrate Conc (mM)	Rate of Reaction ΔA_{405} (10^{-2}) / minute +/- SE		Rate of ρ - nitroaniline production pmole / minute +/- SE	
	- PAPP-A	+ PAPP-A	-PAPP-A	+PAPP-A
1.0	10.0 +/- 0	9.8 +/- 0.03	961.5 +/- 0	942.3 +/- 5.5
0.8	9.8 +/- 0.02	10.0 +/- 0	942.3 +/- 3.9	961.5 +/- 0
0.6	10.0 +/- 0	11.0 +/- 0	961.5 +/- 0	1057.7 +/- 0
0.4	10.0 +/- 0.01	10.0 +/- 0	959.1 +/- 2.4	961.5 +/- 0
0.2	9.4 +/- 0.07	10.0 +/- 0	906.3 +/- 12.6	961.5 +/- 0
0.1	10.0 +/- 0	10.0 +/- 0.01	961.5 +/- 0	959.1 +/-2.4
0.08	8.8 +/- 0.07	8.6 +/- 0.01	843.8 +/- 13.8	834.1 +/- 27.3
0.04	6.5 +/- 0.01	6.7 +/- 0.13	620.2 +/- 2.8	639.4 +/- 25.0
0.02	2.7 +/- 0.01	2.6 +/- 0	262.0 +/- 2.4	250.0 +/- 0
0.01	2.0 +/- 0.02	1.9 +/- 0.04	192.3 +/- 3.9	185.1 +/- 8.2

There was no significant difference in either the rate of reaction or amount of ρ-nitroaniline produced in the presence or absence of PAPP-A.
The results suggest therefore, that PAPP-A, at 62.5nM had no effect on the inhibition of plasmin at varying substrate concentrations

6.3 Trypsin inhibition

6.3.1 Introduction

Trypsin is a monomeric digestive enzyme that is produced by the pancreas. Trypsinogen is converted to trypsin by the action of enteropeptidase, or trypsin itself, in the intestine to produce the active trypsin. Trypsin is found in increasing amounts in plasma in certain diseases of the pancreas, particularly acute pancreatitis (Colowick and

Kaplan, 1981) and in the context of these experiments it was used as a representative serine proteinase.

6.3.2 Method

Trypsin was assayed using a colorimetric substrate, DL-BAPNA, as described in section 2.12.3.

6.3.3 Results

The results were expressed as the rate of reaction in absorbance change per minute and the rate of formation of ρ-nitroaniline calculated as for plasmin in section 5.2.3, see table 6.2.

Table 6.2 The effect of PAPP-A (62.5nM) on trypsin activity (0.45uM) at varying substrate concentrations expressed as the amount of ρ- nitroaniline produced in pmole/ minute +/- SE

Substrate Conc (mM)	Rate of Reaction ΔA_{405} (10^{-2}) / minute +/- SE		Rate of ρ – nitroaniline production pmole / minute +/- SE	
	- PAPP-A	+ PAPP-A	-PAPP-A	+PAPP-A
2.5	1.85 +/- 0.10	1.80 +/- 0.04	177.9 +/- 18.6	173.1 +/- 8.8
2.0	1.85 +/- 0.09	1.95 +/- 0.01	177.9 +/- 17.8	187.5 +/- 2.8
1.0	1.30 +/- 0	1.35 +/- 0.01	125.0 +/- 0	129.8 +/- 2.8
0.8	1.05 +/- 0.01	1.13 +/- 0.01	101.0 +/- 2.8	108.2 +/- 2.4
0.6	0.90 +/- 0	0.86 +/- 0.02	87.0 +/- 1.6	82.5 +/- 3.2
0.4	0.67 +/- 0.01	0.69 +/- 0	63.9 +/- 1.9	66.3 +/- 1.0
0.3	0.56 +/- 0	0.56 +/- 0	53.3 +/- 1.6	54.1 +/- 1.4
0.2	0.4 +/- 0	0.39 +/- 0	38.5 +/- 0	37.5 +/- 1.0

There was no significant difference in either the rate of reaction or amount of ρ-nitroaniline produced in the presence or absence of PAPP-A.

The results suggest therefore, that PAPP-A, at 62.5nM had no effect on the inhibition of trypsin at varying substrate concentrations

6.4 Cathepsin B inhibition

6.4.1 Introduction

Cathepsin B is a lysosomal cysteine proteinase that has been purified from various mammalian tissues e.g. muscle and the liver (Okitani et al., 1988). It is a protein of about M_r 25,000 that has a pH optimum on synthetic substrates of 6.0, but maximal activity against proteins occurs at a pH as low as 3. Cathepsin B loses activity irreversibly above pH 7.0 and is best-assayed fluorometrically (Barrett et al., 1982). Cathepsin B cleaves the hyaluronic acid-binding region from cartilage-proteoglycan and degrades the glycosaminoglycan-attachment region to small fragments (Morrison et al., 1973). Cathepsin B can also cleave the N terminal peptides of collagen that contain the crosslinks within and between molecules (Kirschke et al., 1982).

There is evidence that cathepsin B may play an important role in the proteolytic cascade involved in cartilage breakdown by activation of prometalloproteinases and pro urokinase plasminogen activator (uPA), leading to the breakdown of extracellular matrix components aggrecan and type II collagen (Mort and Buttle, 1997). It has also been shown that the breakdown of cartilage in tissue culture systems can be inhibited by cathepsin B inhibitors, the lipophilic inhibitors being more effective than the hydrophilic inhibitors which suggests that cathepsin B is more important in the breakdown of cartilage components intracellulary than extracellularly. It has been concluded from this work that cathepsin B may act as an intracellular activator of other proteinases that can then degrade the extracellular matrix components (Buttle, 1994).

6.4.2 Method

Cathepsin B was assayed using a fluorogenic substrate, Z-Arg-Arg-βNA, as described in section 2.13.3.

6.4.3 Results

The results were expressed as the change in fluorescence per second, see table 6.3 .

Table 6.3 The effect of PAPP-A (62.5nM) on Cathepsin B activity (9.8nM) using various substrate concentrations

Substrate Conc (mM)	Rate of Reaction $\Delta F_{355-460}$ / second +/- SE	
	- PAPP-A	+ PAPP-A
2.5	184.2 +/- 5.1	179.5 +/- 1.2
2	159.0 +/- 5.8	169.0 +/- 5.3
1.5	170.7 +/- 4.0	168.0 +/- 6.5
1	175.1 +/- 1.4	147.8 +/- 4.1
0.8	140.4 +/- 2.0	146.4 +/- 1.4
0.6	103.0 +/- 1.2	120.3 +/- 1.0
0.3	91 +/- 1.3	80.2 +/- 0.85
0.15	51.1 +/- 0.35	46.0 +/- 0.25

There was no significant difference in the rate of reactions in the presence or absence of PAPP-A (62.5nM) indicating that PAPP-A has no inhibitory effect on Cathepsin B in the assay system.

6.5 Elastase inhibition

6.5.1 Introduction

Elastase is a neutral serine proteinase stored within the primary granules of polymorphonuclear granulocytes. It is released in large quantities at inflammatory sites and is able to cause tissue damage. The active site of human leukocyte elastase is a channel on the protein surface that has extended substrate binding sites and it is in this

channel that the catalytic triad of Ser^{195}, His^{57} and Asp^{102} catalyse amide bond hydrolysis of various proteins including elastin, collagen types III and IV and fibronectin (Janoff, 1985). Elastase also acts on cartilage proteoglycan to remove the hyaluronic acid-binding region and then to fragment the glycosaminoglycan attachment region (Roughley,1977). Circulating elastase is normally found associated with endogenous inhibitors, the major one being α_1 proteinase inhibitor, the remainder being bound to α_2m (Ohlsson et al., 1974). These circulating inhibitors occur in excess even in chronic inflammation and therefore free elastase activity has been found only in the synovial fluid of patients with extremely high granulocyte counts (Kleesiek et al., 1986). Elastase mediated tissue damage in rheumatoid arthritis may result from interference with elastase-α_1 proteinase inhibitor (PI) complex formation at the cartilage surface (Burkhardt et al., 1987, Schalkwijk et al., 1987).

6.5.2 Method

Elastase was assayed using a colorimetric substrate, MeO-Suc-Ala-Ala-Pro- Val-ρNA, as described in section 2.10. Elastase activity was measured with differing PAPP-A concentrations at 62.5uM, 6.25uM and 0.625uM, and differing substrate concentrations (0.05 - 1.0 mM).

6.5.3 Results

The results were determined as the rate of reaction in absorbance change per minute and the rate of formation of ρ-nitroaniline, calculated as for plasmin in section 6.2.3, see table 6.4.

Table 6.4. The effect of PAPP-A (62.5nM) expressed as the amount of ρ- nitroaniline produced in pmole/minute +/- SE on elastase activity (12.5nM) using different substrate concentrations (0.05 -1.0mM). Significant inhibition as determined by the t-test, *p< 0.05, ** p< 0.01, *** p<0.001.

Substrate Conc (mM)	Rate of Reaction ΔA_{405} (10^{-2}) / minute +/- SE		Rate of ρ - nitroaniline production pmole / minute +/- SE	
	- PAPP-A	+ PAPP-A	-PAPP-A	+PAPP-A
1.0	2.25 +/- 0.05	0.83 +/- 0.01	432.6 +/- 14.2	159.6 +/- 2.9 **
0.5	1.76 +/- 0.05	0.43 +/- 0.01	338.4 +/- 15.4	83.4 +/- 2.4 **
0.25	1.57 +/- 0.01	0.31 +/- 0.01	303.2 +/- 2.4	59.6 +/- 1.6 ***
0.1	1.14 +/- 0.02	0.10 +/- 0	218.6 +/- 5.3	19.8 +/- 0.6 ***
0.05	0.83 +/- 0.01	0.08 +/- 0	160.2 +/- 2.9	16.6 +/- 0.6 ***

PAPP-A at 62.5nM significantly inhibited elastase activity at all the substrate concentrations investigated. It was seen that the substrate concentration did affect the % inhibition of the PAPP-A, see table 6.5.

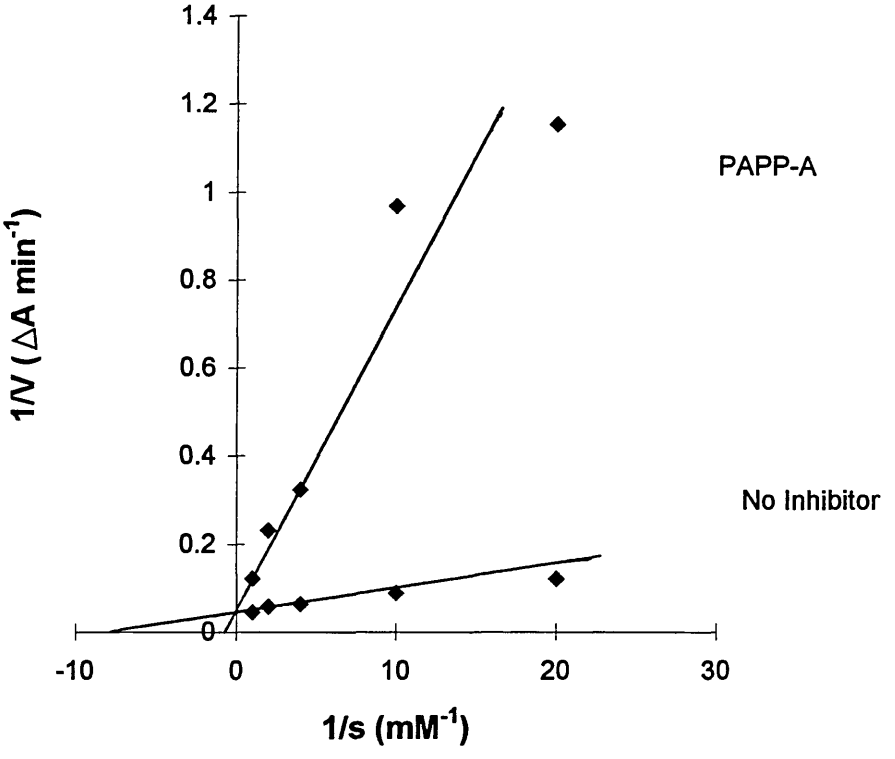
Table 6.5. The effect of varying substrate concentrations (0.05 -1.0 mM) on the inhibition of PAPP-A (62.5nM) at a fixed enzyme concentration (12.5nM)

Substrate Conc (mM)	**% Inhibition**
1.0	63.1
0.5	75.4
0.25	80.3
0.1	90.9
0.05	90.0

The results suggest that PAPP-A has greater inhibitory action on the elastase when the substrate concentration is very low, which is characteristic of competitive inhibition.

The results obtained from the varying substrate concentrations were also graphically expressed on a Lineweaver Burke plot, see figure 6.1. The results again suggest competitive inhibiton since a competitive inhibitor is able to compete with the substrate for the available active site of the enzyme and therefore increases the apparent Km value and the velocity of the reaction is decreased, as seen here.

Figure 6.1 The activity of elastase (12.5nM) in the presence and absence of fixed PAPP-A concentration (62.5nM) at varying substrate concentrations (0.05 -1.0 mM)



PAPP-A incubated at different concentrations (0.625 -62.5nM) with elastase (12.5nM) and fixed substrate concentration (0.5mM) resulted in different rates of production of ρ nitroaniline dependent on the concentration of PAPP-A, the higher the concentration of PAPP-A the greater the inhibition, see table 6.6.

Table 6.6. The effect of differing concentrations of PAPP-A (0.625 -62.5nM) on the % inhibition of elastase (12.5nM) at a fixed substrate concentration (0.5mM)

Concentration of PAPP-A (nM)	Rate of Reaction ΔA_{405} (10^{-2}) / minute +/- SE	Rate of ρ - nitroaniline production pmole / minute +/- SE	% Inhibition
0.0	1.76 +/- 0.05	338.4 +/- 10.2	0
0.625	1.89 +/- 0.02	363.4 +/- 8.0	0
6.25	1.33 +/- 0.04	255.8 +/- 12.2	24.4
62.5	0.43 +/- 0.01	82.6 +/- 3.3	75.6

The Ki was calculated as described in section 2.14 and found to be 6.25 x 10^{-9} M which compares favourably with the Ki determined by Oxvig et al (1994) of 5-10 x 10^{-9} M. Overall, the results suggest that the inhibition of elastase by PAPP-A is competitive, as the degree of inhibition is dependent on the relative concentrations of the substrate and inhibitor and by suitably increasing the substrate concentration the inhibition can be reduced. Whereas non competitive inhibition depends only on the inhibitor concentration and is unaffected by the variation in substrate concentration

6.5.4 Discussion

The results indicate that PAPP-A only seems to have an inhibitory effect on elastase and not on the other proteinases studied. The results obtained from this study agree with those of Oxvig et al. (1994) that PAPP-A is a competitive inhibitor of elastase. Oxvig stated that a four fold molar excess of PAPP-A greatly reduced the activity of elastase. The results obtained in this study indicate that with an approximate five fold molar excess of PAPP-A to elastase, 13nM of elastase and 62.5nM of PAPP-A, PAPP-A has a profound effect on the activity of elastase producing 75.6 % inhibition (table 6.6). A smaller inhibition can also be detected when the molar ratio of PAPP-A to elastase is much smaller.

This study used only small peptide substrates and if PAPP-A were acting via a trapping mechanism (Zorin et al., 1995) it may not be expected to inhibit the

activity was observed with small substrates by Zorin et al, (1995) which was not seen in this study.

The effect of PAPP-A on elastase even with a small peptide substrate may indicate that the mechanism for inhibition by PAPP-A is not by trapping, as the smaller substrates should still be able to gain access to the proteinase and therefore little, if any inhibition should be detected. There are however other explanations that may explain the inhibitory effects of PAPP- A on elastase. PAPP-A is known to contain approximately 19% carbohydrate including GAGs. Travis et al. (1983) reported that GAGs prevent the release and action of HLE. Chondroitin sulphate (CS) and dermatan sulphate (Ds) were chemically oversulphated to give many preparations with varying degrees of sulphation to enable their inhibitory effect on HLE to be investigated. HLE is a basic protein containing a number of arginine residues. The results showed that increased sulphation and oversulphation generally increased the inhibitory effects of GAGs on elastase. The interactions between GAGs and proteins are both specific and non specific and are thought to occur mainly between cationic residues of the side chains of the proteins and anionic residues of the GAGs. The binding ability of GAGs of similar M_r was ; heparin > Ds > CS and this is due to the higher charge density in heparin. PAPP-A, with its high GAG content, could therefore inhibit elastase in the same manner.

Oxvig et al. (1994) also reported that the inhibition of HLE was likely to be due to the electrostatic interactions between the negatively charged GAG on the pro MBP and a set of Arg- residues located close to the active site of elastase.

Chapter Seven

The production of a monoclonal antibody for PAPP-A

7.1 Quantitation of PAPP-A

A variety of techniques have been used to measure PAPP-A. Measurement was initially carried out using immunodiffusion and immunoelectrophoretic techniques because only an antiserum was required, however these techniques were very insensitive (detection limit 10mg/l) and PAPP-A could only be measured in the later stages of pregnancy.

The first ELISA to be established for PAPP-A was a sandwich assay using polyclonal antibodies. The sensitivity was very poor and levels of PAPP-A could only be detected in second and third trimester samples (Macdonald et al., 1984). An ELISA was then developed to detect levels of PAPP-A in the first trimester using plates coated with the commercial polyclonal antibody (DAKO) that had been adsorbed, by negative affinity chromatography, with an immobilised < 300kDa fraction of pregnancy serum obtained by pressure ultrafiltration. The secondary antibody used was the polyclonal rabbit anti-PAPP-A peroxidase conjugate. The sensitivity of the assay was good, detecting PAPP-A levels as low as 0.04mIU/ml (40mIU/l) (0.02 ug/ml) and could detect PAPP-A in normal pregnancy as early as 5-7 weeks (Bersinger et al., 1995).

More recently four double-monoclonal time-resolved immunofluorometric assays (TrIFMAs) were developed for PAPP-A/proMBP complex determination and a TrIFMA was also described using the commercially available polyclonal antibody (DAKO) (Qin et al., 1997). The polyclonal TrIFMA had a working range of 4-1000mIU/l (2-500 ug/l). The monoclonal TrIFMAs have a similar or better sensitivity compared to the polyclonal TrIFMA. In this assay some of the monoclonal assay combinations used have given much higher concentrations of PAPP-A than in the polyclonal assay. This may be attributable to variations in the detection of isoforms of PAPP-A/proMBP or that other complexes containing either or both of the two proteins PAPP-A and proMBP may exist in different stages of pregnancy.

Unfortunately polyclonal antibodies tend not to be absolutely specific for PAPP-A. The polyclonal anti-PAPP-A antibodies raised to date often recognise anti-PAPP-A/proMBP, because PAPP-A isolated from serum and used in antibody production is in the form of PAPP-A/proMBP complex. The commercially available polyclonal antiserum (DAKO) has also been shown to react with haptoglobin (Bueler et al., 1989)

and SP 1 (Bersinger et al., 1995). Therefore the levels of PAPP-A determined using these antibodies may be severely biased by cross reactivity.

More recently it has been reported that a monoclonal antibody to PAPP-A has been successfully produced (Qin et al., 1997). Under both native and denaturing and reducing conditions, western blots have demonstrated that this antibody reacts with the PAPP-A part of the PAPP-A/proMBP complex and not the proMBP part (Qin et al., 1996). It is of the utmost importance that the monoclonal antibodies produced do not recognise the proMBP part of the PAPP-A molecule as at least two other proMBP complexes are present in term pregnant serum, complexes with angiotensinogen and complexes with complement 3dg. (Oxvig et al., 1995). Nothing is currently known about the function of these complexes or their serum concentrations in DS pregnancies.

Bersinger et al. (1999) have produced four monoclonal antibodies that specifically recognize the pure PAPP-A and not the pro MBP with the purpose of using them in an automated DS screening test for testing large numbers of serum specimens. The monoclonal antibodies were tested in an ELISA system and had a reported detection range of 0.06-2.04 mIU/ml. It was reported that in the future, large scale prospective clinical DS screening will be required, with such a project being either present or being launched in many countries. It is believed that the use of highly specific antibodies will significantly contribute to the elucidation of the biological role of PAPP-A and its clinical usefulness in large screening programmes for the detection of abnormal human pregnancies (Bersinger et al., 1999).

The importance of producing a monoclonal antibody to PAPP-A has been described and for the reasons stated an in house monoclonal antibody was produced. The monoclonal antibody was thought to be a valuable asset for the determination of PAPP-A levels in the research undertaken in this work and could also be used in an ELISA system to detect abnormal levels of PAPP-A in first trimester pregnancy blood samples.

7.2 The Preparation of a monoclonal antibody for PAPP-A

7.2.1 Introduction

Most monoclonal antibodies are made in mouse cells and are suitable for diagnostic and many other purposes. In this study mice were used for monoclonal antibody production.

Monoclonal antibodies are produced by a single clone of B cells and thus consist of a single population of identical antibody molecules that are highly specific for a single epitope of an antigen. This is in contrast to polyclonal antibodies which are directed against several epitopes of an antigen and produced by several B cell clones. They thus have more potential for cross reactivity and therefore less specificity. Monoclonal antibodies have three very useful characteristics, their specificity of binding, their homogeneity and their ability to be produced in unlimited quantities.

The immortalisation of monoclonal antibody producing hybridoma cells was first described by Kohler and Millstein in 1975. They used a technique that took advantage of immortal malignant plasma cells (myeloma cells) which can be maintained in culture indefinitely and selected out those unable to secrete immunoglobulin and also deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Myeloma cells and spleen cells were fused using PEG and hybrid cells selected using HAT medium. The only cells that survive are hybrids with the myeloma's ability to continue to divide and the B cells ability to produce antibody and provide HGPRT to ensure survival.

The hybridoma cells producing the specific antibody can be screened by a variety of different methods and the positive ones cloned and then grown up in tissue culture. The monoclonal antibody is secreted into the cell culture supernatant and is relatively dilute (0.01-0.5 mg/ml culture fluid), this supernatant can then be purified to obtain a pure monoclonal antibody.

7.2.2. Primary stages in the preparation of the monoclonal antibody

7.2.2.1 Immunization of the mice

The method was as described in section 2.16.1

7.2.2.2 Fusion of the cells

The method employed was as described in section 2.16.2

7.2.2.3 Results

During the cloning procedure hundreds of supernatants had to be tested to identify any wells that contained cells secreting the antibody for PAPP-A. The screening procedure employed was described in section 2.16.3. The supernatants must be screened on the same day to enable the positive clones, i.e. those clones secreting the most antibody to be further subcultured. Clones producing an absorbance value of 0.8 or above in the screening assay were kept, the others rejected. RPMI alone gave an absorbance of approximately 0.2 in the screening assay (negative control). The mice tail bleed was used as a positive control and this gave an absorbance reading of between 1.4 and 1.9. The positive clones were recloned and tested several times to ensure they were still secreting the antibody. Many of the clones throughout the recloning lost their ability to secrete the antibody and were unstable.

The screening procedure picked up 10 positive clones, see table 7.1, after subcloning and these were further subcloned. The results, indicate that only five of those subclones continued to secrete the antibody, table 7.1. The antibody secreting subclones had all originated from the DF11 clone and the non antibody secreting subclones had all originated from the FC11 clone indicating that these clones had lost their ability to secrete the antibody over time.

Table 7.1 Results obtained from the screening procedure

Subclones	*Absorbance 405nm*	*Source of Clone*
Negative Control	0.067	
Positive Control	1.798	
1	1.713	DF11/E1/C5
2	1.615	DF11/E1/D3
3	1.232	DF11
4	1.586	DF11/D1/B6
5	1.550	DF11/D1/D5
6	0.074	FC11/D2/C5
7	0.141	FC11/A5/D5
8	0.151	FC11/A5/A5
9	0.098	FC11/D2/D2
10	0.091	FC11

Two positive single clones were chosen, numbers 1 and 2 and these were then grown up in cell culture to enable a sufficient amount of cell culture supernatant to be produced containing the antibody to carry out the next stage, which was the purification procedure.

7.2.3 Purification of the monoclonal antibody

7.2.3.1 Method

The method employed was described in section 2.16.4

6.3.3.2 Results

Two single clones were grown up to confluency in large scale culture and the supernatants (800ml) taken from each to be purified. The clones were identified as DF11/E1/C5 and DF11/E1/D3.

The majority of the protein was eliminated in the wash through procedure on the Hitrap column and the antibody was then eluted using the elution buffer. The total volume of antibody obtained was approximately 5ml from each clone and were at concentrations of 1mg/ml and 1.4mg/ml for DF11/E1/C5 and DF11/E1/ D3 respectively.

A Beckmann Electrophoresis gel was performed to determine the purity of the antibody, figure 7.1. The results indicated that the monoclonal antibody produced one band which was present in the immunoglobulin migration region of the gel.

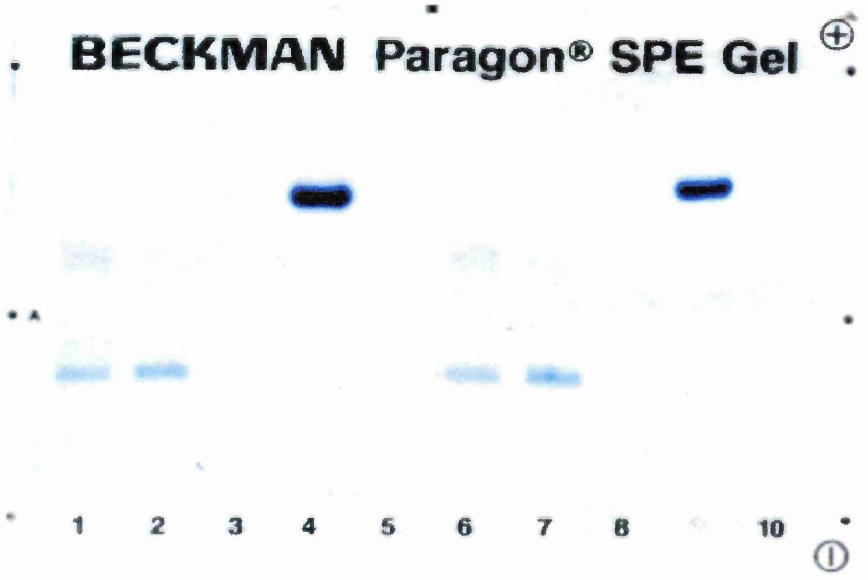


Figure 7.1 An SPE gel, lane 1 is monoclonal DF11/E1/C5, lane 2 is monoclonal DF11/E1/D3, lane 4 is serum albumin and lanes 6, 7 and 10 are identical repeat of the lanes 1, 2 and 4 respectively.

7.3 Comparison of ELISAs for PAPP-A.

Currently available ELISAs were compared with the ELISA developed 'in house' using the 'in house' monoclonal.

7.3.1 Commercial polyclonal assay (DAKO)

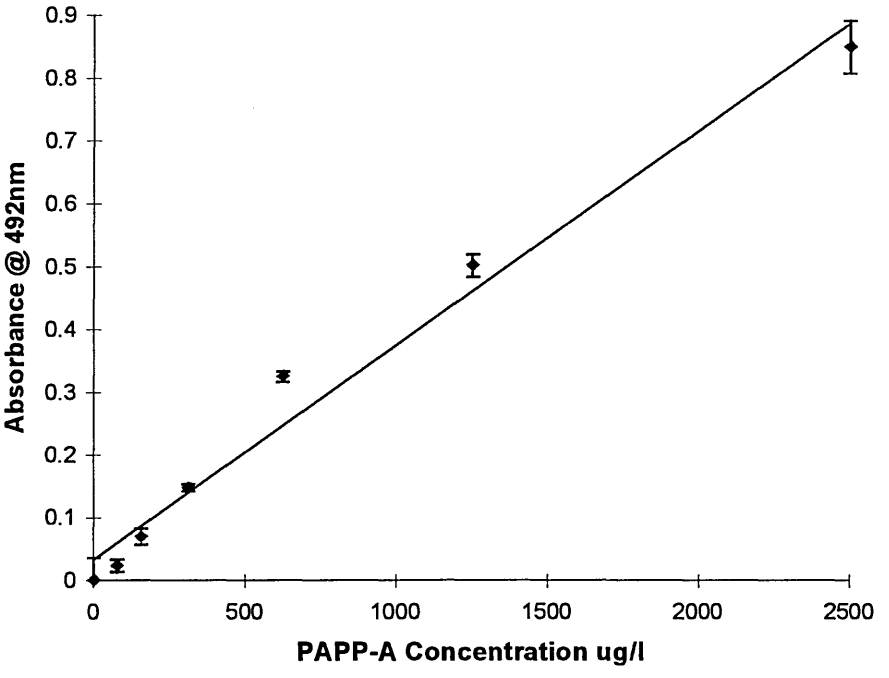
7.3.1.1 Method

As described in section 2.16.1.3

7.3.1.2 Results

The results obtained from the commercial assay suggested that it was detecting PAPP-A in the samples. A typical calibration curve was provided with the protocol and this was poor as the background absorbance reading was quite high, 0.26, and the highest absorbance reading obtained for the standards was 0.7 at a concentration of PAPP-A of 1.5mg/l. The detection range for the assay was between 0.2 –2 mg/l (0.2-2.0ug/ml). PAPP-A levels in serum samples at weeks 11/12 are approximately 1.5ug/ml (3mIU/ml) and in fetal disorders, 0.75ug/ml (1.5mIU/ml) (Bersinger et al., 1999), which would probably be within the detection range of the assay. However levels may be lower earlier in pregnancy as Bersinger also reported values as low as 0.025ug/ml (0.05mIU/ml) in samples obtained at week 7, and therefore these levels would not be easily detected. There is an absorbance difference between 300 and 1500 ug/l of only 0.3 (0.4-0.7) therefore a significant and possibly critical difference in PAPP-A levels even in the early detectable region would not result in a significantly different absorbance reading and therefore may go undetected. The standard curve obtained using late term pregnancy plasma and assuming a concentration of 50mg/l gave a slightly better calibration curve, than the one provided with the protocol, see figure 7.2.

Figure 7.2 A typical standard curve obtained from the ELISA in the range 0 - 2500ug/l



The results obtained suggest that this assay could not be used to determine PAPP-A levels in the early stages of pregnancy as it is not sensitive enough. The detection limits are approximately 200-2000 ug/l, assuming a PAPP-A concentration of 50ug/ml at term. Therefore it could not detect levels of 0.025ug/ml observed by Bersinger et al.(1999) in early pregnancy.

7.3.2 Dr Christiansen's monoclonal assay

7.3.2.1 Method

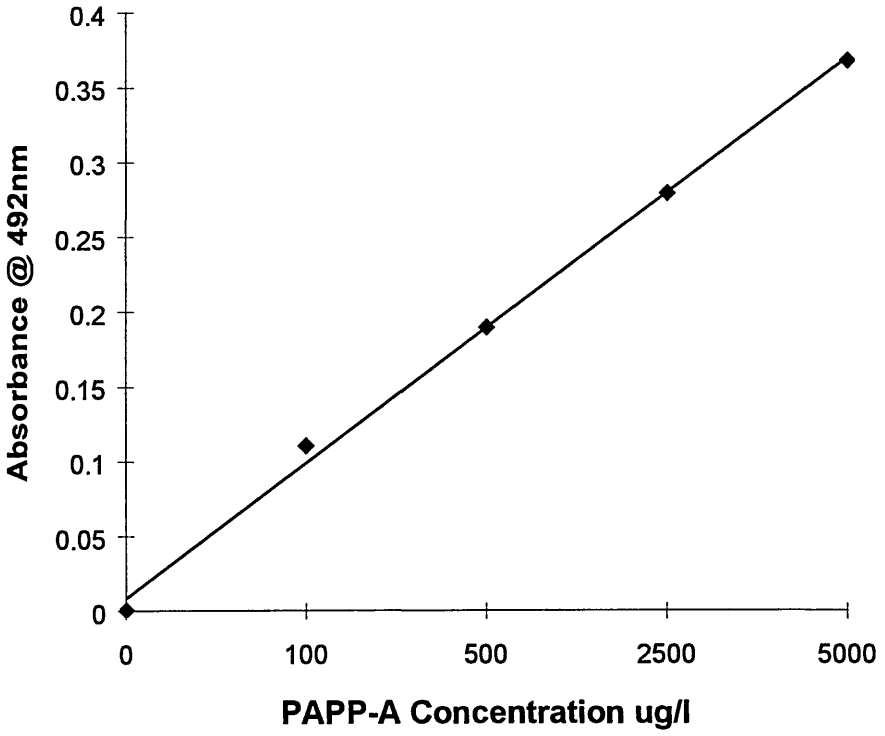
As described in section 2.16.2.3.

7.3.2.2 Results

The results obtained suggested that the assay was detecting PAPP-A in the samples and a reasonable standard curve was produced using the PAPP-A standard provided by Dr Christiansen. The standard curve absorbance readings were not as high as would have

been expected, compared to the standard curve produced using late term pregnancy plasma and diluted to the same concentrations. This suggested the provided standard PAPP-A had degraded or was not at the concentration given. A standard curve was constructed using term plasma, assuming a concentration of 50mg/l, see figure 7.3, and this was used to determine PAPP-A levels in samples from different stages of the purification. The lower detection limit was set at the concentration of PAPP-A giving an absorbance value twice that of the blank. Any lower absorbance reading cannot be assumed to be a true value compared to zero. The detection limits for PAPP-A in this assay were within the range 100-5000ug/l.

Figure 7.3 A typical standard curve obtained for PAPP-A in the range 0- 5000ug/l



7.3.3 The "In House" ELISA

7.3.3.1 Method

As described in section 2.16.3.2.

7.3.3.2 Results

Experiments that were carried out using the 'in house' monoclonal antibody and substituting this into the protocol of Dr Christiansens assay gave a very good standard curve, see figure 7.4. The assay was performed using late term pregnancy plasma for the standard and the samples obtained from the stages in the purification process. The standard curve was linear in the range 0 -1600ug/l with the detection limits 100-1600ug/l.

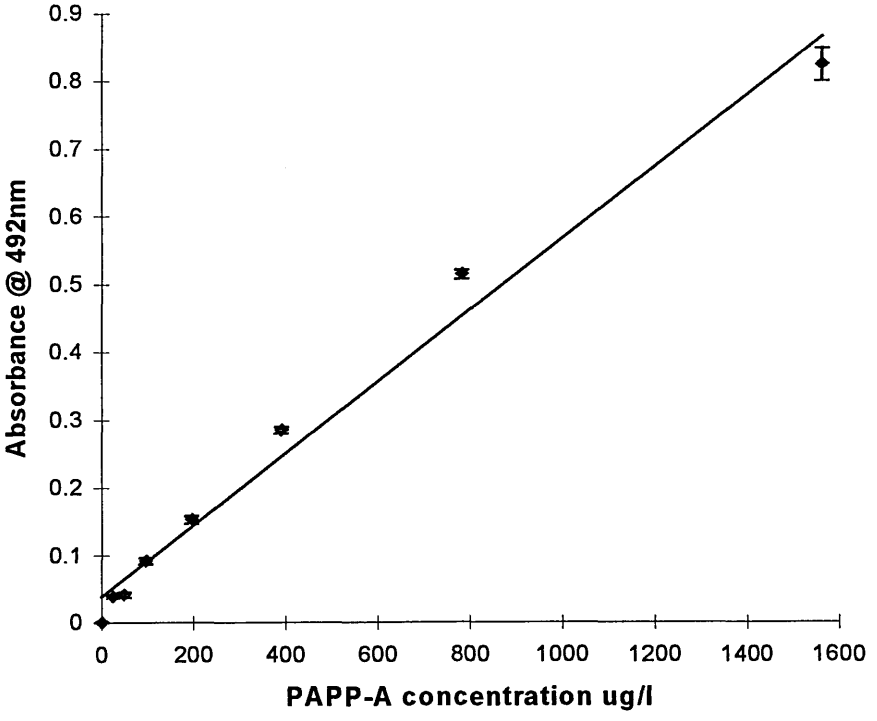


Figure 7.4 A typical standard curve for PAPP-A in the range 0-1600ug/ml.

7.3.4 Comparison of the 'in house' monoclonal ELISA with Dr Christiansens monoclonal ELISA

7.3.4.1 Method

The methods were carried out for both assays as described in sections 2.16.2.3 and 2.16.3.2 respectively. The protocols were identical with the substitution of Dr Christiansens monoclonal with the 'in house' monoclonal into the protocol provided by Dr Christiansen. The fractions obtained from the gel filtration stage of the purification were tested to compare Dr Christiansen's assay with the assay using the monoclonal antibody produced 'in house'.

7.3.4.2 Results

Both of the assays detected PAPP-A in the fractions. Dr Christiansen's assay however indicated a much broader PAPP-A containing peak than the assay using the in house monoclonal, see figure 7.5.

The results from Dr Christiansens assay suggest that PAPP-A is present in fractions 6-38 (an absorbance of less than 0.5 being rejected) and the results from the 'in house' assay suggest that PAPP-A is present in fractions 2-26. There is one major PAPP-A peak, as expected with the 'in house assay', whereas Dr Christiansen's assay contains many PAPP-A peaks. The elution profile is very poor for this assay and it does not seem to reflect the elution of PAPP-A from the column compared to the rocket immunoelectrophoresis results which showed distinct rockets only for fractions 8-26, see figure 3.3.

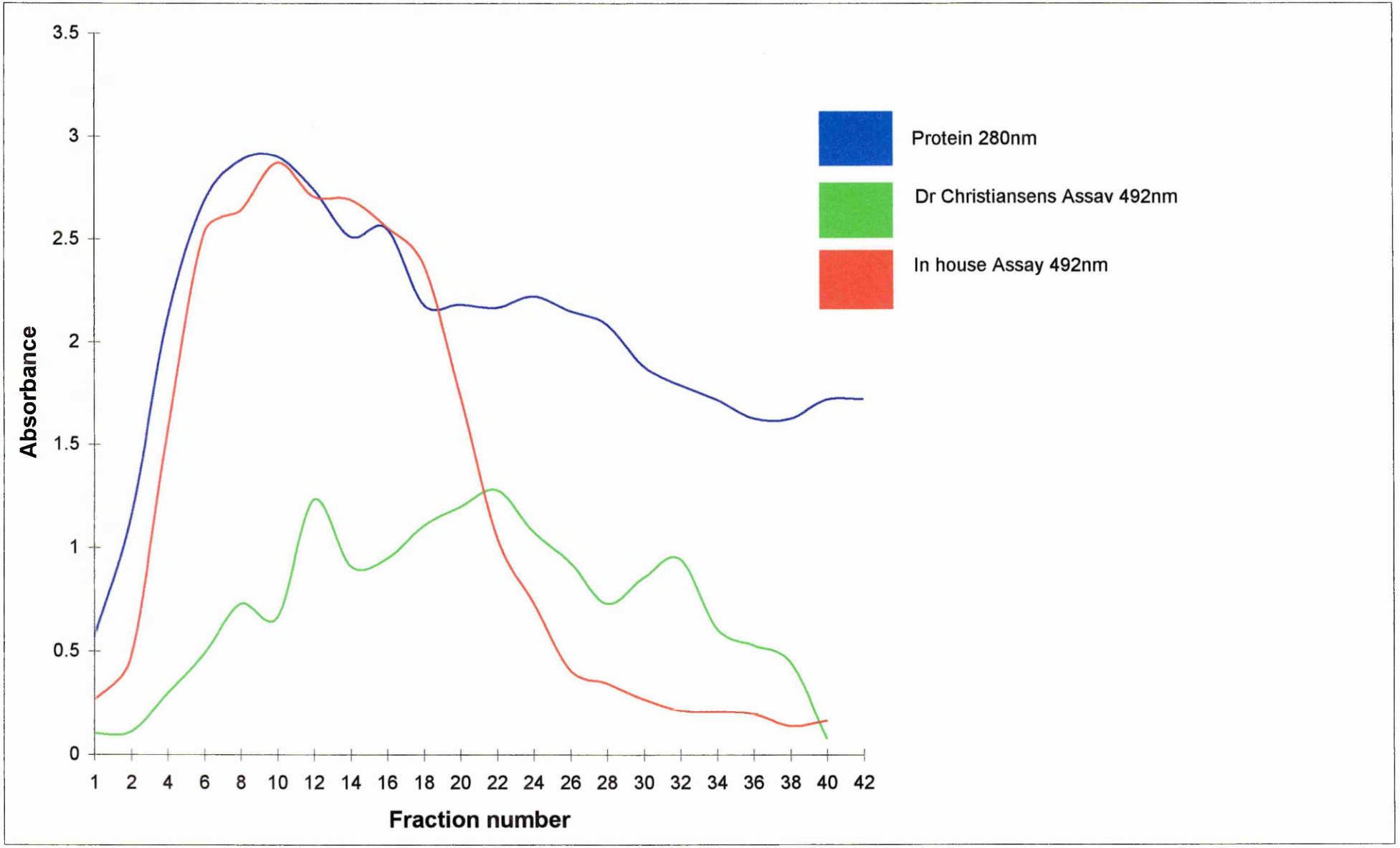


Figure 7.5. Absorbance traces obtained from the ELISA methods at 492nm and from protein analysis at 280nm.

7.4 Discussion

Two monoclonal antibodies were obtained from the preparation procedure and the purity was confirmed on a paragon electrophoresis gel. The antibodies were aliquoted and stored at -20^0C and these antibodies were used in the 'in house' assay.

The DAKO ELISA using the two polyclonal antibodies gave poor results. The sensitivity was very poor, with levels below 200ug/ml being undetected and the absorbance reading for the blank was high (0.26) suggesting there was non specific binding. Two polyclonal antibodies, one a derivative of the other, were used in the ELISA possibly enabling polyspecific binding to occur. Therefore the readings obtained may not be a true reflection of PAPP-A alone, other factors present in the standards or samples contributing. The ELISA would be suitable to determine the concentration of PAPP-A in pure samples but would not be suitable for the determination of PAPP-A in crude samples due to potential interference from contaminants present.

The ELISA protocol and reagents, very kindly donated by Dr Christiansen gave a reasonable standard curve, however the values were low, even after long incubation periods with the substrate. On repeating this assay with substitution of the 'in house' monoclonal for that donated by Dr Christiansen, higher absorbance values were obtained. The time of colour development was also much quicker and the absorbance values were much higher, for example the maximum absorbance obtained for the standard at 500ug/ml was 0.4 as opposed to 0.28 for Dr Christiansens ELISA. Detection levels for the ELISAs were very similar, both assays detecting levels as low as 100ug/l (200 mIU/l assuming 100 IU/l at term).

The results from the standard curves indicate that the 'in house' assay and Dr Christiansen's assay may be used as a potential screening assay for samples obtained from week 8 onwards in pregnancy. Samples obtained before week 8, should not be screened using the assays described as they would not be sensitive enough to detect any differences between normal and abnormal levels of PAPP-A in serum.
The results obtained from the elution profiles from Dr Christiansen's assay suggest that the monoclonal antibody may not be specific for PAPP-A and may be detecting other proteins in the fractions. At present it cannot be confirmed that the monoclonal

8.1 Discussion

The aim of this study was to determine PAPP-A's involvement, if any, in the remission of RA in terms of immunomodulatory and inflammomodulatory effects. There have been many studies of the purification and characterisation of PAPP-A, mostly controversial. The effect of PAPP-A on proteinases has been studied previously and the results obtained in this study confirmed the finding of some of the most recent work carried out on PAPP-A. There has been no published data however, on the effect of PAPP-A on cytokine and PGE_2 production by cell lines and the ability of PAPP-A to bind to cytokines and also to cartilage, which makes the data presented here unique, see table 8.1.

A major part of this work was the development of a purification protocol for PAPP-A. A 4 stage process was developed which produced pure PAPP-A as determined by SDS PAGE and western blotting. The PAPP-A isolated, did not have the pro MBP attached, as there was no band corresponding to this detected on SDS PAGE, as detected by other workers (Oxvig et al., 1995a). Whether proMBP was ever attached to the PAPP-A purified and has become detached on purification is uncertain and could not be clarified as at present there are no commercial monoclonal antibodies available to proMBP. PAPP-A therefore may not be in its normal physiological, circulating form and therefore the actions seen for the pure PAPP-A may not truly reflect its actions in vivo. This however, is true for all the work on PAPP-A to date as purification procedures may have structurally slightly altered the PAPP-A molecule. Alternatively there may be different isoforms of the PAPP-A/proMBP complex or other complexes containing either or both of the two proteins PAPP-A and proMBP that may exist in different stages of pregnancy (Qin et al., 1997). The PAPP-A species purified may depend on the protocol used. The protocol described in this thesis may favour purification of uncomplexed PAPP-A. It has also been reported that other proMBP complexes are present in term serum, complexes with angiotensinogen and complement 3dg (Oxvig et al., 1995b) and as yet nothing is currently known about the function of these complexes and their serum concentration in DS pregnancies.

This project was totally dependent on an available source of PAPP-A and on occasion was slowed due to lack of availability of pregnancy plasma. Continuation of this work in the future would include the investigation of a possible alternative source(s) of

PAPP-A. One potential source of PAPP-A would be from secreting endometrial cells, Bersinger et al.(1997) observed small amounts of PAPP-A production in endometrial explant cultures obtained in the secretory phase of the normal menstrual cycle. The supernatant obtained from these cultures would still have to undergo purification to to obtain pure PAPP-A. Another source of PAPP-A would possibly be from cell lines as it has been reported that fibroblasts, obtained from human foreskin produce PAPP-A (Lawrence et al., 1999). Short term cultures of trophoblastic and decidual explants have also been reported to produce PAPP-A (719 +/- 172 ng/ml) in vitro (Barnea et al., 1986).

The results indicate that PAPP-A does stimulate the production of PGE_2 and also IL-1, IL-6 and TNFα in THP 1 cells. These cytokines have been reported to have an involvement in the pathogenesis of RA (Harris, 1990, Ridderstad et al., 1991). PAPP-A however had no effect on the stimulation or inhibition of PGE_2 and IL-6 production by the MG-63 cell line. Further work needs to be carried out to determine the actual mechanism of stimulation of the cytokine production in the THP 1 cells by PAPP-A. One possibility, as discussed earlier (section 4.3), is the involvement of the CD14, LPS receptor. This could be tested by incubating the cells with an anti CD14 antibody which blocks the CD14 receptor sites on the cell surface to see whether the effects of PAPP-A would then be inhibited.

Studies of cellular binding of labelled PAPP-A to cell lines may also help in determining whether there are specific receptors for PAPP-A and their distribution. Responsiveness of cell lines to PAPP-A in terms of their cytokine production may also indicate the presence of PAPP-A receptors and their distribution.

PAPP-A does not appear to inhibit the actions of cytokines by binding to IL-1β or TGFβ. PAPP-A therefore does not act in the same way as α_2m by inactivating them by complex formation.

The large numbers of neutrophils entering the synovial joint in RA should bring in large amounts of proteinases. However on direct assay of active proteinases in rheumatoid synovial fluid, little activity has been found probably due to inactivation of

proteinases by inhibitors in the synovial fluid (Harris et al., 1969). However neutrophil proteinases may still be involved in cartilage breakdown in two ways, one being that the proteinases escape inhibitors in the synovial fluid that would render them inactivate by adhering to the cartilage surface and acting locally. Another theory is that of 'frustrated phagocytosis' which is direct release of enzymes onto the cartilage surface by the cells adhering to it. Microscopic evidence has shown that neutrophils adhere to the cartilage surface and then release enzymes, directly leading to the erosion of tissue (Henson, 1973; Cochrane, 1977; Mohr et al., 1980). A molecule that may coat the surface of the cartilage perhaps preventing binding of neurophils or their enzymes could protect cartilage. PAPP-A is known to bind to cartilage and may form such a protective layer on the cartilage. Alternatively its elastase inhibitory activity may prevent cartilage damage.

The observed stimulation of resorption by PAPP-A in explant cultures could result from degradation of PAPP-A in the presence of enzymes released from chondrocytes, releasing proteoglycan that may be detected in the DMB assay and augment the apparent proteoglycan release. It may also be produced by the breakdown products of PAPP-A stimulating cartilage breakdown in a similar way to fibronectin fragments, as discussed in section 5.2.

Further work would be to look at the effect of PAPP-A on IL-1 stimulated cartilage resorption, since the IL-1 stimulatory pathway is different from that of the Ret pathway (Buttle et al. 1993). In addition effects of PAPP-A fragments on resorption could be studied, in particular those produced by aggrecanase digestion.

The proteinase inhibitory activities of PAPP-A have been studied by a number of workers over the years but still remain controversial. In this study inhibition of elastase has been demonstrated but no inhibition seen for plasmin, trypsin or cathepsin B using peptide substrates. This agrees with the work carried out by Oxvig et al. (1994). Elastase, though involved in the pathogenesis of RA, is not solely responsible for connective tissue breakdown. Thus the role of PAPP-A in reducing cartilage breakdown in RA during pregnancy by proteinase inhibition may be limited. Further work in this area would be to try and establish if PAPP-As effects on any of the proteinases studied would be different using substrates with varying M_r values. This

investigation would either confirm or refute the proposed theory that PAPP-A may be acting via a trapping mechanism (Zorin et al., 1995).

Overall the results obtained indicated that PAPP-A does not have have a key role in the remission of RA which occurs in pregnancy.

Generally the functions of PAPP-A are not clear even in pregnancy and further investigations may uncover possible roles. One possibility may be in the maintenance of a successful pregnancy until term. PAPP-A has been reported to be present in seminal plasma and present at ovulation, it may be immunosuppressive in vivo and serve to blunt the maternal immune response to sperm antigens and following fertilisation to the fertilised ovum. This hypothetical immunoregulatory role would continue throughout pregnancy to protect the developing fetus by the continued presence of PAPP-A in the maternal circulation. However as discussed in this thesis, its immune functions are by no means clear-cut.

In view of recent publications, PAPP-A shows most promise in its use in the detection of first trimester fetal abnormalities (Wald et al., 1996; Bersinger et al 1999). Development of the 'in house' monoclonal antibody for potential use in a screening assay for fetal abnormalities in first trimester serum samples from pregnant women will continue.

Table 8.1 A summary of the activities of PAPP-A.

	Parameter	*Effect*
THP 1 cell line	PGE production	Significant ⇑
	IL-1β production	Significant ⇑
	IL-6 production	Significant ⇑
	TNF α production	Significant ⇑
MG-63 cell line	PGE production	None
	IL-6 production	None
Cytokine Binding	IL-1β	No binding
	TGFβ	No binding
Proteinase activity	Cathepsin B	None
	Elastase	Significant ⇓
	Plasmin	None
	Trypsin	None
Cartilage Resorption (Ret stimulated)	% GAG release	Significant ⇑
Cartilage Binding	Human cartilage sections	Binding

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Abbreviated Terms

α PAG	Pregnancy associated globulin
α_2m	α_2 macroglobulin
AAPV-ρNA	Methoxysuccinyl-L-alanine-L-alanine-L-proline-L-valine-ρ-nitroaniline
AC	Amniocentesis
ADAM	A disintegrin and a metalloproteinase
AFP	α-fetoprotein
Ag	Antigen
ANOVA	Analysis of variance
APS	Ammonium persulphate
ATCC	American type culture collection
BAN	N-tert-butoxy-carbonyl-L-alanine- ρ-nitroaniline
BCA	Bichinchoninic acid solution
Boc-L-glu-L-lys-L-lys-MCA	N-tert-butoxy-carbonyl-L-glutamic acid-L-lysine-L-lysine-4-methyl coumaryl-7-amide
BSA	Bovine serum albumin
BTN	N-butoxy-L-tyrosine-ρ-nitroaniline
BTS	Blood transfusion service
C_3	Complement component 3
CD 14	Cluster of differentiation antigen 14
Ci	Curie
CN-Br-act.sep.4B	Cyanogen bromide activated sepharose 4B
cpm	Counts per minute
CS	Chondroitin sulphate
Cu	Copper
CVS	Chorionic villus sampling
DAB	Diaminobenzidine
DL-BAPNA	Nα- Benzoyl-DL-arginine-ρ-nitroaniline
DMB	1,9-dimethylmethylene blue
DMEM	Dulbecco's Modified Eagles Medium

DMSO	Dimethyl sulphoxide
Ds	Dermatan sulphate
DS	Downs syndrome
DTT	Dithiothreitol
ECACC	European collection of animal cell cultures
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked-immunosorbant-assay
FCS	Foetal calf serum
FDA	Federal drug agency
FPLC	Fast protein liquid chromatography
GAG	Glycosaminoglycan
GM-CSF	Granulocyte/macrophage colony stimulating factor
H_2O_2	Hydrogen peroxide
HB-EGF	Heparin binding epidermal growth factor
hCG	Human chorionic gonadotrophin
HCl	Hydrochloric acid
HGRT	Hypoxanthine-guanine phosporibosyl transferase
HLE	Human leukocyte elastase
hPL	Human placental lactogen
HRP	Horse radish peroxidase
IEC	Ion exchange chromatography
IGF	Insulin like growth factor
IgG	Immunoglobulin G
IL 1	Interleukin 1
IL 6	Interleukin 6
IL	Interleukin
ISF I	Immunosupressive factor I
ISF II	Immunosupressive factor II
Ki	Inhibitor constant
LDL	Low density lipoprotein
LDLRP	Low density lipoprotein receptor related protein
LIF	Leukaemia inhibitory factor

LPS	Lipopolysaccharide
LRP	Lipoprotein receptor related protein
MBP	Major basic protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
M_r	Relative molecular mass
NaCl	Sodium chloride
NSAIDS	Non steroidal anti-inflammatory drugs
NSB	Non specific binding
OPD	o-Phenylenediamine dihydrochloride
PA	Plasminogen activator
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAPP	Pregnancy associated protein
PAPP-A	Pregnancy associated protein A
PBS	Phosphate buffered saline
PG	Proteoglycans
PGE_2	Prostaglandin E_2
PHA	Phytohaemagglutinin
PI	Proteinase inhibitor
PMA	Phorbol 12-myristate 13-acetate
PP	Placental proteins
PRL	Prolactin
Pro MBP	proform major basic protein
PTI	Pancreatic trypsin inhibitor
PVDF	Polyvinyl difluoride
PZP	Pregnancy zone protein
Ret	all-trans-retinoic acid
RA	Rheumatoid arthritis
RIA	Radioimmunoassay
RPMI	Roswell Park Memorial Institute
SAAVN	N-Succinyl-L-anlanyl-L-alanyl-L-valine-ρ-nitroaniline

SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SP 1	Pregnancy specific glycoprotein
TBS	Tris buffered saline
TBST	Tris buffered saline with tween
TC	Total counts
TEMED	N,N,N,N'- tetramethylethylenediamine
TGFβ	Transforming growth factor β
TIMP	Tissue inhibitor of matrix metalloproteinase
TNFα	Tumour necrosis factor α
TrIFMA	Time resolved immunofluorometric assays
Tris	Tris [hydroxymethyl] aminomethane
UE3	Unconjugated estriol
uPA	Urokinase plasminogen activator
w/v	weight per volume
WHO	World health organisation