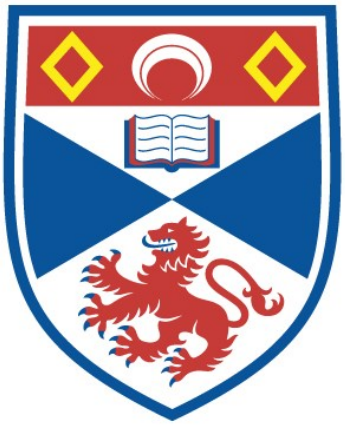
MORPHOGENESIS OF THE ELASTIC FIBRE: A STUDY IN BOVINE LIGAMENTUM NUCHAE AND HUMAN FOETAL AORTA

Anna Maria Jaques

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A Thesis Presented by Anna Maria Jaques

to

The University of St Andrews

in Application for

The Degree of Doctor of Philosophy

1987

DECLARATION.

I hereby declare that the following thesis is based on work carried out by me, that it is my own composition and that no part of it has been previously presented for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Professor A. Serafini-Fracassini.

CERTIFICATE.

I hereby certify that Anna M. Jaques has spent nine terms engaged in research work under my direction and that she has fulfilled the conditions of Ordinance General No. 12, and Resolution of the University Court, 1967, No. 1, and that she is qualified to submit the accompanying Thesis for the degree of Doctor of Philosophy.

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This thesis is dedicated to my husband Bryon Charles Jaques and my parents Gilbert and Nancy Campbell.

ABBREVIATIONS

AAN	α-Aminoacetonitrile
BAPN	β-Aminoproprionitrile
BSA	Bovine Serum Albumin
DEAE	Diethylaminoethyl cellulose
DMSO	Dimethyl Sulphoxide
DTT	Dithiothreitol
Ed(s)	Editors
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Foetal Calf Serum
FECTS	Federation of European Connective Tissue Society
HPMA	2-Hydroxypropyl methacrylate
PAGE	Polyacrylamide Gel Electrophoresis
PAS	Periodic Acid / Schiff Reagent Stain
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulphate
TBS	Tris Buffered Saline
TEMED	NNN'N'-Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane

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CHAPTER ONE.

LITERATURE REVIEW.

1.1. INTRODUCTION

A mammalian elastic fibre can be schematically visualised as consisting of a core of elastin surrounded by a layer of 11nm microfibrils - usually referred to as the microfibrillar component. These fibres are widely distributed in connective tissues and are particularly abundant in anatomical locations such as arteries, lung and skin in which a high degree of reversible extensibility is required for their physiological function. Changes due to senescence or disease in the functioning of these tissues are associated with extreme degradation of the components of the elastic fibres and/or with their disordered synthesis. The resulting loss of physiological function is observed in major human degenerative diseases such as atherosclerosis and pulmonary emphysema.

Progress towards a fuller understanding of the pathogenesis of these diseases has been inhibited by difficulties in the biochemical characterisation of the elastic fibres and a lack of information concerning elastogenesis. Although considerable advances have been made in the molecular biology of elastin with the isolation and partial sequencing of the elastin gene, there still remains major deficiencies in our understanding of the factors which control elastin biosynthesis, particularly the mechanism of elastin fibrillogenesis and its role in the morphological organisation of elastic tissue.

The studies described in this thesis were undertaken in order to further our understanding of elastin fibrillogenesis and in particular to examine the role of the elastin-associated microfibrils in this process. This introductory chapter attempts to put these studies into perspective by reviewing the literature on the morphology and biochemistry of the two components of the elastic fibre namely elastin and elastin-associated microfibrils, and by outlining present theories of elastin fibrillogenesis.

1.2. THE MORPHOLOGY OF THE ELASTIC FIBRE.

The protein elastin, which is similar in mechanical properties to polymeric rubbers (Gosline, 1976), is responsible for the reversible extensibility displayed by elastic fibres present in the ligamentum nuchae of ungulates and the arteries, lungs, skin and elastic cartilage of mammals. Examination of these connective tissues by conventional histological techniques have shown that elastic fibres have three distinct morphologies:-

(a) In the tunica media of the adult aorta, elastic tissue forms concentric layers of fenestrated lamellae, approximately 3um in thickness, which are interconnected by bridges of elastic fibres. The adult human aorta consists of 60-65 lamellae (Ayer, 1964). Smaller longitudinal fibres predominate in the tunica intima which is subjacent to the endothelial surface.

(b) In elastic cartilage there is a three-dimensional network of elastic fibres, 1-2um in diameter, extending throughout the matrix (Sheldon and Robinson, 1958; Serafini-Fracassini and Smith, 1974).

(c) In the ligamentum flavum and the lung, elastic fibres are small (diameter <10um), variable in length and tend to branch and fuse in an irregular manner. In ligamentum nuchae, which is subject to unidirectional stress, the elastic fibres have a preferred orientation parallel to the major ligament axis.

In ultrastructural studies, elastic fibres are seen to be comprised of two distinct components: a core of polymeric elastin

which appears amorphous and stains lightly with cationic stains such as lead citrate and uranyl acetate; and a peripheral mantle of microfibrils, 10-14nm in diameter, which display a strong affinity for heavy metal stains (Fahrenbach et al., 1966; Greenlee et al., 1966).

Quintarelli et al. (1973), suggested that the electron-transparent appearance of elastin is due to either the fixatives sterically altering metal-binding sites or the plastic-embedding material blocking and concealing available sites on the tissue. However, it should be noted that in calf ligamentum nuchae (Fahrenbach et al., 1966; Kewley et al., 1978), rabbit ear cartilage (Cox and Peacock, 1977) and rat aorta (Thyberg et al., 1979), newly synthesised elastin in immature elastic fibres stains heavily with conventional electron microscopic stains and that the intensity of staining decreases as the fibre matures. This has been attributed to changes in the quaternary structure of elastin which either hinder metal-ion diffusion (Cliff, 1971) or result in the transfer of ionisable groups from an aqueous to a mainly hydrophobic environment with the consequent alteration of their ion-binding characteristics (Serafini-Fracassini et al., 1978).

Although the elastic fibre core is usually described as amorphous, in certain conditions it has revealed a fibrillar substructure. In 1963, Gotte and Serafini-Fracassini first reported the presence of thin filaments in negatively stained preparations of purified elastic fibres after prolonged

sonication. Fahrenbach et al. (1966) found that tangled filaments were also present in immature elastic fibres of foetal bovine ligamentum nuchae. Cliff (1971) showed that prolonged treatment (72 hours) of rat aortic tissue with 2% osmium tetroxide at 4°C before embedding, produced an open meshwork of branched and tangled fibrils. Katsuda et al. (1982) noted that a similar appearance could be produced in elastic lamellae with periodic acid at 18°C. In the latter two studies, the fine fibrils or filaments had an average diameter of 4nm.

An alternative approach has been provided by the analyses of the substructure of mechanically disrupted elastic fibres and of coacervates of insoluble elastin, tropoelastin - the biosynthetic precursor of insoluble elastin (see page 9), and synthetic elastin peptides. Cox et al. (1973) in a study of mature elastin, solubilised by partial oxalic acid hydrolysis and referred to as α-elastin (Partridge et al., 1955), observed that negatively stained coacervates of this material form filaments 5nm in diameter. Similarly, coacervates of tropoelastin and of synthetic polypenta- and polyheptapeptides consisting of typical repeating elastic sequences, were also shown to contain fibrils composed of parallel aligned filaments approximately 5nm in diameter (Cox et al., 1974; Volpin et al., 1976). In negatively contrasted preparations of highly purified elastin, Gotte et al. (1976) observed that the fibres consist of a lateral array of 4nm filaments which have a beaded appearance with an axial periodicity of 3.5 - 4.5nm, varying according to the amount of

longitudinal stretching in the sample. These findings were interpreted as indicating that each filament is comprised of a helical arrangement of two polypeptide chains with cross-linking regions bonding the filaments into larger fibrillar aggregates.

These results, concerning the dimensions and periodicity of the primary filament, were confirmed by small angle X-ray diffraction and electron microscopic studies of highly purified bovine ligamentum nuchae elastin by Serafini-Fracassini et al. (1976,1978). However, the identification under the electron microscope of isolated primary filaments appeared to be incompatible with the primary filaments being joined together within the fibril as a continuous reticulum stabilised by covalent cross-links. This observation led Serafini-Fracassini et al. (1976,1978) to postulate an alternative arrangement in which the cross-links are restricted to the primary filament and the fibrils, composed of such primary filaments, held together by non-covalent interactions.

Pasquali-Ronchetti et al. (1979) and Fornieri et al. (1982), using cryotechniques to examine elastic fibres, found no ordered structure in longitudinal or transverse freeze-fracture replicas of unstretched specimens. However, fibres stretched to 150-200% of their resting length showed a filamentous structure with a three-dimensional meshwork of filaments, each filament being composed of globular subunits 5nm in diameter. These findings are particularly significant because the specimens were fully hydrated, this being an essential prerequisite for the functional

integrity of elastin (Weis-Fogh and Anderson, 1970; Gosline, 1978).

Recently, in order to gain further insight into the quaternary structure of the primary filament, Serafini-Fracassini et al. (1985) isolated and characterised multichain peptide fragments of bovine ligamentum nuchae elastin. These were produced from highly purified elastin by elastase digestion in the presence of sodium dodecyl sulphate. Chromatographic fractionation of the digest yields two peptide fractions, with the major fraction (F2) having a molecular weight distribution between 25,000 and 34,000 and a chain weight, assessed by N and C terminal analysis, of approximately 6,000. These results are consistent with F2 being a multichain molecule comprising of four peptide chains linked by three polyfunctional amino acids. This model interprets the beaded filament described earlier as consisting of four peptide chains, with the beads arising from the juxtaposition of four folded intercross-linked regions belonging to adjacent chains, and the intervening narrow segments consisting of four tightly-packaged α-helical regions.

1.3. ELASTIN

1.3.1. Fibrillar Elastin.

Elastin, present in fully developed fibres and lamellae, is a chemically inert protein which forms a supramolecular structure stabilised by inter-chain bonds involving polyfunctional amino acids. Techniques for the isolation of this insoluble material have therefore relied on the removal of all other tissue components. This was first achieved by either autoclaving or extracting the tissue with hot dilute alkali (Lowry et al., 1941; Lansing et al., 1952). When prepared in this way, elastin has a very characteristic amino acid composition, as shown in Table 1.1. (column 1). Glycine, alanine, valine and leucine together comprise approximately 80% of the total amino acid residues. Glycine alone accounts for about 30% of total residues and the valine content is the highest in any known protein. Purified elastin contains 1-1.5% hydroxyproline but no hydroxylysine, tryptophan, cysteine, methionine or histidine (Gotte et al., 1963). Fibrillar elastin also contains the following lysine-derived polyfunctional amino acids:- desmosine, isodesmosine, aldol condensation product, dehydrolysinonorleucine, lysinonorleucine, dehydromerodesmosine and merodesmosine, all of which are responsible for the cross-linking of the peptide chains.

The amino acid compositions of insoluble elastins isolated from different bovine tissues have been shown to be remarkably similar (Gotte et al., 1963). Sandberg (1976) however, has pointed out that extraction by autoclaving or with hot alkali, may lead to a "common composition" due to degradation of more labile amino acid residues. Less drastic methods involving a combination of treatments with enzymes, chaotropic solutions and mild hydrolytic agents are therefore now applied (Steven and Jackson, 1968; Ross and Bornstein, 1969; Richmond, 1974; Serafini-Fracassini et al., 1975b; Rasmussen et al., 1975; Starcher and Galione, 1976). The amino acid composition of elastin obtained by these methods (Rucker and Tinker, 1977a; Spina et al., 1979; Sandberg et al., 1981b; Foster, 1982) has been shown to contain more polar amino acids (Table 1.1. column 2).

1.3.2. Tropoelastin.

Tropoelastin, the soluble precursor of mature elastin, can be isolated by preventing the enzyme lysyl oxidase from cross-linking elastin lysyl residues. Since this enzyme requires copper as a cofactor (Siegel et al., 1970), the establishment of dietary copper deficiency results in its inhibition. Soluble elastin then accumulates in developing elastic tissues in sufficient amounts to be extracted and characterised. Tropoelastin has been isolated in this way from the aorta of copper-deficient pigs (Sandberg et al., 1969; Smith et al., 1972)

and chicks (Rucker et al., 1973) and from the ligamentum nuchae of copper-deficient calves (Whiting et al., 1974). Elastin cross-linking can also be inhibited by the lathyrogens β-aminoproprionitrile (BAPN) and α-aminoacetonitrile (AAN) which irreversibly inactivate lysyl oxidase probably by covalent binding (Siegel, 1979). This method has been used to extract tropoelastin from lathyritic chick aorta (Sykes and Partridge, 1974; Foster et al., 1975) and the aorta and ear cartilage of lathyritic pigs (Foster et al., 1980a).

Tropoelastin has a molecular weight of 72,000-74,000 Daltons, (72-74-kDa) and contains approximately 850 amino acid residues (Sandberg et al., 1977). It is soluble in cold salt solutions but undergoes reversible coacervation within the temperature range of 27-37°C, as a function of concentration (Sandberg et al., 1969).

Tropoelastin has been shown to be the precursor of cross-linked elastin by several criteria:-

(a) Its amino acid composition, shown in Table 1.1. (column c), is virtually identical to that of insoluble elastin with the exception of a higher lysine content and the absence of polyfunctional amino acids (Whiting et al., 1974).

(b) Antibodies prepared to oxalic acid-soluble elastin i.e. (α-elastin) cross-react with tropoelastin (Daynes et al., 1977; Mecham and Lange, 1982b).

(c) ^{14}C-lysine labelled tropoelastin added to smooth muscle cells in tissue culture results in the appearance of the label in

the desmosine and isodesmosine of the newly deposited elastin (Narayanan and Page, 1976).

TABLE 1.1. AMINO ACID COMPOSITION OF BOVINE LIGAMENTUM NUCHAE TROPOELASTIN AND MATURE INSOLUBLE ELASTIN.

SOURCE:	Mature Elastin[A] (Neuman) 1949	Mature Elastin[B] (Spina et al.) 1979	Tropoelastin (Whiting et al) 1974.
Hydroxyproline	16.0	8.5	8.0
Aspartic Acid	4.0	5.7	6.0
Threonine	9.0	9.2	8.0
Serine	7.0	8.6	9.0
Glutamic Acid	17.0	15.1	15.0
Proline	165.0	114.0	92.0
Glycine	274.0	336.2	316.0
Alanine	173.0	224.0	220.0
Valine	159.0	129.9	147.0
Half-Cysteine	2.0	0.0	0.0
Methionine	0.5	0.0	0.0
Isoleucine	36.0	23.6	20.0
Leucine	79.0	58.6	55.0
Tyrosine	10.0	5.8	6.0
Phenylalanine	39.0	28.9	32.0
Hydroxylysine	0.0	0.0	0.0
Lysine	**3.0**	**3.2**	**49.0**
Histidine	0.5	0.4	trace
Arginine	6.0	5.7	5.0
α-Hydroxylysine	N.D.	0.6	0.0
Aldol Condensation Product	N.D.	3.7	0.0
Dehydrolysinonorleucine	N.D.	0.0	0.0
Lysinonorleucine	N.D.	2.1	0.0
Dehydromerodesmosine	N.D.	0.1	0.0
Merodesmosine	N.D.	0.5	0.0
Isodesmosine	N.D.	5.4	0.0
Desmosine	N.D.	10.2	0.0
Amide	N.D.	16.6	N.D.

A :- Mature Elastin prepared by Autoclaving.
B :- Mature Elastin prepared by collagenase and guanidine hydrochloride treatment.

Values are expressed as residues per 1000 total amino acid residues and have been corrected for hydrolytic losses.

1.3.3. Molecular Models of Elastin.

Sequence data from tryptic peptides of tropoelastin (Foster et al., 1973; Gerber and Anwar, 1974; Sandberg et al., 1977) indicate the presence within the molecule of long runs of sequence of hydrophobic amino acid residues with frequently repeating tetrapeptide (-gly-val-pro-gly-), pentapeptide (-val-pro-gly-val-gly-) and hexapeptide (-ala-pro-gly-val-gly--val-) sequences. These areas are separated by much shorter polyalanyl sequences in the form of α-helices which contain the lysyl residues involved in cross-linking (Mammi et al., 1968; Mammi et al., 1970).

Recently, the published nucleotide sequences and translated amino acid sequences for the fourteen exons at the 3' portion of the bovine elastin gene have shown that the 'hydrophobic' and 'cross-link' regions of elastin are encoded by separate exons which appear to alternate with each other, except in the region closest to the carboxy terminus. The hydrophobic exon 13 contains the hexapeptide -ala-pro-gly-val-gly-val- repeated five times in pig elastin and four times in bovine elastin (Cilia et al., 1985a). While the polyalanyl regions are in an α-helical conformation, the repeating hydrophobic sequences probably adopt the β-turn as the dominant conformational feature, as it has been shown that synthetic polymers of the tetra-, penta- and hexapeptides form a right-handed β-spiral structure (Urry and Long, 1977; Urry, 1978).

Various models which have been proposed for the quaternary structure of elastin are briefly outlined below:-

A 'corpuscular model' proposed by Partridge (1966) and modified by Weis-Fogh and Anderson (1970) envisages elastin as a two-phase system consisting of a lattice of cross-linked globular domains of elastin surrounded by free aqueous spaces.

The second model views elastin essentially as a 'random network' of cross-linked tropoelastin chains similar to that found in polymeric rubbers (Hoeve and Flory, 1974).

Sandberg et al. (1981b) proposed an 'oiled-coiled' model which views the extensible segments as being formed by the broad left-handed spirals of the repeating tetrapeptide -gly-val--pro-gly interspersed with the α-helical crosslinked regions.

Both the 'oiled-coiled' and 'corpuscular' models are not consistent with nuclear-magnetic resonance observations (Torchia and Piez, 1973) and do not allow for volume changes during deformation (Hoeve and Flory, 1974).

In 1983, Urry proposed a 'fibrillar model' for elastin in which right-handed β-turns of polypentapeptide -val-pro-gly-val--gly-, are suspended within a particular hydrophobic construct, called a 'librating' peptide segment. These 'suspended segments' which have recently been shown to be capable of undergoing large amplitude librations (Urry, 1987) are thought to be responsible for the elasticity of the protein. This model strongly relies on hydrophobic interactions within an aqueous environment for the maintenance of elasticity and is supported by the ultrastructural

observations of fibrillar forms of elastin-derived materials, as discussed in Section 1.2.

1.3.4. Biosynthesis of Elastin.

Studies on elastin deposition during the foetal development of bovine ligamentum nuchae have indicated that an intense period of elastin biosynthesis begins near the onset of the third trimester of gestation and diminishes in early postnatal life (Cleary et al., 1967; Kewley et al., 1978; Serafini-Fracassini, 1984).

In the aorta, however, elastin synthesis commences earlier prenatally (Haust et al., 1965; Kadar et al., 1971) and also considerable variation in the levels of collagen and elastin synthesis can be observed in different regions of the aorta (Hill and Davidson, 1986). In the rat, the ratio of elastin to collagen is two to one in the upper thoracic aorta, one to one at the level of the diaphragm and one to two in the abdominal aorta (Wirtschafter and Sandberg, 1968). Also, in one day old piglets, elastin synthesis has been found to be maximal in the upper thoracic aorta while levels of collagen synthesis are highest in the lower abdominal aorta (Davidson et al., 1985). These results show that there is considerable phenotypic variation within the smooth muscle cells of the aortic media and suggest that this is expressed as a function of their distance from the heart.

As mentioned earlier (Page 10), biosynthetic labelling studies have shown that tropoelastin is the tissue intermediate

in the formation of insoluble elastin (Narayanan et al., 1976; Rosenbloom and Cywinski, 1976; Rosenbloom et al., 1980) and recent reports on the cell-free translation of elastin mRNA in the developing chick aorta (Burnett and Rosenbloom, 1979; Burnett et al., 1980; Foster et al., 1980b; Karr and Foster, 1981), sheep ligamentum nuchae (Davidson et al., 1981; Davidson et al., 1982a+b) and sheep lung (Shibahara et al., 1981) have confirmed that tropoelastin is the primary translation product.

Measurements of elastin mRNA levels by in vitro translation (Davidson et al., 1982a,b+c) and by hydridisation with elastin specific cDNA probes (Burnett et al., 1981, 1982a+b; Davidson et al., 1984a+b) have shown that the changes in elastin synthesis seen during embryonic development of the chick, sheep and pig are largely governed by the mRNA content of the tissue. Whether the site of control which affects mRNA levels is in the number and transcription rate of elastin genes, the processing and transport of $mRNA_{ELASTIN}$ precursors or the rate of degradation of $mRNA_{ELASTIN}$ remains unknown.

Recent analyses of the 3'region of the sheep elastin gene have revealed some interesting aspects of tropoelastin biosynthesis (Yoon et al., 1984; Yoon et al., 1985; Cicila et al., 1985a+b). There is a strongly conserved 974 base pair untranslated sequence at the 3'end which appears to be cleaved off rapidly during biosynthesis. The translated carboxy terminal region contains two cysteine residues which may be involved in cross-linking the tropoelastin molecules through disulphide bonds

or may form disulphide linkages with other components of the extracellular matrix, including the microfibrillar component.

Tropoelastin is probably synthesised in the rough endoplasmic reticulum and packaged and secreted via the Golgi apparatus. During, or immediately after extrusion into the extracellular matrix, some of the lysyl residues located in the alanine-rich sequences are converted into α-amino adipic δ-semialdehyde (allysine) by oxidative deamination. This reaction is catalysed by the enzyme lysyl oxidase (molecular weight 32-kDa) which is only soluble in chaotropic solutions (Siegel, 1979) and when exposed to aqueous environments rapidly aggregates to a series of oligomeric species that exceed a molecular weight of $1x10^6$ Daltons (Kagan et al., 1979). During growth, lysyl oxidase levels appear to be subject to tissue-specific age-dependent fluctuations that overlap those displayed by the rates of elastin and collagen synthesis (Brody et al., 1979). It has been postulated that in vivo the binding of the enzyme to tropoelastin is facilitated by the formation of small clusters of lysyl residues, generated by the juxtaposition of the polyalanyl sequences of several adjacent elastin chains. This suggests a charge-based substrate-directed modulation of the enzyme activity, supported by the results of the sequencing of the elastin gene which shows the lysine residues embedded in exceedingly basic regions (Yoon et al., 1985).

Peptidyl aldehydes are very reactive and condense spontaneously with one another or with the α-amino groups of an

unmodified lysyl residue to form the structurally related polyfunctional amino acids. Although the exact sequence of events is still uncertain, a likely pathway is illustrated in Figure 1.1.

The metabolic turnover of mature cross-linked elastin appears to be extremely slow, with the half-life of rat aortic elastin estimated at approximately 40 years (Rucker and Tinker, 1977). However, some matrix elastin is continually degraded and replaced by newly synthesised protein, particularly in a variety of pathological conditions which include atherosclerosis and emphysema (Ayer, 1964; Sandberg, 1976; Sandberg et al., 1981b). In a study of elastase-induced emphysema in hamsters, elastin synthesis was shown to be reactivated in mature tissues by destruction of the elastin (Kuhn et al., 1976). Mecham (1981a) has suggested that cellular control of elastin turnover may be achieved by a number of different mechanisms:-

(a) modulation of the amount, activity and substrate affinity of lysyl oxidase by inhibitors or activators;

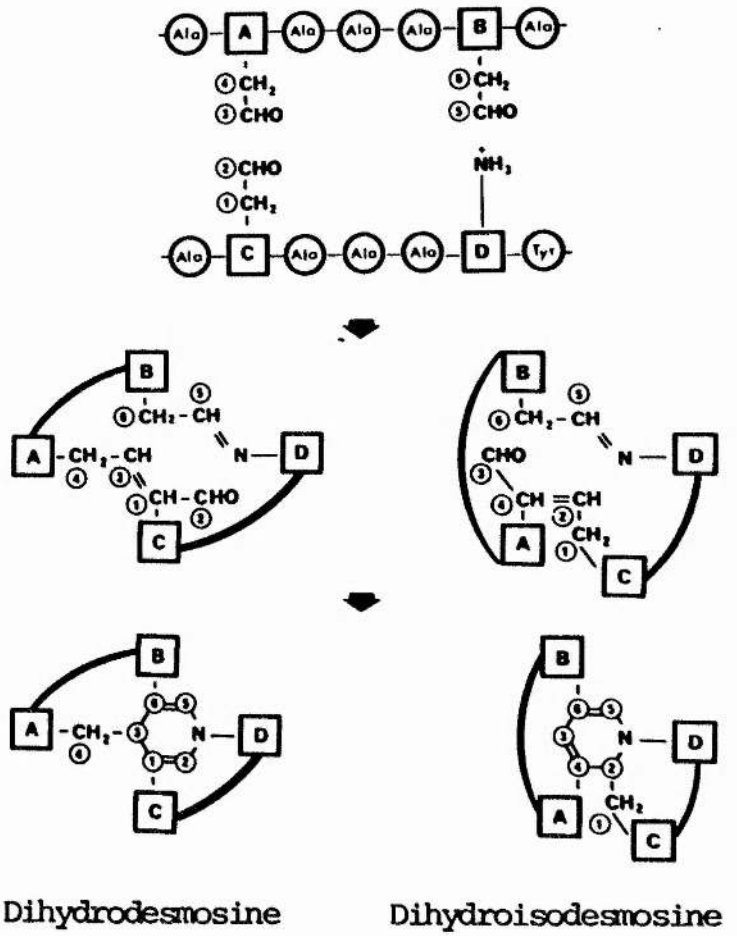
(b) alteration of the interactions between tropoelastin and other components of elastic tissue matrices;

(c) modulation of the activity of extracellular proteins that degrade elastin precursors or elastic fibre constituents (Kagan et al., 1981; Werb et al., 1982).

Another possible control mechanism was suggested by Senior et al. (1980,1982), whose studies showed that tropoelastin and elastase-digested elastin peptides can act as chemoattractants

for fibroblasts, and therefore participate in directing reparative elastin synthesis.

FIGURE 1.1. Schematic representation of a pathway leading to the formation of dihydrodesmosine and dihydroisodesmosine (Foster et al., 1974).



1.3.5. Elastin Synthesis In Vitro.

Although elastin is synthesised *in vivo* as tropoelastin, this macromolecule is quickly cross-linked into insoluble polymeric elastin which cannot then be dissociated into its component subunits (Rucker and Tinker, 1977). Since this process imposes constraints on studies of the chemical composition of precursor molecules and the steps involved in the process of elastic fibre formation, *in vitro* studies of elastin synthesis have proved very helpful.

A number of cell types have been shown to synthesise either tropoelastin or insoluble elastin in tissue culture. These include vascular smooth muscle cells (Ross, 1971; Abraham *et al.*, 1974; Narayanan *et al.*, 1976; Burke and Ross, 1979; Oakes and Batty, 1982), endothelial cells from human umbilical cord veins (Jaffe *et al.*, 1976), cloned rat lung endothelial cells (Cantor *et al.*, 1980), calf pulmonary artery endothelial cells (Mecham *et al.*, 1983), auricular chondrocytes (Quintarelli *et al.*, 1979; Moskalewski, 1981) and *ligamentum nuchae* fibroblasts (Mecham, 1981b; Mecham *et al.*, 1981; Mecham *et al.*, 1984a+b).

In vitro, these cells vary considerably both in the amount of elastin synthesised and in the form of the protein found in the media and cell layer. For example, rabbit auricular chondrocytes (Starcher and Mecham, 1981; Moskalewski, 1981) and rabbit aortic smooth muscle cells (Snider *et al.*, 1981) rapidly synthesise mature cross-linked elastin whereas foetal calf

ligamentum nuchae fibroblasts (Mecham et al., 1984a+b) and calf aortic smooth muscle cells (Schwartz et al., 1980) produce tropoelastin but no insoluble elastin under the culture conditions used in these studies.

There is also great variation in the ability of different cell types to maintain differentation in culture. Rabbit auricular chondrocytes rapidly lose their ability to synthesise mature elastin if subcultured (Moskalewski, 1981) whereas Abraham et al. (1977) have reported that a line of pig aortic smooth muscle cells retained the ability to synthesise elastin over 65 subcultures. Species differences (Sandberg et al., 1981a), degree of development of the tissue (Mecham et al., 1983) as well as cell density and growth phase (Giro et al., 1984) are also important in determining elastogenic capabilities in vitro.

Recent advances in methodology for the identification of tropoelastin have enabled accurate detection and quantitation of the precursor and facilitated the evaluation of the effects of tissue culture conditions on elastin biosynthesis (Foster et al., 1981). The use of immunoassay (Mecham and Lange, 1980; Mecham et al., 1984a; Giro et al., 1984) and immunoprecipitation (Foster et al., 1980b; Davidson et al., 1981) have confirmed that culture conditions have a major effect on elastin synthesis in vitro.

Ascorbate supplementation significantly suppresses deposition of insoluble elastin in cultures of rat heart smooth muscle cells (Scott-Burden et al., 1979; De Clerck and Jones, 1980) and rabbit pulmonary artery smooth muscle cells (Dunn and

Franzblau, 1982) whilst promoting synthesis of collagen. Faris et al. (1984) showed recently that ascorbate levels at a concentration of 2ug/ml which is much lower than that normally used in tissue culture experiments (50ug/ml) is sufficient to substantially reduce insoluble elastin synthesis and lysyl oxidase activity in rabbit aortic smooth muscle cells, while collagen synthesis is maximally stimulated. A decrease in ascorbate concentration to 0.5ug/ml allowed insoluble elastin synthesis to proceed at a normal level. Ascorbate appears to have no effect on the synthesis of tropoelastin as reported by Mecham et al. (1981) in a study on the supplementation of bovine ligamentum nuchae fibroblasts in tissue culture with 50ug/ml ascorbate.

Other pharmacological agents also affect elastin biosynthesis in vitro. For example, the irreversible lysyl oxidase inhibitor BAPN, at a concentration of 50ug/ml, has been shown to inhibit cross-linking of newly synthesised tropoelastin in cultures of chick arterial smooth muscle cells (Narayanan et al., 1976). Also, Sandberg et al. (1981a) reported that BAPN at 10uM is toxic to rat aortic smooth muscle cells and decreases soluble elastin synthesis to low levels. However, another lathyrogen AAN at 50 ug/ml was shown to have no effect on total tropoelastin synthesis by cultures of foetal calf ligamentum nuchae fibroblasts (Mecham et al., 1981).

Dexamethasone (0.1uM), on the other hand, stimulates elastin biosynthesis in foetal ligamentum nuchae fibroblast cell cultures

(Mecham et al., 1981.) and embryonic chick aorta organ cultures (Burnett et al., 1982a). This effect of steroid in cell culture is similar to stimulation of elastin biosynthesis in embryonic chick aorta by the glucocorticosteroid hydrocortisone (150ug/egg) reported by Eichner and Rosenbloom (1979). It may therefore be important to examine the effects of other corticosteroids on elastin synthesis in vitro.

It is apparent that very careful selection of cell type with reference to species, age of donor tissue, phase of growth and length of time in tissue culture, as well as adequate specification of culture conditions, must be undertaken in a study of elastin biosynthesis in vitro. Caution must also be exercised in the general interpretation of results obtained from different model systems.

The culturing of elastin-producing cells should prove extremely useful in the elucidation of the role played by components of the extracellular matrix in regulating the formation of the elastic fibre. In the studies reported in Chapter Five of this thesis, use has been made of foetal calf ligamentum nuchae fibroblasts and human aortic smooth muscle cells to study the morphogenesis of the elastic fibre in vitro.

1.4. ELASTIN-ASSOCIATED MICROFIBRILS

1.4.1. Ultrastructural Studies of Elastin-Associated Microfibrils.

Elastin-associated microfibrils have been studied by high-resolution electron microscopy in human, sheep, pig and bovine aortic fibres (Fanning et al., 1981). A beaded periodicity consisting of alternating dark and light staining areas was observed, as previously described by Low (1962). In longitudinal sections, the microfibrils were described as having a lucent central axis with a linear array of dark-staining particles arranged along their surface either as parallel lines or spirals. In transverse section, the microfibrils were reported to be circular with a poorly stained central core, 4-5nm in diameter, surrounded by 4-6 discrete spherical electron-dense subunits. Unfortunately, the published electron micrographs are of insufficient resolution to allow an objective confirmation of this report.

The different staining characteristics of elastin-associated microfibrils in comparison to amorphous elastin was the first factor to stimulate interest in the possibility that their chemical composition and function may be distinct from that of elastin. Yu and Lai (1970) demonstrated that elastin-associated microfibrils of developing rat aortic elastic fibres stain intensely with ruthenium red which suggested that microfibrils

contained a glycoprotein. Luft (1971) however, argued that the matrix components most likely to bind to the ruthenium red are glycosaminoglycans and that any polyanionic material would also react non-specifically with the dye. Birembaut et al. (1982) showed that subendothelial microfibrils of rabbit aorta, exposed by prior collagenase digestion, bind the lectins Concanavalin A and Ricinus Communis as well as ruthenium red - an observaton which supports the presence of a glycoprotein component. However, as these authors were primarily interested in the interaction of platelets with microfibrils after induced damage to the endothelium, it is possible that the glycoprotein reacting with the lectins had been adsorbed from the bloodstream. However, Fanning and Cleary (1985) have shown that under normal circumstances, elastin-associated microfibrils of the aortic tunica media react with alkaline bismuth after periodic acid oxidation (a proceedure analogous to the PAS stain for glycoproteins in light microscopy). Electron microscopic observations therefore support the view that a glycoprotein moiety is indeed intimately associated with elastin-associated microfibrils.

1.4.2. Biochemical Characterisation of Elastin-Associated Microfibrils.

Although a number of attempts have been made to isolate and characterise elastin-associated microfibrils, most have failed to yield a homogeneous preparation which could confidently be

described as being of microfibrillar origin. The method for isolating microfibrils used by Ross and Bornstein (1969) has served as the prototype for many later investigations. These authors extracted foetal ligamentum nuchae with 5M guanidine hydrochloride followed by collagenase and finally 5M guanidine hydrochloride solution containing the sulphydryl reducing agent dithiothreitol (DTT). The resulting extract has an amino acid composition markedly different from that of elastin and collagen, being rich in polar amino acids and lacking hydroxyproline, hydroxylysine and the cross-linking residues such as desmosine and isodesmosine (see Table 1.2. column 1). The reported cysteine content of 70-80 residues/1000 residues is comparatively high and led to the postulate that the insolubility of the microfibrillar protein is due to disulphide bonding. The relationship of this extract to microfibrils was inferred from electron microscopic examination of tissue before and after extraction with DTT in which they showed that this proceedure led to a decrease in the amount of microfibrils present in the matrix. Unfortunately no electrophoretic analysis of the final product was undertaken in this work or in the following publication which showed that the 'microfibrillar component' contained hexose and hexosamine and was therefore claimed to contain one or more glycoproteins (Ross and Bornstein, 1970).

An immunological approach was then undertaken by several groups in an attempt to elucidate the composition of the microfibrils. Kewley et al. (1977a) modified the extraction

proceedure of Ross and Bornstein to include a more extensive pretreatment protocol. The foetal calf ligamentum nuchae was sequentially extracted with 6M guanidine hydrochloride (step G) followed by treatment with purified collagenase (step C) repeated in the order GCGCG. The "microfibrillar protein" was then solubilised by a final extraction with 6M guanidine hydrochloride containing 0.1M 2-mercaptoethanol (step M). The amino acid composition of this extract is similar to that reported by Ross and Bornstein (1970). However, on analysis by polyacrylamide gel electrophoresis, this extract appeared to be extremely heterogenous (Sear et al., 1977) with the most prominent protein band (apparent molecular weight 135-kDa) staining with PAS reagent. An antiserum raised in rabbits against this heterogenous extract showed the presence of at least three precipitin lines in immunodiffusion tests. Using the major precipitin complex as antigen, a secondary immunisation proceedure was undertaken and an antiserum was produced which showed a single precipitin line on immunodiffusion against the original extract. This antisera did not react with serum proteins, proteoglycans or elastin either solubilised by oxalic acid or pepsin treatment. Immunofluoresence studies revealed strong binding of the antibody to partially purified elastic fibres (residue GCGCG) but no reactivity could be observed to elastic and collagen fibres after treatment with 2-mercaptoethanol. Using an indirect immunoperoxidase technique for electron-microscopic immunolocalisation, intense staining of

elastin-associated microfibrils was observed as well as less, but significant, binding to collagen fibres. Further immunofluorescent studies of the tissue localisation of this 'anti-microfibrillar antibody' indicated that it is localised widely in a variety of tissues including bovine ligamentum nuchae, aorta, spleen and kidney (Kewley et al., 1977b). The authors interpreted these findings as establishing an immunological relationship between elastin-associated microfibrils and the morphologically similar microfibrils, described by Hsu and Churg (1979), present in basement membranes and other non-elastic tissues. However, proper consideration should be given to the heterogeneity of the immunogen and the poorly characterised 'monospecific' antiserum before drawing any conclusions from this study.

Subsequent to this work, Cleary et al. (1981) have described the production of antisera against a GCGCGM extract of bovine ligamentum nuchae using the proceedure described by Kewley et al. (1977a) with the exception that a cocktail of protease inhibitors was used throughout the extraction proceedure and preparation of the antigen. Ultrastructural examination of the residues after each extraction indicated that a proportion of the microfibrils associated with elastic tissue were removed prior to the final reductive guanidine hydrochloride extraction (step M). A polyclonal antiserum produced in rabbits to this final extract (GCGCGM) gave a single precipitin line in immunodiffusion to the 'microfibrillar antigen' and also to earlier guanidine

hydrochloride extracts. However, when the antiserum was tested for specificity by the more sensitive ELISA technique, activity (undetected by immunodiffusion) against bovine serum albumin and fibronectin was discovered. These workers removed contaminant activity against these proteins by affinity chromatography and showed by immunofluorescence that the purified antiserum bound avidly to the elastic fibres of aorta, lung, skin, perichondrium of ear cartilage and mesangium of the renal glomerulus and peritubal regions. Ultrastructural localisation of ferritin-labelled antibodies in sections of nuchal ligament revealed strong antibody localisation to elastin-associated microfibrils as well as occasional binding to thin filaments located among collagen fibres (Prosser et al., 1984). Cleary and Gibson (1983), in a review on microfibrillar proteins, have stressed that immunological identification of materials claiming to be microfibrillar in origin is necessary. The monospecificity of the anti-microfibrillar antisera described above is therefore debatable, because a heterogenous antigen was employed. Western blots, which would indicate the number of components within the immunogen that react with the antisera, were not undertaken by any of the above groups.

In 1981, using the Ross and Bornstein protocol (1969), modified to include protease inhibitors to all buffers, Serafini-Fracassini et al (1981a+b) obtained an extract which, when analysed by polyacrylamide gel electrophoresis, revealed the presence of a number of proteins. The fastest moving PAS

positive band was isolated in pure form by preparative gel electrophoresis yielding a glycoprotein of apparent molecular weight 34-kDa which possessed amino-oxidase activity towards both peptidyl-lysine and free lysine. The amino-acid composition of this structural glycoprotein 'SGP' is shown in Table 1.2. column 2. This glycoprotein showed a marked tendancy to aggregate in the absence of guanidine hydrochloride and mercaptoethanol and could be induced to precipitate from solution by exposure to copper ions in 8M urea, followed by dialysis in distilled water. Electron microscopic examination of the precipitate showed cylindrical tactoids which had a diameter of 10-11nm, identical to that of native microfibrils.

Kawaguchi (1982) also reported the isolation of two glycoproteins from ligamentum nuchae with an apparent molecular weight of 35-kDa. These two fractions A and B are similar in terms of apparent molecular weight on SDS-PAGE and amino acid composition to the 34-kDa glycoprotein of Serafini-Fracassini et al. (1981a) described above (Table 1.2. column 3). Kawaguchi therefore suggested that glycoproteins A and B may be derived from elastin-associated microfibrils.

Very recently, three separate papers have been published on the immunolocalisation of three distinct antibody preparations to elastin-associated microfibrils. Gibson et al. (1986), have established by immunoblotting and immunoelectron microscopy, an association between an acidic glycoprotein (31-kDa) and elastin-associated microfibrils in bovine elastic tissue. The

glycoprotein, referred to as MAGP, was isolated from the ligamentum nuchae of 210- to 250-day-old foetal calves treated with guanidine hydrochloride and optionally with bacterial collagenase prior to the final extraction with guanidine hydrochloride containing dithiothreitol. The amino acid composition of MAGP, reported in Table 1.2. (column 4) shows an exceptionally high content of glutamic acid and high levels of proline and cysteine. The carbohydrate content, estimated to be approximately 4%, contains N-acetylgalactosamine, glucose, galactose and mannose as the major sugars present.

Antibodies raised against chromatographically-purified MAGP were shown by electron microscopy, using an indirect immunogold method, to localise specifically to microfibrils present in 250-day-old foetal bovine aorta embedded in Lowycryl K4M. Immunoblotting with the anti-MAGP identified in the reductive guanidine extract of the tissue, two additional reactive proteins of higher molecular weight (60-kDa and 300-kDa respectively). These two larger macromolecules are considered to be MAGP aggregates rather than precursors, since they were not detected in the fibroblast culture medium.

Schmitt et al. (1986) have shown by immunogold electron-microscopy that a monoclonal antibody (HB8) to human dermal fibres binds to the microfibrillar component of elastin and oxytalan fibres in the skin. The precise biochemical nature of the antigens identified by HB8 are still unknown.

Also using monoclonal antibodies, Sakai et al. (1987) have just isolated a 350-kDa glycoprotein called 'fibrillin' which is synthesised by fibroblasts from human skin explants. In an indirect immunofluoresence study, fibrillin appears to be distributed in the connective tissue matrices of the human skin, lung, kidney, vasculature, cartilage, tendon, muscle, cornea and ciliary zonule. Ultrastructurally, the fibrillin-specific monoclonal antibodies were found to bind specifically to elastin-associated microfibrils in the dermis, and the localisation of the colloidal gold conjugates suggested that the fibrillin may be arranged periodically along the individual microfibril and that this periodicity may be aligned along a bundle of microfibrils. It is extremely interesting that the periodicity of this epitope appeared to match the interstitial collagen band periodicity.

TABLE 1.2. AMINO ACID COMPOSITION OF LIGAMENT GLYCOPROTEINS EXTRACTED UNDER DISSOCIATIVE CONDITIONS.

	Microfibrillar Protein Preparation	SGP 34,000	Glycoprotein A 35,000	MAGP 31,000
	(Ross and Bornstein)	(Serafini-Fracassini et al.)	(Kawaguchi)	(Gibson et al.)
Amino Acid	1969	1981	1982	1986
Hydroxyproline	-	0	12	-
Aspartic Acid	114	119	121	68
Threonine	55	44	48	40
Serine	59	61	69	45
Glutamic Acid	111	103	118	222
Proline	70	47	65	106
Glycine	120	112	106	45
Alanine	59	72	66	49
Half-Cysteine	80	27	51*	68
Valine	54	56	49	69
Methionine	16	14	11	4
Isoleucine	45	33	37	22
Leucine	57	84	76	72
Tyrosine	30	43	39	51
Phenylalanine	32	48	46	22
Hydroxylysine	-	-	1	-
Lysine	37	47	31	26
Histidine	14	18	14	34
Arginine	45	55	37	56

* = Measured as S-carboxymethylcysteine
Values are expressed as residues/1000 total amino acid residues.

1.4.3. Elastin-Associated Microfibrils in Tissue Culture.

The difficulties involved in attempting to purify, from tissue matrices, proteins which are related to elastin-associated microfibrils have prompted investigation into cell culture as an alternative source of microfibrillar materials. Workers have used either vascular smooth muscle cells or nuchal ligament fibroblasts to examine the possibility of a precursor of the microfibrils being secreted into the medium.

Ross (1971) first demonstrated that guinea-pig aortic smooth muscle cells synthesise both components of elastic fibres *in vitro*. Cell layers were harvested and extracted, according to the Ross and Bornstein method (1969), with 5M guanidine hydrochloride and collagenase, but with no reducing step included. The insoluble residue was shown by electron microscopy to be rich in microfibrils and its amino acid composition was similar to that of the 'microfibrillar protein' reported by Ross and Bornstein (1969, 1970).

Muir *et al*. (1976) was the first group to attempt the isolation of a homogeneous protein preparation from the extracellular matrix laid down by monkey smooth muscle cells in culture. On examination by electron microscopy, the extracellular matrix of the cultures was shown to contain large amounts of elastin-associated microfibrils, elastic fibres, collagen and basement membrane material. Cultures were incubated with ^{3}H-cysteine to label newly synthesised macromolecules, and

then the cell layer was extracted sequentially with (a) 1M NaCl plus 0.1% Triton X-100; (b) 5M guanidine hydrochloride; and (c) 5M guanidine hydrochloride with 25mM DTT. Polyacrylamide gel electrophoresis showed a major ^{3}H-cysteine labelled glycoprotein with a molecular weight of 270-kDa in all three extracts of the cell layer as well as in the medium, but it was the only radioactively labelled protein present in the reductive extract of the cell layer. The authors suggested that this glycoprotein may represent a soluble precursor subunit of microfibrils. Subsequent work by Burke and Ross (1979) however, showed that this glycoprotein is in fact fibronectin.

Schwartz et al. (1980, 1982), examined calf aortic smooth muscle cells under scorbutic conditions in tissue culture and observed that microfibrils were the only matrix component identifiable by electron microscopy. No amorphous elastin was observed in supplemented or unsupplemented cultures. Harvested cell layers from scorbutic cultures were finally extracted with buffered 1% Sodium Dodecyl Sulphate (SDS)/0.33M mercaptoethanol. The resulting heterogenous extract contained two major bands which were identified by polyacrylamide gel electrophoresis. The smaller of the proteins (45-kDa) was identified as actin and the larger (200-kDa) as fibronectin.

Jones et al. (1979) described the isolation of a 250-kDa molecular weight fucosylated glycoprotein from cultured rat heart smooth muscle cells by extensive extraction with 8M urea buffer containing 1% SDS and 1% mercaptoethanol. No further

characterisation of this material was carried out although it was stated that the glycoprotein was similar to both the microfibrillar component of Muir et al. (1976) and to fibronectin.

In the initial studies of Sear et al. (1978) on the synthesis of microfibrils, foetal ligamentum nuchae fibroblasts were maintained in culture under scorbutic conditions. Electron microscopic examination of the cell layer showed large numbers of microfibrils but no insoluble elastin, even after five weeks in culture. The cells were incubated with ^{3}H-fucose and newly synthesised glycoproteins secreted into the medium were detected by polyacrylamide gel electrophoresis. Five major fucosylated species were found. Two of these could be immunoprecipitated from labelled media by the 'anti-microfibrillar protein' antiserum produced by Kewley et al. (1977a) (see section 1.4.2.). These two glycoproteins, with apparent molecular weights of 150-kDa and 300-kDa, were called MFP1 and MFP2 respectively (Sear et al., 1981a+b). The 'microfibrillar preparation' used by Kewley et al (1977a) as the immunogen, contains a number of PAS-positive bands, one of which corresponded in electrophoretic mobility to MFP1. It was therefore proposed that MFP1 is related to elastin-associated microfibrils and that MFP1 and MFP2 are immunochemically related or physically associated with one another.

In further studies of these glycoproteins by Sear et al. (1981b), MFP1 was shown to contain 4-hydroxyproline and

4-hydroxylysine and was susceptible to digestion by highly purified collagenase. Also, ascorbate supplementation of cultures produced a doubling of the synthesis of MFP1 whereas MFP2 synthesis was unaffected. Neither glycoprotein was precipitated from labelled media by anti-fibronectin antisera. The authors concluded that MFP1 (150-kDa) is a new collagenous glycoprotein which is part of the elastin-associated microfibrils and that MFP2 (300-kDa) is associated with MFP1 in an unknown manner, possibly as a component or organiser of microfibrils. MFP1 has subsequently been shown to be the undegraded tissue form of collagen type VI by Knight et al. (1984) and Ayad et al. (1985). Despite the elegance of this work, it is clear that the antibody preparation on which it is based is polyspecific and therefore any conclusions regarding the role played by MFP1 and MFP2 in the formation of microfibrils must be viewed critically.

Lamberg et al. (1980) attempted to specifically isolate microfibrils from calf ligamentum nuchae fibroblasts by a non-immunological method using saturated fluoroscein mercuric acetate as an extractant. The resulting product, when examined by electron microscopy, was claimed to be a microfibrillar-rich extract. This preparation of microfibrils was reported to be susceptible to digestion with trypsin but not with elastase, collagenase or hyaluronidase and its amino acid composition was similar to that of Ross and Bornstein's 'microfibrillar protein'. However, the use of amino acid analyses as a basis for describing

an extract or component as being microfibrillar in origin is insufficient evidence (Cleary and Gibson, 1983).

Using anti-MAGP antisera, Gibson et al. (1986) have shown by Western blotting analysis that the media from confluent cultures of nuchal ligament fibroblasts, derived from 120- and 250-day old foetal calves, contain the 31-kDa MAGP. Such a source of highly purified undenatured MAGP should help in the study of its synthesis, structure and function in elastic tissues.

1.5. MORPHOGENESIS OF THE ELASTIC FIBRE.

With the isolation and characterisation of the mammalian elastin gene (Cicila et al., 1985a+b; Yoon et al., 1984+ 1985; Emmanuel et al., 1985) a great deal of information has been obtained on the biosynthesis of elastin (section 1.3.4.). However, it is now evident that the morphogenesis of the elastic fibre is a more dynamic process than was previously believed and involves complex interactions between the components of the elastic fibre itself, the surface of elastogenic cells and the extracellular matrix.

1.5.1. Transport of Elastin to the Extracellular Matrix.

Little is known at present about the mechanism by which the elastin precursor is exported into the extracellular matrix from the elastogenic cell and assembled in the elastic fibrils, bearing in mind the fact that tropoelastin coacervates in vitro under physiological conditions. From ultrastructural studies, it is thought that tropoelastin may be packaged in the Golgi complex and then secreted via 'acanthasomes' (Fahrenbach et al., 1966; Thyberg et al., 1979). These large 'spiny vesicles' were originally described by Fahrenbach et al. (1966) as a vehicle for the secretion of protein-polysaccharide complexes. Immunochemical detection of tropoelastin within the cell was attempted by Damiano et al. (1981) but the intracellular immunoferritin staining was extremely sparse with a lot of

non-specific electron-dense material scattered throughout the cytoplasm - despite biochemical evidence that these cells were rapidly synthesising elastin. Recently, these workers have repeated their study on the secretion of elastin in the embryonic chick aorta using an improved peroxidase anti-peroxidase method to detect the primary elastin antibody. Specific antibody staining was visualised in the cisternae of the endoplasmic reticulum, in the Golgi apparatus and in vesicles that formed on the trans side of the Golgi. Some of these smaller vesicles appeared to fuse into larger vesicles which, as the authors suggested, may have a storage function. Both types of vescicles were seen fusing with the cell plasma membrane - a feature which suggests that elastin is secreted by exocytosis (Damiano et al., 1984). Although the immunoperoxidase technique is an extremely sensitive method for detecting specific binding, the resulting stain tends to diffuse and mask underlying organelles. An equivalent study using an immunogold stain would possibly give clearer results.

One possible mechanism for the transport of the elastin precursor to the site of elastic-fibre formation is suggested by Baccarani-Contri et al. (1985). Using various cytochemical cationic stains in an electron microscopic study, they discovered an interesting association between proteoglycans and elastic fibres. The proteoglycans found on the elastic fibres of lathyritic chicks appeared to be lateral branches of matrix proteoglycans lying on the external surface of the elastic

fibres. This, the authors suggested, implies that proteoglycans are bound to tropoelastin by electrostatic interactions and therefore prevent coacervation of the tropoelastin molecules at physiological temperatures. These workers stated that the reason why this association is undetected in the normal aorta is that lysyl oxidase competes for the positively charged groups on the tropoelastin and probably possessing a higher affinity for these sites, displaces the proteoglycans and consequently cross-links the peptide chains. At a recent FECTS meeting, Pasquali-Ronchetti presented electron micrographs of chick aorta which, using the immunogold-labelling technique, showed the association of proteoglycan and tropoelastin within the extracellular matrix. The disperse distribution of gold particles in the extracellular matrix was interpreted by Pasquali-Ronchetti as showing that tropoelastin is 'sprayed' into the matrix and held there by electrostatic interactions to proteoglycans until it is cross-linked by lysyl oxidase to form elastic fibres (unpublished results).

On the other hand, electron microscopic studies have repeatedly shown that there is a close inter-relationship between elastogenic cells and immature elastic fibrils, the latter frequently being situated within infoldings of cell surface (Greenlee et al., 1966; Takagi and Kawase, 1967; Vaccaro and Brody, 1978). In foetal bovine ligamentum nuchae tissue, fibroblasts displaying morphologies suggestive of active protein synthesis appear to envelop a high proportion of the growing

fibrils (Serafini-Fracassini, 1984). These observations suggest that elastogenesis requires the establishment of an intimate contact between the plasma membrane of the elastogenic cell and the site of fibre formation. Chapter Two of this thesis outlines the results of an *in vivo* study on the distribution of elastin and its organisation into elastic fibres in the extracellular matrix of the human foetal aorta. Chapter Five includes the results of *in vitro* studies on an extracellular matrix component which may play a part in initiating elastogenesis.

1.5.2. Role of Microfibrils in the Formation of the Elastic Fibre.

Another major question in the morphogenesis of the elastic fibre is the role played by the elastin-associated microfibrils. The temporal and morphological relationship of microfibrils to elastin in developing elastic tissues was firmly established by Fahrenbach *et al*. (1966), and Greenlee *et al*. (1966), in separate electron microscopic studies of the elastogenesis of foetal *ligamentum nuchae*. They showed that microfibrils appear in foetal life as well-defined bundles preceding the development of amorphous elastin. These bundles are usually arranged with their long axis parallel to that of the ligament and are often found in close apposition to fibroblasts, sometimes occupying infoldings of the cell membrane (Kewley *et al*., 1978). Definitive elastin first appears in the form of small clumps of amorphous material within individual bundles of microfibrils, and these foci of

elastin deposition subsquently coalesce to form true elastic fibres (Serafini-Fracassini, 1984).

This sequence of events in elastin fibrillogenesis seems to occur in a variety of tissues from different species:- human aorta (Haust et al., 1965); rat flexor digital tendon (Greenlee and Ross, 1967); embryonic chick aorta (Takagi, 1969; Kadar et al., 1971); rat aorta (Albert, 1972); chick lung (Jones and Barson, 1971) and foetal lamb pulmonary septum (Fierer, 1977). A similar process also occurs during in vitro formation of elastic fibres by foetal calf ligamentum nuchae fibroblasts (Jones et al, 1980), guinea pig smooth muscle cells (Ross, 1971) and aortic tissues from the rat (Hinek and Thyberg, 1977), rabbit (Toselli et al., 1981) and pigeons (Wight et al., 1977). These observations suggest that microfibrils have a crucial role in elastic fibre formation and there is a great deal of speculation on this subject.

Ross et al. (1977) and Cleary et al (1981), have suggested that microfibrillar aggregates take the orientation of the definitive elastic fibres, thus directing the morphogenesis of elastic tissue by acting as a 'scaffold' onto which elastin is deposited. Since the microfibrils have a net negative charge it could be speculated that they immobilise tropoelastin (positively charged) until cross-linking takes place. The study on the ultrastructural alterations which occur in elastic fibres of chicks rendered lathyritic by treatment with BAPN, by Pasquali-Ronchetti et al. (1981) supports this view. The authors

reported that newly-synthesised elastin is deposited on pre-existing elastic fibres as button-like appendices which lacked the normal complement of microfibrils, despite the presence of microfibrils around unaffected portions of the same fibre. They interpreted this as confirmation of the role of microfibrils in directing elastin morphogenesis but also implied that deposition of elastin may continue in their absence, although in a disordered fashion.

Another possibility is that the microfibril may serve as a registration peptide (Sandberg, 1976). In this model, microfibrils align tropoelastin molecules in precise register so that their cross-linking regions are juxtapositioned prior to oxidative deamination by lysyl oxidase. This alignment of tropoelastin molecules appears to be an important prerequisite for cross-linking (Narayanan et al., 1978) and therefore lysyl oxidase may be bound to the microfibrils in a co-ordinate complex thus generating the observed ultrastructural periodicity described in section 1.4. The ultrastructural appearance of elastic tissues in experimental copper deficiency, in which super-abundant microfibrils are found to surround sparse amounts of electron-dense elastin (Waisman and Carnes, 1967) is consistent with this hypothesis. Although microfibrils are present, normal elastic fibres cannot be found due to a deficiency in lysyl oxidase activity leading to poorly cross-linked elastin. Similar elastic fibres have been found in patients with Menkes Syndrome (Oakes et al., 1976) - a human

genetic disease caused by defective intestinal absorption of copper leading to a state of copper deficiency (Danks, 1977; Kivirikko and Peltomen, 1982).

A third model envisages microfibrils coalescing directly to form amorphous elastin (Fanning et al., 1981). These authors suggest that microfibrils consist of a glycoprotein coat surrounding a central core of tropoelastin. This packaging may facilitate extracellular transport and aggregation of the soluble elastin after which the peripheral coat is stripped and the tropoelastin core is cross-linked into amorphous elastin. Consistent with this theory, is the observation that microfibrils lose their dense-staining peripheral coat to varying degrees and assume staining characteristics similar to those of amorphous elastin. The authors also suggest that variations in diameter exhibited by the microfibrils may be due to differing degrees of cross-linking of tropoelastin chains contained within them - a theory compatible with the observation reported by Cleary and Gibson (1983) that microfibrillar diameters increase in copper deficient animals.

An interaction between the elastin precursor and the microfibrils was also suggested by Cicila et al. (1985a+b) having discovered that the basic C-terminal region of tropoelastin contains two cysteinyl residues. The microfibrils appear, because of their overall negative charge and relatively high content of cysteinyl residues, to be suited to the function of binding tropoelastin until, as part of the elastin

post-translational modifications, the C-terminal extension is removed.

Finally, it should be noted that lysyl oxidase has been shown to be associated extracellularly with the developing elastic fibre (Kagan et al., 1974). In Kagan's recent paper on the ultrastructural immunolocalisation of lysyl oxidase in vascular connective tissue (Kagan et al., 1986) lysyl oxidase was shown to be associated with microfibrillar deposits before and after the appearance of amorphous elastin within the area circumscribed by the microfibrils. Whether the enzyme is secreted with tropoelastin and in what way it is complexed to the microfibrils is unknown. In this respect it is of interest that the putative microfibrillar component, with an apparent molecular weight of 35-kDa, isolated from bovine ligamentum nuchae exhibits peptidyl-lysyl oxidase activity (see section 1.4.2.) (Serafini-Fracassini et al., 1981a).

1.5.3. Role of the Extracellular Matrix in Elastic Fibre Formation.

At present very little is known about the factors which affect developmental control of elastin gene expression. For example, it is unknown what causes, during development of the mammalian aorta, the elastogenic smooth muscle cells to produce mainly elastin in the upper section of the vessel, whereas the same cell-type synthesises predominantly collagen in the abdominal portion.

The extracellular matrix is thought to influence tissue development and cellular differentiation in most vertebrate tissues (Bissell et al., 1982). Recent studies by Mecham et al. (1984b) suggest that in vitro at least, the extracellular matrix simultaneously induces elastin synthesis and the appearance of chemotaxis to elastin peptides by bovine foetal ligamentum nuchae fibroblasts. In one set of experiments (Mecham, 1981b), these ligament cells were cultured either directly on plastic or on killed foetal ligament tissue and, although soluble elastin was synthesised in both systems, only the fibroblasts grown on dead ligament tissue formed insoluble elastin. This work was extended in a later study which showed that undifferentiated (non elastin-producing) ligament cells from early gestation animals initiate elastin synthesis when grown on an extracellular matrix substratum prepared from the ligamentum nuchae of foetal calves in late gestation (last trimester) (Mecham et al., 1984a). Thus, direct cell-matrix contact appears to be important in mediating elastin biosynthesis.

A new family of extracellular matrix cell-attachment glycoproteins has been identified as the molecular mediators of cell-matrix interactions (Kleinman et al., 1981+1984; Hay, 1984; Yamada, 1983; Yamada et al., 1984). These glycoproteins stimulate the adhesion of cells to substrates, affect the biological activities that the cells express, and regulate the formation of the matrix itself. These glycoproteins include fibronectin, laminin, vitronectin and chondronectin, each of

which shows a distinct histological distribution and activity toward different cell types. Fibronectin promotes the adhesion and spreading of fibroblasts (Yamada et al., 1978), certain epithelial cells (Johansson et al., 1981), chondrocytes (West et al., 1979) and various transformed cell lines (Ali et al., 1977) and binds to all collagens. Laminin, a basement membrane glycoprotein, mediates the attachment and spreading of epithelial cells to plastic or glass substrates (Couchman et al., 1983) and is specific for type IV collagen. Chondronectin is a glycoprotein that specifically mediates the attachment of chondrocytes to cartilage type II collagen in vitro (Hewitt et al., 1982) and vitronectin is an attachment-promoting glycoprotein present in mammalian serum but distinct from fibronectin (Hayman et al., 1982). These glycoproteins also affect the behavior of their responding cells. Both fibronectin and laminin alter cell morphology, growth, differentiation and migration in vitro and are considered to exert these activities in vivo during tissue development and wound healing. Lastly, because of their interaction with collagen and proteoglycans, the attachment glycoproteins influence the organisation of the extracellular matrix and thus influence the form and function of the tissues.

As matrix molecules are non-diffusible, the effects of the extracellular matrix in modulating biosynthetic activities may be due to structural changes of the cell itself within the matrix.

If the response of cells to their local environment is mediated by the adoption of different shapes, then the cytoskeleton must play a central role in gene expression (Bissell et al., 1982). One example of close association between the extracellular matrix and the cytoskeleton is the alignment of cell surface fibronectin and heparin sulphate proteoglycan with actin microfilament bundles in the cytoplasm of spreading fibroblasts (Woods et al., 1984).

A model has been outlined by Watt (1986) to explain how signalling between the matrix and the cytoskeleton could be transduced by the plasma membrane. Receptors for individual matrix components within the cell membrane are envisaged as interacting directly with actin or with actin-attachment proteins in the plane of the membrane. The resulting modulation of actin assembly would then produce changes both in cell shape and in the synthesis, assembly and organisation of other cytoskeletal proteins. As polyribosomes and elements of the Golgi and rough endoplasmic reticulum are specifically associated with the cytoskeleton, disassembly of microtubules or microfilaments could ultimately affect gene expression at the transcription or translation level.

In this context, Frisch and Werb (1983) have shown that changes in cell shape influence tropoelastin gene expression in rat aortic smooth muscle cells, an interesting observation which is consistent with the above hypothesis.

1.6. AIMS OF INVESTIGATION.

This study has used a combined biochemical, immunological and electron-microscopic approach to study the morphogenesis of the elastic fibre. The specific aims were as follows:-

(1) To examine elastic fibre formation in the human foetal aorta with particular reference to the deposition of newly-synthesised elastin. To this purpose, an antiserum to human elastin was used as a label in an immunogold electron microscopic investigation.

(2) To raise an antiserum against the 35-kDa glycoprotein which probably represents a component of elastin-associated microfibrils (described on Page 28) and to study its localisation in connective tissues by immunohistochemical techniques.

(3) To examine the process of elastin fibrillogenesis <u>in vitro</u> with cultures of foetal calf <u>ligamentum nuchae</u> fibroblasts and human foetal aortic smooth muscle cells, using the anti-elastin and anti-35k-GP antisera as probes and the purified 35k-GP in cell-adhesion experiments.

CHAPTER TWO.

AN IMMUNOELECTRONMICROSCOPIC STUDY OF ELASTIC FIBRE FORMATION IN THE HUMAN FOETAL AORTA.

2.1. INTRODUCTION.

One of the most characteristic features in the morphogenesis of elastic tissues is the close inter-relationship that exists between elastogenic cells and immature elastic fibrils. This has led to the suggestion that elastogenesis may require the establishment of an intimate contact between the plasma membrane of the elastogenic cell and the surface of the growing elastic fibril; and that the biosynthetic episode triggered by this cell-fibril contact would result in the deposition of a finite but continuous layer of elastin at the cell-fibre interface, should such contact cause a polarisation of the secretory process and induce the cell to perform morphogenetic movements.

The studies in this Chapter were undertaken to show that, in keeping with this model, newly synthesised elastin is deposited on pre-existing elastin surfaces in spiral arrangements during the final stages of aortic development.

2.2. MATERIALS AND METHODS

2.2.1. Materials.

Human foetal aortae were obtained from the Department of Pathology, Royal Sick Childrens Hospital, Edinburgh; bovine ligamentum nuchae and calf skin from St Andrews slaughterhouse; and trout from M^{c}Kenzie fish farm, Auchterarder.

Goat anti-rabbit IgG-labelled with colloidal gold (5, 15 and 20nm) were bought from Janssen Pharmaceutica, Beerse, Belgium; Epoxy resin kit from Fluka AG, Buchs, Switzerland; DEAE-cellulose from Whatman, Merseyside; and polystyrene microtitre plates from Flow laboratories, Rickmansworth, Herts. Fibronectin, goat anti-rabbit IgG peroxidase, guanidine hydrochloride (Analar grade), Freunds complete and incomplete adjuvants and all other reagents were obtained from Sigma Chemical Company, Poole, Dorset.

2.2.2. Preparation of Elastin and Collagen.

Elastin was purified from human aortae (from 16-week old foetus, 16-year old female, 70 year old male), adult bovine ligamentum nuchae and trout bulbus arteriosus by the method described below (Serafini-Fracassini et al., 1975; Spina et al., 1979):-

The tissues were washed in 1% NaCl at 4°C for 24hr, defatted by treatment with ethanol, acetone and ether, homogenised to a fine

powder and sequentially extracted with 5M guanidine hydrochloride -0.4% EDTA-0.1M TRIS, pH7.4 (guanidine HCl buffer) to remove muscle proteins, collagen and proteoglycans. This was followed by extraction for 48 hours in guanidine HCl buffer containing 2% mercaptoethanol. Finally, in the case of the human and bovine material, the preparation was treated with collagenase which had been purified by affinity chromatography and by passing through a column of alkali-treated elastin (Serafini-Fracassini et al., 1975). The material was suspended in 20ml of 0.01M $CaCl_2$ (pH 7.5) and 500ug of highly purified collagenase was added. The suspension was stirred at 37°C in a Radiometer pH stat set at pH 7.5, until no further activity was apparent.

Soluble elastin was obtained from preparations of purified human, bovine and trout elastin by refluxing in 0.25M oxalic acid at 98°C for 5 x 45 minute periods (Partridge et al., 1955). After each stage, the residue was collected by centrifugation and the supernatant analysed for protein content by the method of Bradford (1976) using crystalline bovine serum albumin as a standard. The fourth and fifth supernatants, which contained approximately 89% of the starting material, (70% in the case of the trout) were pooled, neutralised, dialysed against water at 4°C and freeze-dried.

Acid-soluble collagen was extracted from calf skin by the method of Steven and Tristam (1962). Briefly, the tissue was washed extensively in phosphate buffered saline (PBS) and ground to a fine powder. The collagen within the skin was extracted

with 0.1M acetic acid and then precipitated selectively from the acetic acid extract by the dropwise addition of 30% (w/v) sodium chloride solution with continuous stirring. Precipitation of a fine fibrous mass was complete at a final concentration of 7% sodium chloride. After centrifugation, the precipitate was redissolved in 0.1M acetic acid and the protein was precipitated again by the addition of sodium chloride. This procedure was repeated five times to remove non-collagen contaminants.

2.2.3. Amino Acid Analyses.

Purity of the bovine, trout and human elastin was assessed by comparison of the amino acid composition of the sample with the corresponding published data (Serafini-Fracassini et al., 1975b; Spina et al., 1979; Spina et al., 1983). Amino acid analysis was carried out by hydrolysing samples in constant boiling hydrochloric acid (1mg/ml) at 110°C for 24hrs. Samples were taken to dryness and amino acid analysis performed on a Locarte amino acid analyser. Control values for amino acids were obtained from a standard solution of amino acids. Amino acids were quantitated manually from chromatograms.

2.2.4. Preparation of Anti-Elastin Antiserum.

In order to maximise the number of possible antigenic sites, the immunogen consisted of both soluble and insoluble elastin. Finely comminuted insoluble human foetal elastin (100-200 mesh fraction) was suspended (1mg/ml) in a 0.1% solution of soluble

human elastin (α-elastin) in 0.1M phosphate buffer and emulsified in an equal volume of Freunds complete adjuvant. Four Wild Dutch rabbits, which had been pre-bled, were immunisd by multiple-site subcutaneous injections on the back - each rabbit receiving 2mls of emulsion in six divided doses. Animals were boosted every two weeks with a further 2mg of soluble and insoluble elastin mixture in Freunds incomplete adjuvant. Sera were checked after each bleed and only those of high titre were used in the experiments which followed.

2.2.5. Preparation of IgG Antiserum.

Rabbit IgG was prepared from the pooled antisera of rabbits numbered 1, 3 and 4 by precipitation in a 50% ammonium sulphate solution followed by chromatography on DEAE-cellulose as described by Fahey and Terry (1978). The buffers used in the extraction of IgG from the serum by ion-exchange chromatography are shown in Table 2.1. IgG passed through the column in the 0.01M phosphate buffer and the remaining serum proteins were then eluted in the 0.3M phosphate buffer. The resulting IgG fraction was concentrated by ultrafiltration and stored at -20°C in the presence of 0.02% NaN_3.

2.2.6. Immunochemical Characterisation of Anti-Elastin Antiserum by ELISA.

Titres were determined by indirect enzyme linked immunosorbent assay (ELISA) using the method of Rennard et al.

(1980). The constituents of the buffers used in the ELISA are shown in Table 2.1. Antigens were dissolved in 'binding' buffer (pH9.6) and 100ul added to each well of a polystyrene microtitre plate which was incubated for one hour at 37°C to allow passive adsorption of antigen onto the plate surface. These microtitre plates could then be stored indefinitely at 4°C. Antigen-coated plates were washed three times with 'washing buffer' and then incubated for one hour at room temperature with 'blocking buffer'. This buffer is to prevent non-specific binding of serum protein to the wells. After washing once, serial doubling dilutions of each antibody preparation under test were made in washing buffer and 100ul added to each well. Controls containing buffer only or antigen only were incubated in each test in addition to known positive and negative sera. The plates were incubated for three hours at 37°C then washed three times. Goat anti-rabbit IgG-peroxidase conjugate diluted to working strength in washing buffer (1:1000) was added to each well and the plate incubated for a further one hour at 37°C. After washing and drying as before, 100ul of the enzyme substrate dissolved in a pH5 phosphate-citrate buffer was added to each well and the colour development was allowed to proceed for 30 minutes at room temperature. The reaction was stopped by the addition of 50ul 3M NaOH to all wells. Absorbances at 492nm were read on a Titertek Multiskan, Eflab Oy, Helsinki Finland.

The resulting titre was determined by subtracting the absorbance of the buffer-only well (blank) from that of the test

wells and comparing this value with the absorbance of wells containing pre-immune rabbit serum at a comparible dilution (negative). Samples were considered positive if the absorption was at least double that of the corresponding negative.

Antisera were tested by ELISA for cross-reactivity with the following plasma and connective tissue proteins: bovine serum albumin (BSA), fibronectin, fibrinogen, human elastin from the aortae of a foetus, 16 year old and a 70 year old, trout bulbus arteriosus elastin, bovine ligamentum nuchae elastin, acid-soluble collagen and putative microfibrillar component 35-kDa-GP.

2.2.7. Preparation of Tissue for Electron Microscopy.

From aortae excised from aborted human foetuses (14, 16, 17 and 23 weeks old), small blocks of tissue were fixed with 2% gluteraldehyde, post-fixed with OsO_4, dehydrated and embedded in epoxy resin (see Figure 2.1.). The formulation of the epoxy resin used was 10ml Epon 812 (resin), 10 ml dodecenyl succinic anhydride - DDSA (hardener), 1.2ml hexahydrophthalic anhydride - HHPA (plasticer) and 0.6ml benzyl dimethylamine - BDMA (accelerator). Ultrathin sections were cut (800-1300 Å) with a glass knife on a Reichart OM-U2 ultramicrotome, Austria, and transferred onto bare nickel 300-mesh grids.

TABLE 2.1. BUFFERS USED IN ANTIBODY PURIFICATION AND QUANTITATION.

DEAE Column Buffers:-	
A. 0.01M K H_2PO_4	To Elute Pure IgG
B. 0.3M K H_2PO_4	To Elute IgA, IgM, transferrin, albumin and other serum proteins
ELISA Buffers:-	
Binding Buffer:-	0.05M Carbonate Buffer (pH 9.6).
Washing Buffer:-	0.1% BSA + 0.05% Tween-20 in 0.1M Phosphate Buffer (PBS) (pH 7.4).
Blocking Buffer:-	1% BSA + 5% Normal Rabbit Serum + 0.05% Tween-20 in PBS (pH 7.4).
Phosphate Citrate Buffer:-	24.3ml 0.1M Citric Acid + 25.7ml 0.2M Na_2HPO_4 + 50ml distilled H_2O (pH 5). Add 80mg ortho-Phenylene Diamine + 60ul H_2O_2 to this buffer.

FIGURE 2.1. PROTOCOL FOR EMBEDDING SPECIMENS IN EPOXY RESIN.

Specimens fixed 2 hr at 4°C with 2% gluteraldehyde in 0.1 M phosphate buffer pH7.4 (PBS).

↓

Gluteraldehyde-fixed specimens rinsed with PBS.

↓

Post-fixed with 1% (w/v) OsO_4 in PBS, 30 minutes.

↓

70% (v/v) ethanol, 15 minutes.*

↓

95% (v/v) ethanol, 15 minutes.*

↓

2 changes of 100% ethanol, 20 minutes each.*

↓

Infiltrated with 50:50 mixture of resin:100% ethanol, 1 hour.*

↓

Infiltrated ovenight with undiluted epoxy resin.*

↓

Resin changed and infiltrated for further 3 hours.*

↓

Embedded in BEEM capsules and polymerised overnight at 60°C.

* These steps were carried out with specimens in capped bottles, being rotated at approximately 2 revolutions per minute.

2.2.8. Immunogold Staining of Sections.

This method, described in detail by Roth (1984), is as follows:-

(a) The grids were placed onto drops of 1% BSA in 0.1M phosphate buffer (pH7.4) for 10 minutes at room temperature in order to minimise non-specific antibody binding.

(b) Grids were incubated on drops of either normal pre-immune rabbit IgG (control) or rabbit anti-elastin IgG pre-adsorbed on human aortic elastin (control), or rabbit anti-human elastin IgG, all in 0.1M phosphate buffer (pH7.4) at 4°C. Since high dilutions and long incubation times minimised background and enhanced specific staining, overnight incubations at an antibody dilution of 1:5000 were used.

(c) Grids were rinsed for 3x5 minutes with 0.1% BSA in 0.1M phosphate buffer (pH7.4.)

(d) Grids were incubated on drops of goat anti-rabbit IgG conjugated to colloidal gold at a dilution of 1:30 in 0.1% BSA in 0.1M phosphate buffer (pH 8.2) for one hour at room temperature.

(e) Grids were rinsed for 3x5 minutes in 0.1% BSA in 0.1M phosphate buffer (pH8.2.)

(f) Grids were stained with 1% uranyl acetate for 15 minutes and 1% lead citrate for 3-5 minutes as described by Venable and Coggeshall (1965).

Grids were examined in a Phillips EM 301, Holland at 60kv and photographs taken at instrumental magnifications varying from 7,200 to 45,000

2.3. RESULTS.

2.3.1. Amino Acid Composition of Human Foetal Elastin.

In order to assess the purity of the immunogen, the amino acid composition of the human foetal elastin used to immunise rabbits was compared to the amino acid analysis of human elastin (Spina et al., 1983). Serine and threonine values have been corrected for hydrolytic losses by the addition of 10% and 5% respectively to experimentally determined values. By comparing the composition of the two proteins in Table 2.2., it can be concluded that this elastin preparation was a contaminant-free immunogen.

TABLE 2.2.
AMINO ACID COMPOSITION OF HUMAN AORTIC ELASTIN.

Amino Acid	Foetal Aortic Elastin	Adult Aortic Elastin Spina et al., 1983
Hydroxylysine	N.D.	1.6
Lysine	21.5	13.2
Histidine	3.7	2.2
Arginine	16.8	19.2
Hydroxyproline	45.7	38.2
Aspartic Acid	16.0	15.5
Threonine	11.4	10.4
Serine	16.4	13.9
Glutamic Acid	31.9	33.3
Proline	129.8	120.0
Glycine	344.0	347.8
Alanine	175.7	188.2
Half-cysteine	0.8	3.3
Valine	99.5	96.0
Methionine	0.0	2.3
Isoleucine	11.0	17.4
Leucine	41.5	44.0
Tyrosine	11.5	12.4
Phenylalanine	14.7	16.4
Isodesmosine	2.8	4.4
Desmosine	4.9	8.0
Lysinonorleucine	N.D.	1.2

Values are expressed as residues/1000 total amino acid residues.

2.3.2. Immunochemical Characterisation of Anti-Elastin Antiserum.

The indirect ELISA method was used to study the specificity of the anti-elastin antiserum. Optimal coating of the plate with human α-elastin was found to be 100ng/ml as determined by checkerboard titration technique. All antigens were coated onto microtitre plates at this concentration.

Significant titres to human α-elastin were first obtained in the sera of rabbits No.1, 3, and 4, eight weeks after the initial immunisation. Rabbit No.2 failed to produce a significant anti-elastin response and as a result only the antisera of 1,3, and 4 were pooled and purified.

As shown in Figure 2.2.(a), a titre of 1:10,000 was routinely obtained for the rabbit anti-human elastin antiserum when tested against human α-elastin from either foetal, 16-year old or 70-year old aortae. No specific binding of the antibody was observed to bovine or trout α-elastin, as shown in Figure 2.2.(b).

A similar negative response was obtained with acid-soluble collagen [shown in Figure 2.2.(b)], bovine serum albumin, fibronectin and microfibrillar-associated glycoprotein 35-kDa-GP.

FIGURE 2.2.(a).

Reactivity of anti-human elastin antiserum against: human α-elastin from (⊡) foetal; (★) 16 year old; (⊠) 70 year old aortae. Reactivity of pre-immune rabbit serum against human foetal aortic elastin (✳). In calculation of titre, A_{492} readings were considered positive when double the corresponding values for pre-immune serum. All antigens used at 100ng/ml.

FIGURE 2.2.(b).

Reactivity of anti-human elastin antiserum against: α-elastin from (⊡) human foetal aorta; (✳) bovine ligamentum nuchae; (⊠) trout bulbus arteriosus and against acid-solubilised collagen (▲). All antigens coated onto plate at 100ng/ml.

2.3.3. Ultrastructural Localisation of Anti-Elastin Antibodies.

The most prominent feature in the media of the 14-week old human foetal aorta was the absence of any structure identifiable as elastin. However, in selected areas, such as that shown in Figure 2.3., the 5nm colloidal gold was deposited among and in close proximity to bundles of microfibrils. The microfibrils themselves were not decorated with the gold particles.

Although such an arrangement was still discernible in the 16- and 17-week old aorta (Figures 2.4. and 2.5. respectively), the majority of the 20nm colloidal gold was bound to elastin which appeared to be in the process of forming fibrils, arranged in layers, separated by the cellular component. As shown in Figure 2.6., most of the developing fibrils were in close proximity to the cell membrane of the elastogenic cells.

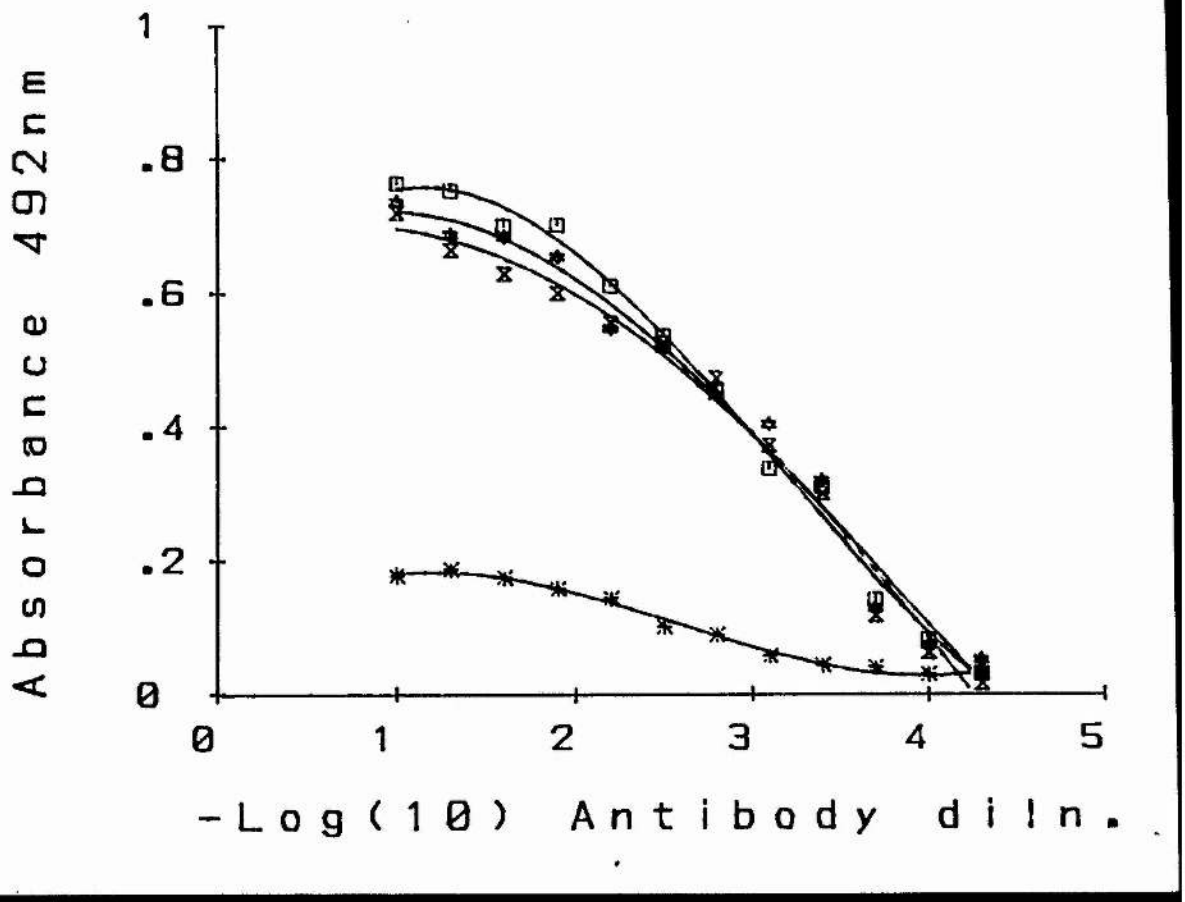
Figures 2.7. and 2.8. show areas of the media from a 23-week old foetal aorta in which elastin was arranged in well-defined layers, characteristic of the fully developed aorta. However, at variance with the adult tissue, the elastic component did not form fenestrated membranes but was still in fibrillar form, as seen in Figures 2.9. and 2.11., in which the direction of sectioning was at right angles to that of Figures 2.7. and 2.8.

FIGURE 2.3.

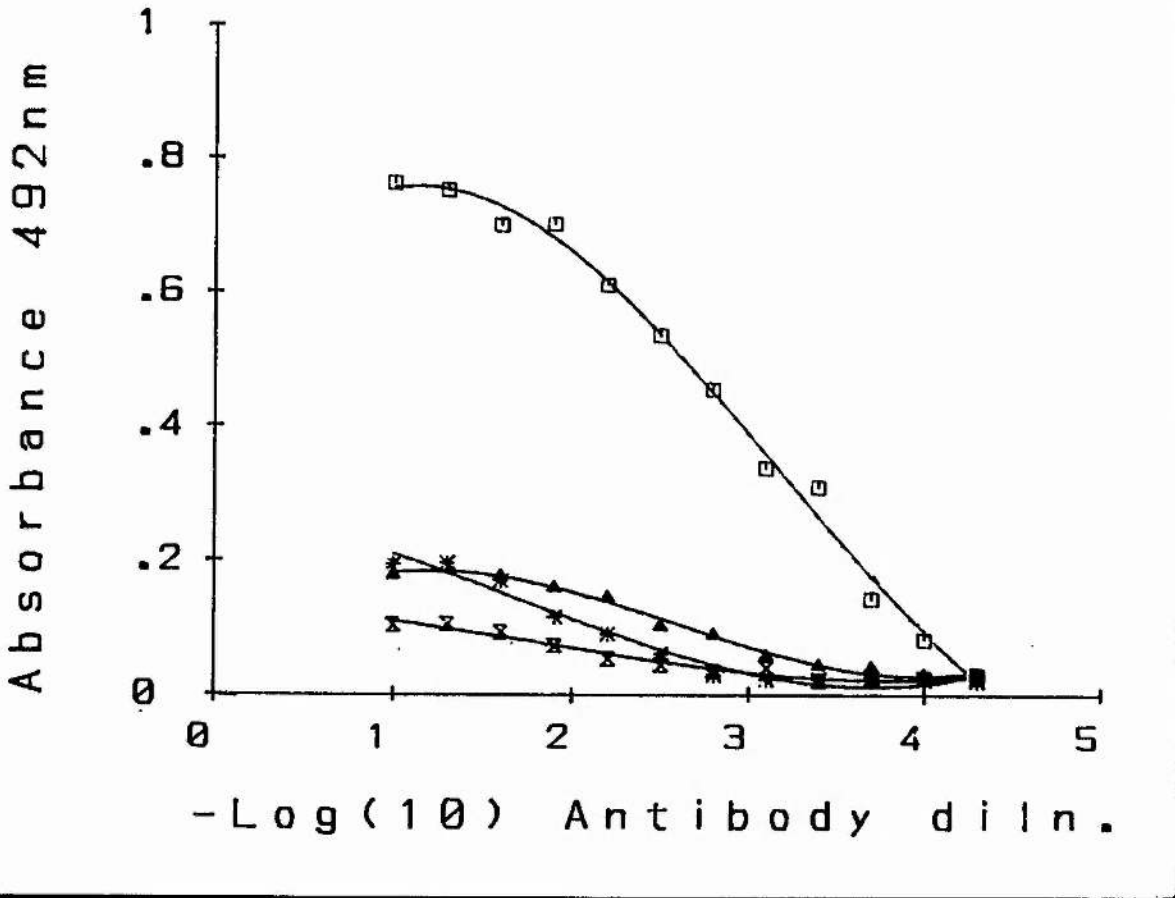
14-week old human foetal aortic tissue treated with anti-human elastin IgG as primary antibody. The 5nm colloidal gold indicates that elastin, although not morphologically distinct, is present in association with the microfibrillar component. Bar 0.1um; x 160,000

FIGURE 2.4.

16-week old human foetal aortic tissue treated with anti-human IgG as primary antibody. The 20nm colloidal gold binds to distinct loci of amorphous material (arrows) and to areas where the elastin has coalesced to generate the central core of the newly formed elastic fibril. Bar 0.1um; x 43,000.


Absorbance 492nm
1
.8
.6
.4
.2
0
0
1
2
3
4
5
-Log(10) Antibody diln.

2.2.a


Absorbance 492nm
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.4
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1
2
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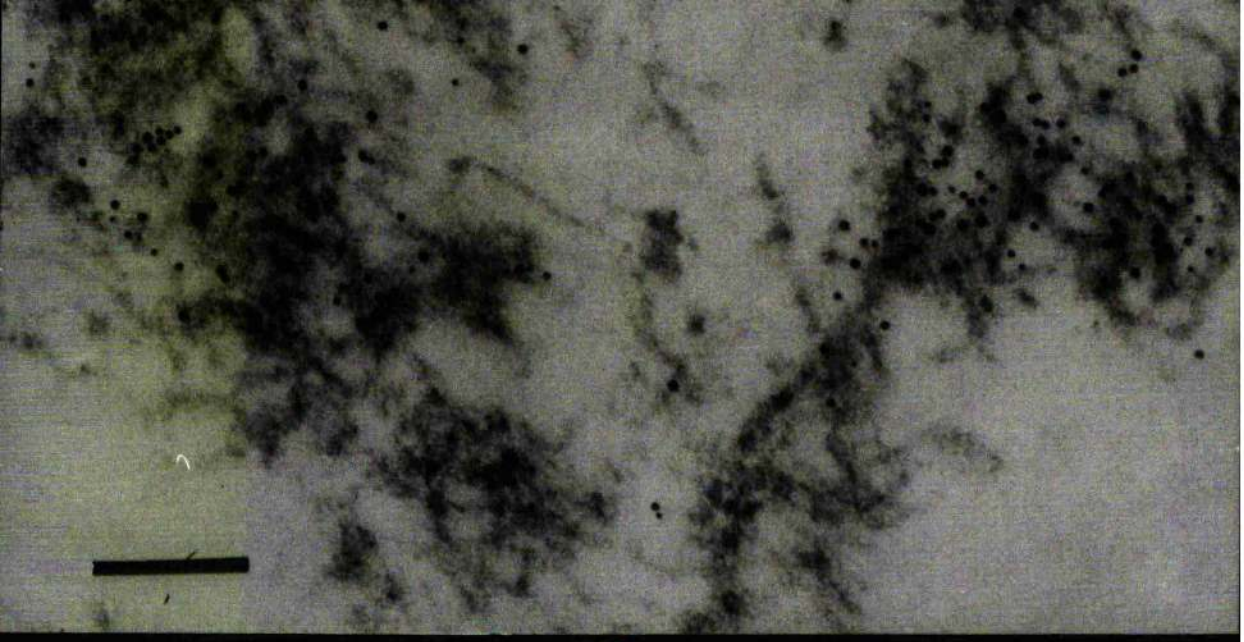
4
5
-Log(10) Antibody diln.

2.2.b



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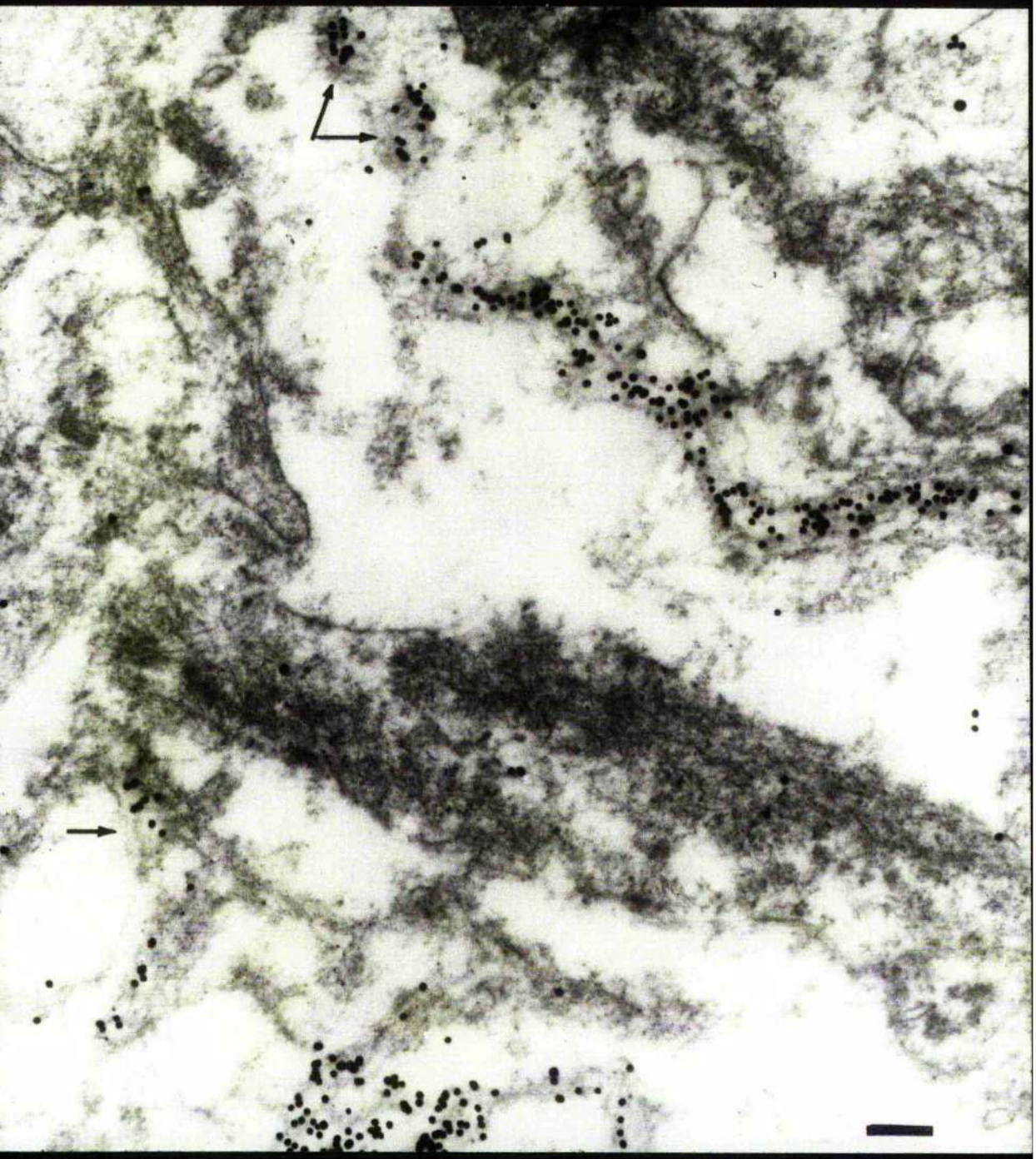


2.4

FIGURE 2.5.
17-week old human foetal aortic tissue treated with anti-human elastin IgG as primary antibody. Cross-section of the aortic wall. Elastin begins to form layers of fibrils (arrows). Bar 1um; x 25,000.

2.5.

FIGURE 2.6.

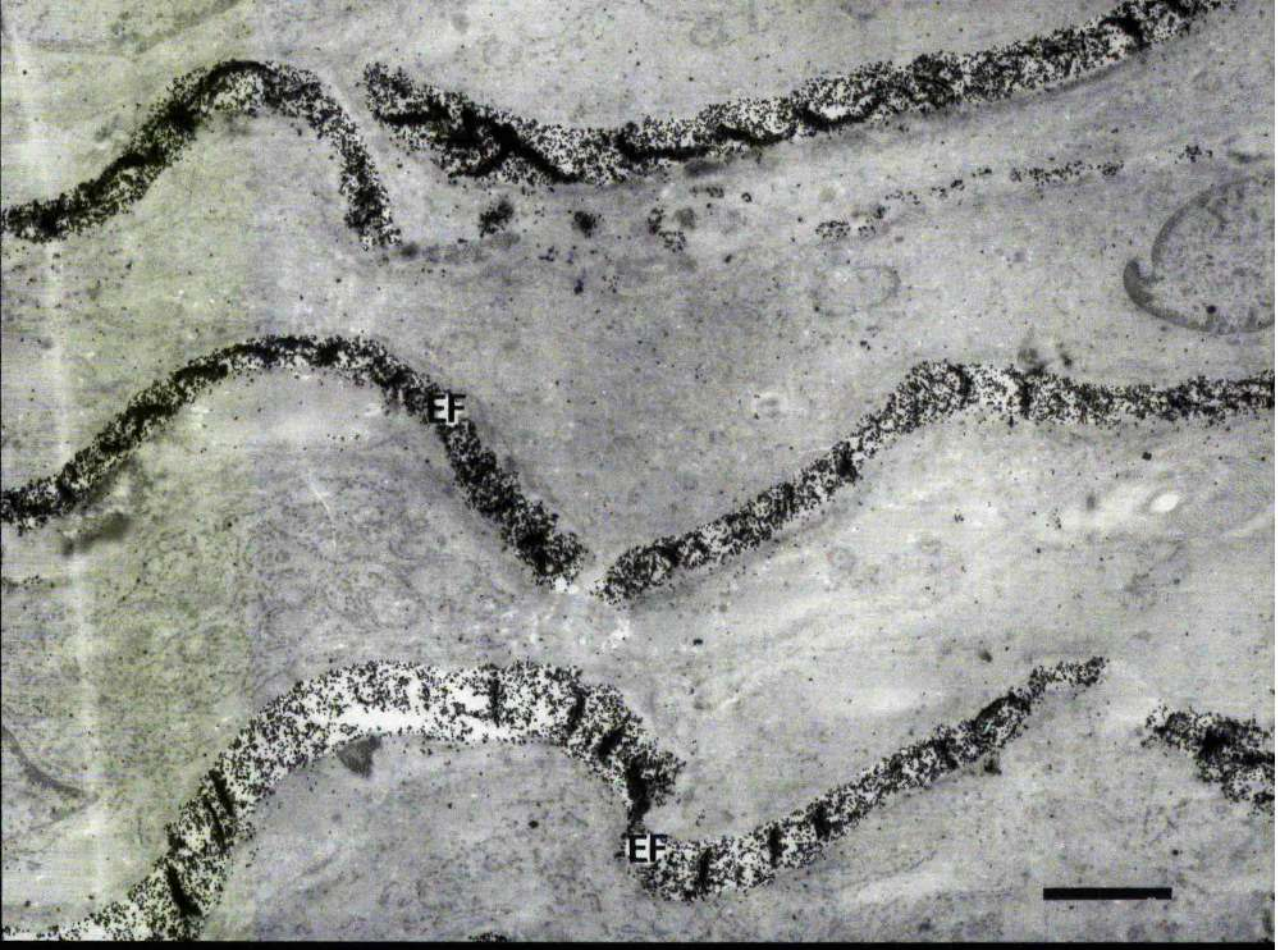
17-week old human foetal aortic tissue treated with anti-human elastin IgG as primary antibody. The distribution of 15 nm gold clearly demonstrates that there is a close inter-relationship between elastogenic cells and immature fibres, the latter being situated within infoldings of the cell surface. Bar 0.5um; x 30,000.

FIGURE 2.7.

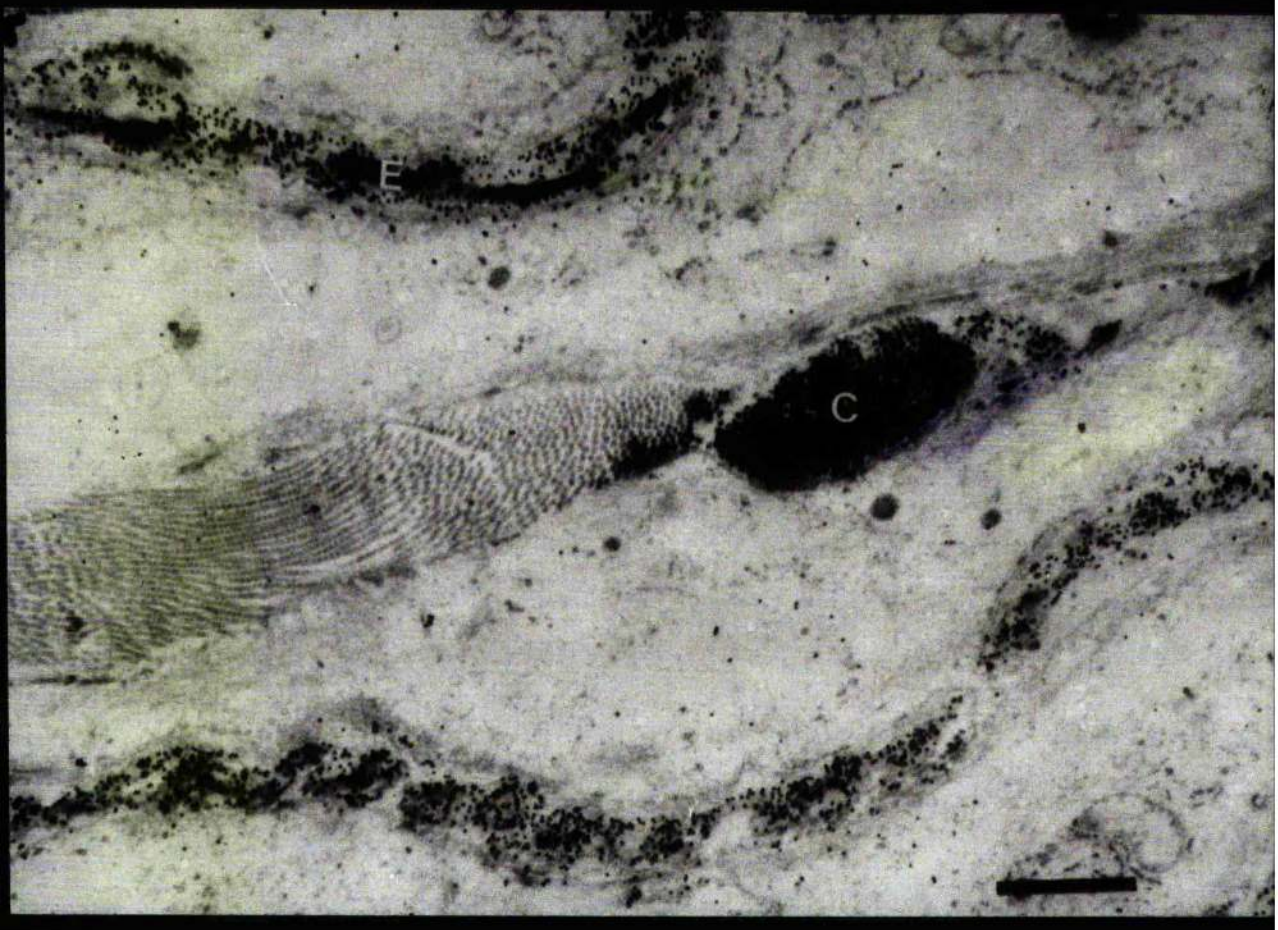
Human foetal aortic tissue (23-week old) treated with anti-human elastin IgG as primary antibody. Cross-section of the aortic wall. The binding of 20nm colloidal gold is restricted to elastic fibrils (EF). A helical pattern is discernible both in the uptake of contrast and colloidal gold binding. Bar 2um; x 8,000.

FIGURE 2.8.

23-week old human foetal aortic tissue treated as above. An electron micrograph taken at higher magnification to show the helical patttern of cationic and immunogold stains. C, collagen; E, elastic fibre. Bar 1um; x 15,000.



2.7.



2.8.

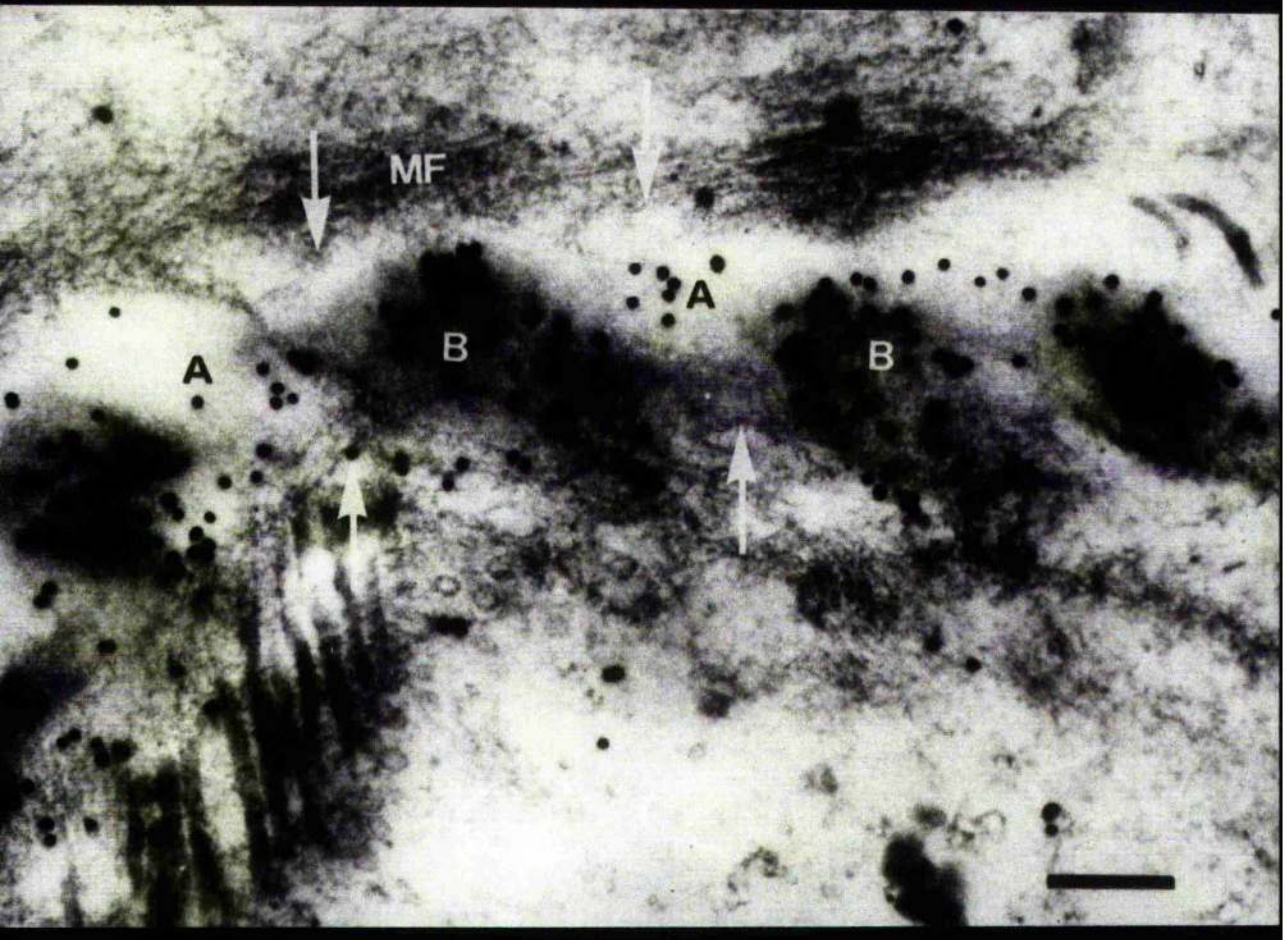
FIGURE 2.9.

Human foetal aortic tissue (23-week old) treated with anti-human elastin IgG as primary antibody. A radial section of the aortic wall showing an area where the immunogold binds to the peripheral circular section of each fibril. Bar 1um; x 18.000.

FIGURE 2.10.

23-week old human foetal tissue treated with anti-human elastin IgG as primary antibody. Cross-section of the aortic wall. A = electron-transparent region of elastic fibril; B = electron-dense area of elastic fibril; MF, microfibrillar component. Arrows define boundaries of elastic fibril. Bar 0.2um; x 65,000.

2.9.



2.10.

In these radial sections of the aortic wall (Figures 2.9. and 2.11.), the colloidal gold was seen to bind specifically to the peripheral circular sector of each fibril over an area which was also more intensely stained with electron-dense ions than the underlying core. While in the cross-section of the 17-week old aortic wall the colloidal gold was uniformly deposited on the elastin substrate, in the 23-week old specimens it formed, on each fibril, discrete clusters which coincided with electron-dense areas. These clusters appeared to exhibit some degree of regularity in their distribution and orientation which conformed, in most areas, with a helical pattern (Figures 2.7. and 2.8.).

This arrangement is particularly evident in Figure 2.10. which was taken at a higher instrumental magnification. In this figure, the microfibrillar component (MF) clearly defines the boundaries (indicated by arrows) of an elastic fibril composed of electron-transparent (A) and electron-dense (B) areas. The anti-human elastin antibodies are preferentially concentrated in those areas of the fibril which show maximal uptake of electron-dense ions. Figure 2.11. shows the cross-section of a large fibril which exhibits an uneven peripheral distribution of both colloidal gold and electron-dense contrast. This morphology is interpreted as representing the cross-section of a helical pattern similar to that shown in Figure 2.10.

When rabbit anti-human elastin IgG, pre-adsorbed on human aortic elastin, or normal pre-immune rabbit IgG, were employed as a primary antibody in immunoelectron microscopy, no specific immunological binding was observed (Figure 2.12.).

No antibody binding could be detected within the cell by etching with 5% periodic acid, 10% hydrogen peroxide, or 1% sodium methoxide to reveal further antigenic sites hidden by the resin.

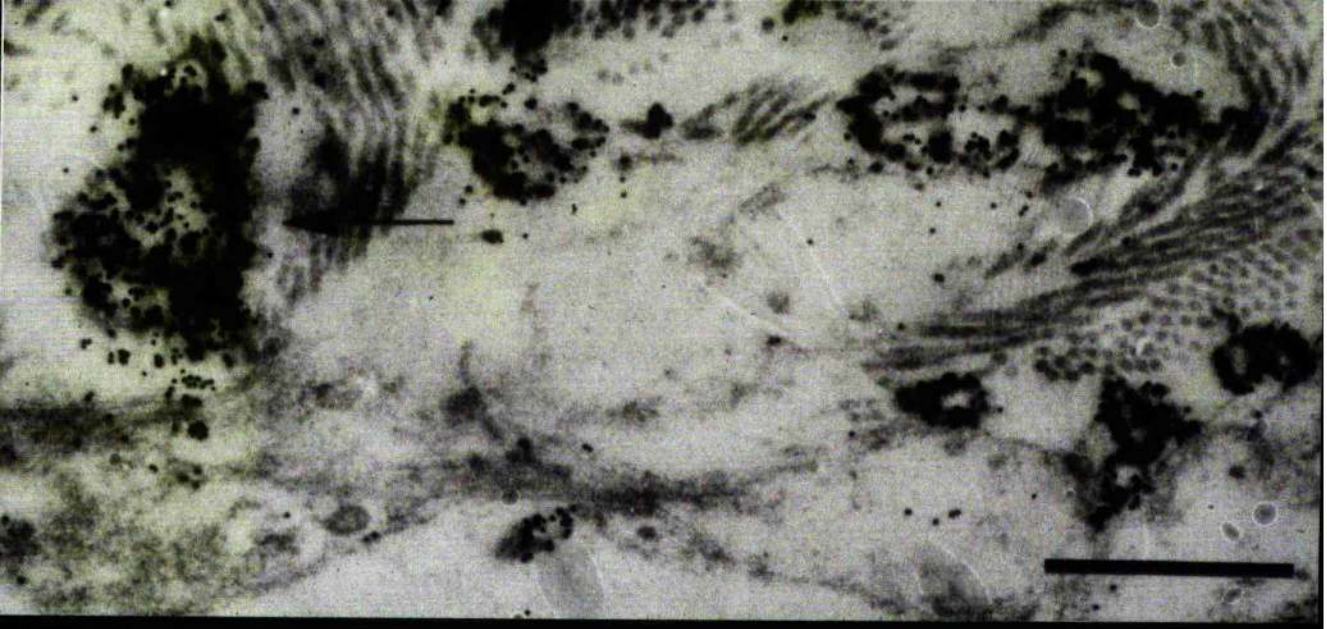
Of interest, with respect to the elastogenic cells however, was the appearance of spiral arrangements of the cell membrane around the growing elastic fibres as shown in Figures 2.13. and 2.14. The cells completely enclose a very high proportion of the developing fibres (Figure 2.13.) and occasionally produce spiral arrangements of their cell membrane (Figure 2.14.). The gap between the plasma membrane and the fibre is often just sufficient to accommodate the microfibrillar layer (see arrows in Figures 2.13. and 2.14.). Frequently where a close contact is established, numerous vesicles can be seen associated with the plasma membrane (Figure 2.15.). Moreover, cells exhibit, albeit to a variable extent, cytoplasmic polarisation of the endoplasmic reticulum, cell organelles and vesicles in the area of cell-fibre interaction (Figure 2.15); and within the cytoplasm, in close proximity to the cell membrane, an alignment of microfilaments and/or microtubules as indicated in Figure 2.16.

FIGURE 2.11.

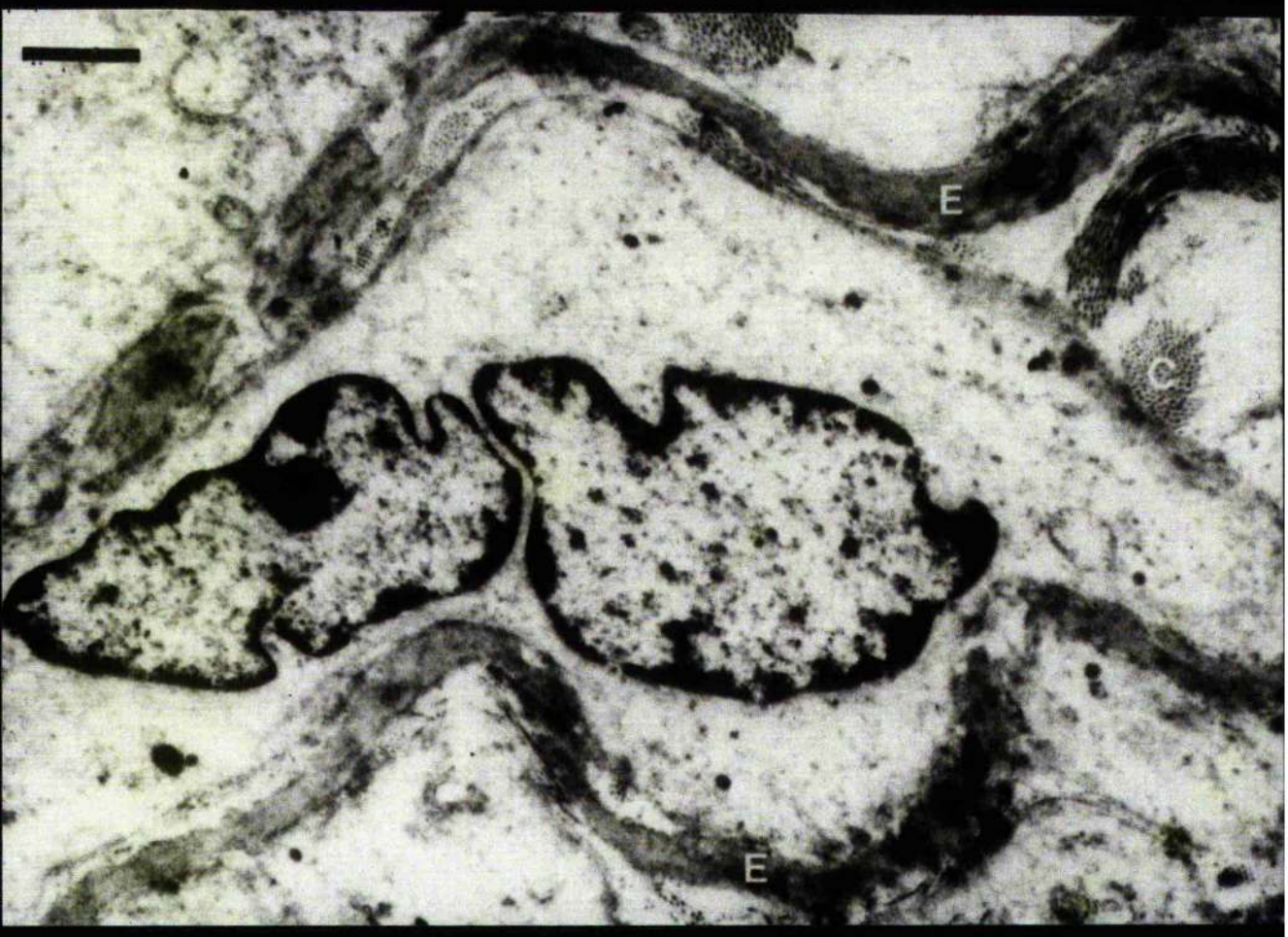
Human foetal aortic tissue (23-week old) treated with anti-human elastin IgG as primary antibody. A radial section of aortic wall showing a large fibril (arrow) exhibiting an uneven peripheral distribution of both colloidal gold and electron dense contrast. Bar 1um; x 25,000.

FIGURE 2.12.

Human foetal aortic tissue (23-week old) treated with normal pre-immune rabbit IgG as primary antibody. No significant binding of colloidal gold is present within the tissue. E, elastic fibril; C, collagen fibres. Bar 1um; x 12,000.



2 .11.



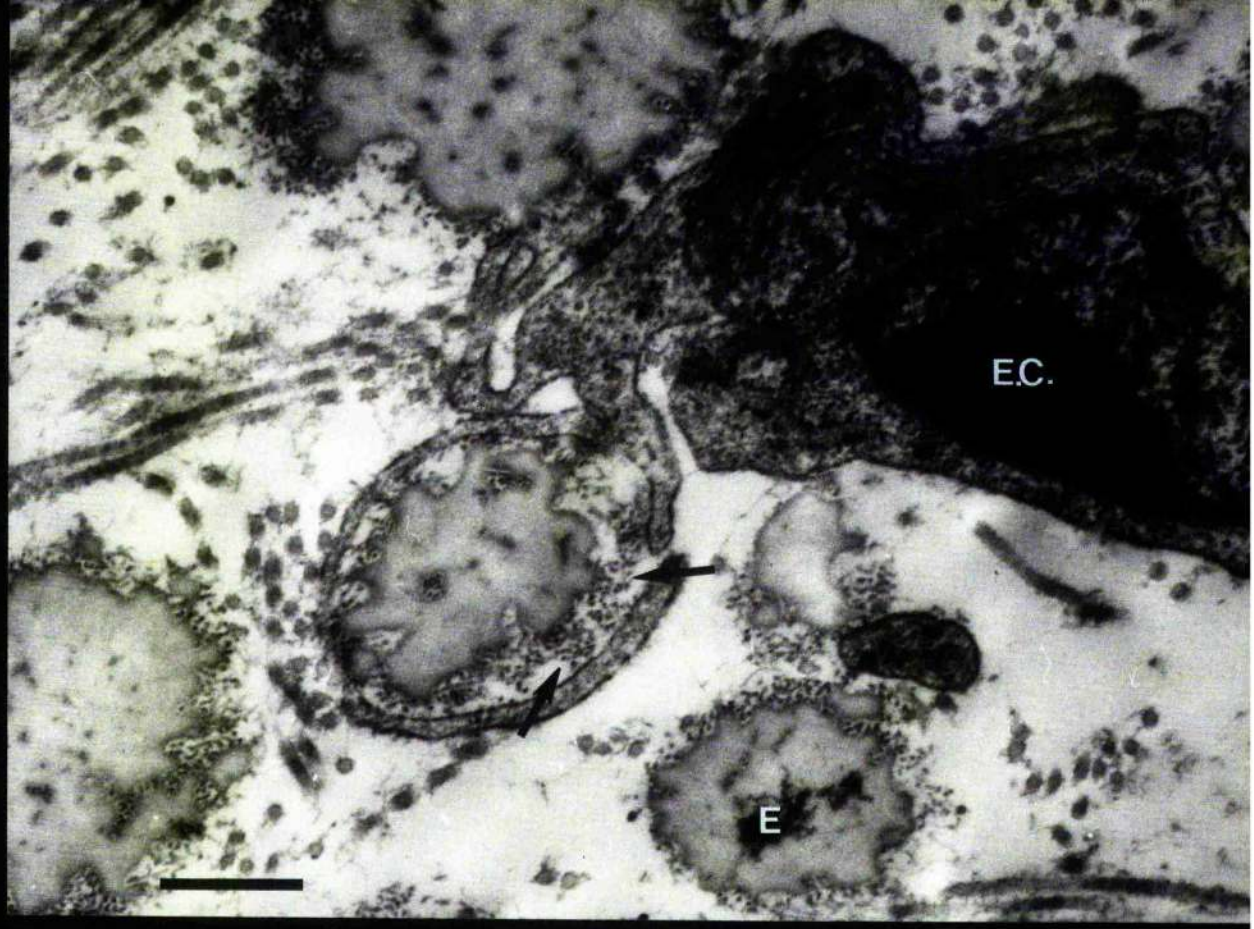
2.12.

FIGURE 2.13.

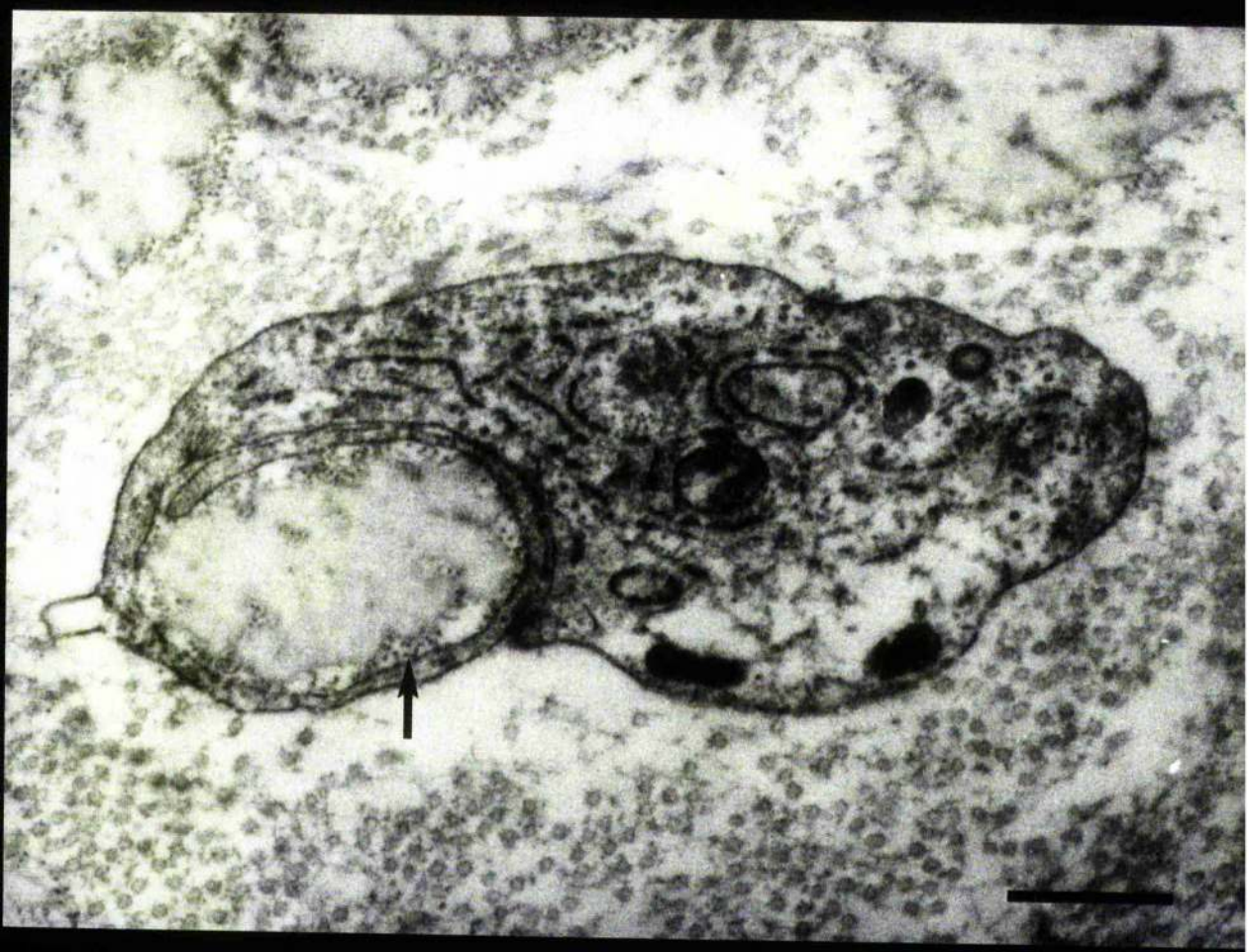
The elastogenic cell (EC) in this transverse section completely encloses an elastic fibre. Note the microfibrillar component (arrows) in close contact with the plasma membrane. Section was stained with uranyl acetate and lead citrate only. Bar 0.5um; x 32,000.

FIGURE 2.14.

This electron micrograph shows the transverse section of a elastogenic cell wrapped around an elastic fibre. The arrow indicates an area of close contact between the microfibrillar component and the cell membrane. Section stained with uranyl acetate and lead citrate only. Bar 0.5um; x 35,000.



2 .13.



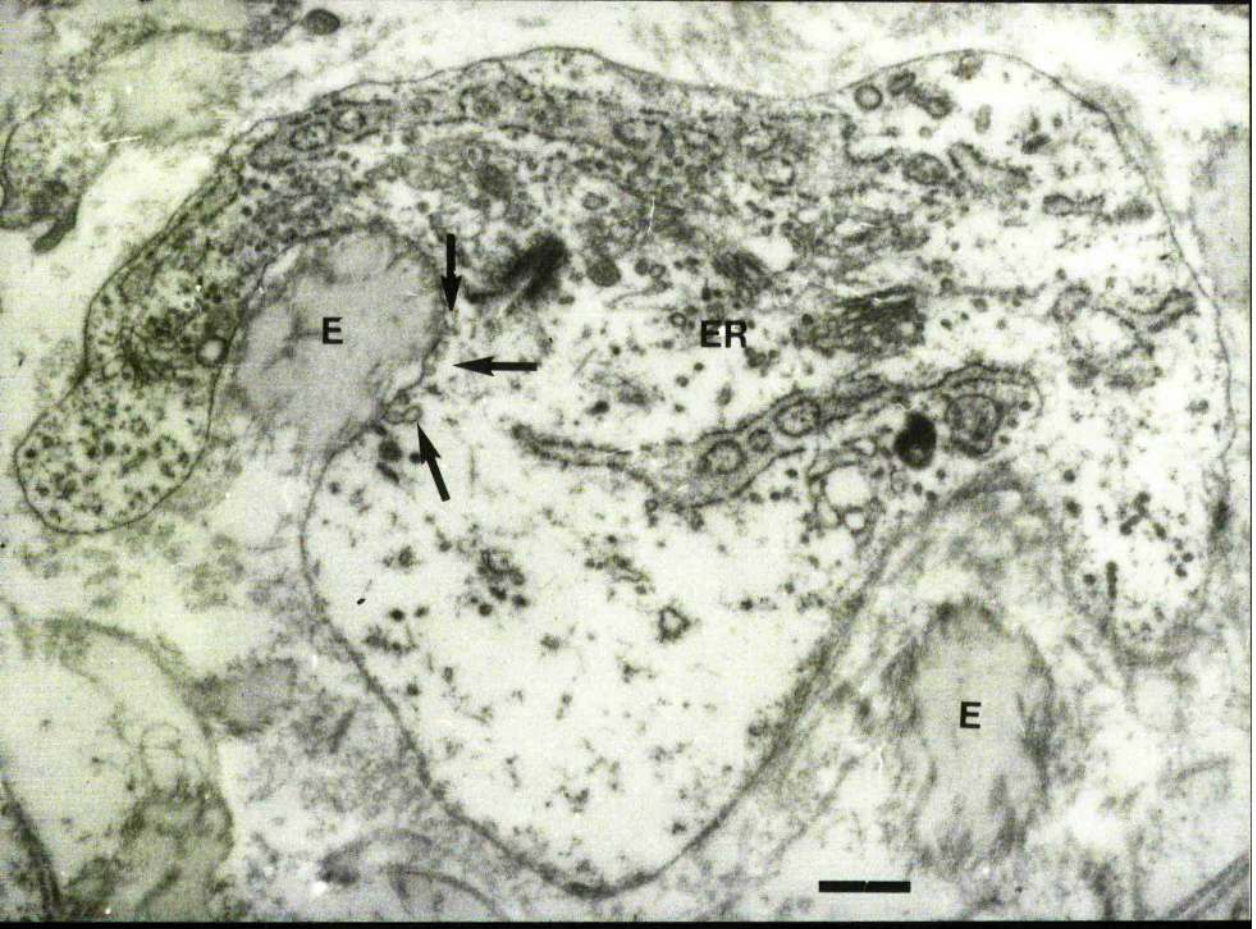
2 .14

Figure 2.15.

This transverse section of an elastogenic cell demonstrates the preferential distribution of the endoplasmic reticulum (ER) and cell organelles in the area adjacent to the infolding of the plasma membrane that contains an elastic fibre (E). Note the discharging vesicles (arrows) in the area of contact. Section stained with uranyl acetate and lead citrate only. Bar 0.5um; x 21,000.

FIGURE 2.16.

Electron micrograph of an elastogenic cell enclosing an elastic fibre (E). In this area, vesicles are present (v) and there is an alignment of microfilaments (mf) at the plasma membrane. Section stained with uranyl acetate and lead citrate only. Bar 0.5um; x 31,000.



2.15.

2.16.

2.4. DISCUSSION

The observations reported on the distribution of elastin in the tunica media of human foetal aorta are in agreement with the model first proposed by Fahrenbach et al. (1966) for the biogenesis of elastic fibres. Moreover, the pattern of immunogold deposition in the 14-week old sample proves that the elastin precursor accumulates in the proximity of the microfibrillar component at a stage of tissue development in which the concentration of elastin in the matrix is not sufficient to produce, even in these discrete areas, supramolecular networks of a size detectable by electron microscopy. This is also in agreement with the observation that the early deposition of elastin in embryonic tissues occurs between and around the microfibrillar component and that eventually elastin takes the shape already assumed by the microfibrillar aggregates (Ross et al., 1977; Cleary et al., 1981).

However, a more important contribution to our understanding of the morphogenesis of elastic tissue is provided by the images obtained with the 23-week old tissue which exhibits a structural organisation similar to that of the fully developed aorta while still retaining the elastic component in fibrillar form. In this tissue, both the uptake by elastin of electron-dense metal ions and the binding of anti-elastin antibodies conform to a spiral pattern in the majority of the elastic fibrils examined.

The uptake of metal ions by elastin is dependent not only on the number of amino acid residues with ionisable side-chains present in the protein, but also on the degree of its structural organisation (Serafini-Fracassini, 1982). As previously mentioned in Chapter One (Page 4), during the early stages of morphogenesis, elastic fibres exhibit in conventionally stained tissue sections, some affinity for heavy metal ions. However, concomitantly with the progressive acquisition by the constituent protein of its definitive conformation and the establishment of the inter-chain cross-links, these fibres modify their staining characteristics and rapidly become almost transparent to electrons (Kewley et al., 1978). Therefore, where a developing fibril shows the coexistence of electron-transparent and electron-dense areas, it is plausible to assume that the latter correspond to the distribution of the newly deposited and still uncross-linked elastin. In this respect, it should be noted that the intensity of electron-dense ion uptake by elastin is not dependent on the age of the tissue examined, but is a function only of the rate of tropoelastin synthesis, since the time required for the formation and stabilisation of cross-links within the elastic fibril is probably constant. Therefore, the occurence of intensely stained elastic fibrils should be maximal in the later stages of foetal development when the rate of elastin biosynthesis is known to increase markedly and abruptly (Eichner and Rosenbloom, 1979). This interpretation is also consistent with the morphology exhibited by the 23-week old

elastic fibrils which in cross-section show a core, almost totally transparent to electrons and an intensely stained peripheral circular sector.

The rabbit anti-human elastin antiserum used in this investigation bound equally well to human elastin purified from aortae of different age groups, varying considerably in amino acid composition as a result of the accumulation of significant amounts of polar proteins with ageing (Spina et al., 1983). These antibodies also showed a remarkable degree of species specificity, being able to differentiate between human and bovine elastin which are compositionally closely related. As Mecham and Lange (1980, 1982a+b) have shown that in elastin, determinants related to cross-linking domains show species cross-reactivity, whereas determinants located in the intervening regions are species specific, it is plausible to assume that our antibodies were elicited to epitopes located in the intercross-linked segments. These domains become partially inaccessible to large macromolecules once the protein is fully cross-linked and thus less antibody staining is observed (Barnard et al., 1982). Conversely, the enhanced antibody binding in foetal tissue is probably indicative of the presence of a largely uncross-linked substrate.

As the helical pattern of electron-dense staining and immunogold deposition co-exists with an equally specific and discontinuous distribution of both immunogold and cationic stain in transverse sections of elastic fibrils, it is difficult to

visualise how such a pattern could result from the differential shrinkage of the elastin component due to contraction of the specimen prior to or during fixation. It should also be noted that excision of a fully developed human aorta results primarily in a deformation in the longitudinal direction (Hesse 1926) which should not produce any significant artifact in fibrils arranged with their major axis on a plane perpendicular to the longitudinal axis of the vessel.

In the light of these interpretations, the identification in the 23-week old tissue of super-imposable discontinuous patterns of ion-uptake and immunogold binding by elastic fibrils suggest that, during this stage of development, newly synthesised elastin is not deposited uniformly on pre-existing fibril cores. This observation is not consistent with the model proposed by Pasquali-Ronchetti in which the elastin precursor is transferred from the elastogenic cell to the site of fibre formation by passive diffusion through the extracellular matrix. Conversely, it is in keeping with the view that biogenesis of elastic matrices requires the establishment of a special dynamic relationship between elastin-producing cells and the surface of the growing elastic fibrils. This is supported by the electron micrographs in the Results Section of this Chapter which show many fibres, at various stages of development, in close contact with the elastogenic cell - the plasma membrane often in direct contact with the microfibrillar layer. This alternative model not only overcomes the conceptual difficulties related to the

diffusion of tropoelastin, which is insoluble in a physiological mileu, but also provides an insight into the mechanism responsible for the generation of supra-molecular structural organisations performing well-defined mechanical functions.

Detailed information on the three-dimensional relationship of the structural components of the aortic wall, namely elastin, collagen and microfibrils is unfortunately lacking. However, ultrastructural data obtained on mammalian yellow ligaments shows the existence of a well-defined multicomponent system designed to resist unidirectional stresses (Serafini-Fracassini et al., 1977). In these ligaments, the elastic fibres are not directly inserted into the vertebral laminae. Their mechanical coupling is provided by the collagen fibres which, while anchored at both ends to the supporting bone, form spiral sheets that envelop individual elastic fibres and prevent their slippage even at high degrees of induced deformation. The formation of this spiralling collagen envelope could be visualised to occur concurrently with the deposition of the final layer of elastin on the fully grown elastic fibres, should concomitant translational and rotational morphogenetic movements of specialised biosynthetic cells be a common feature of the elastogenic phase of development of elastic matrices.

The anti-elastin antiserum was unable to detect tropoelastin within the elastogenic cells, despite various etching procedures. There are a number of possible reasons for this:-

(a) The tropoelastin epitopes may have been destroyed during the embedding of the tissue, particularly during the polymerisation of the resin at 70°C.

(b) The amount of tropoelastin within the cells may have been too low to be detected above background by the indirect immunogold technique. The use of a tertiary or even quaternary antibody conjugated to colloidal gold could be used in future to amplify any specific binding by the primary antibody. This has already been undertaken by Damiano et al. (1984), in a peroxidase-antiperoxidase study of tropoelastin within the endothelial and medial cells of the embryonic chick aorta (Chapter One, Page 39).

(c) Another possibility is that the antigenic determinants are hidden from the anti-elastin antiserum by an intracellular component bound to the tropoelastin molecules to prevent coacervation in vivo. One possible candidate suggested by Baccarani-Contri et al (1985) are negatively-charged proteoglycans which may bind to tropoelastin by electrostatic forces and prevent precipitation at physiological temperatures.

(d) Finally, the anti-α-elastin antiserum may not cross-react with tropoelastin. Daynes et al. (1977) have shown by radioimmunoassay that tropoelastin possesses specific antigenic determinants which are no longer present after assembly into mature elastic fibres and vice-versa, during

the formation of insoluble elastin, structural changes result in new antigenic determinants e.g. the cross-links.

CHAPTER THREE.

ISOLATION AND CHARACTERISATION OF THE 35k-Da GLYCOPROTEIN COMPONENT OF ELASTIN-ASSOCIATED MICROFIBRILS.

3.1. INTRODUCTION.

The electron microscopic observation that the microfibrillar component of elastic fibres is solubilised by chaotropic solutions containing a reducing agent (Ross and Bornstein, 1969) provided a procedure that has been widely adopted in subsequent studies aimed at the identification of the constituent protein(s). As described in Chapter One (Sections 1.4.2. and 1.4.3.), many groups have attempted to link the isolation and characterisation of putative microfibrillar protein(s) present in such extracts with immunochemical investigations. However, in most of these studies the immunogens have consisted of either single fractions, the purity of which is suspect, or total extracts. As a result, several of the antisera have been found to be directed against ubiquitous components of the connective tissue matrix e.g. fibronectin and collagen-like proteins.

In this laboratory, a major glycoprotein component has been isolated from guanidine hydrochloride extracts of bovine ligamentum nuchae and characterised in terms of purity, molecular weight (35,000 Daltons), compositional parameters and enzymatic activity. Upon dialysis in the presence of Cu^{2+}, this glycoprotein (referred to as **35k-GP**) was found to aggregate and form fibrils with a uniform diameter of 11nm, an observation that led to the suggestion that it represents one of the components of elastin-associated microfibrils.

This Chapter reports on the production and characterisation of a polyclonal antiserum to 35k-GP and its use in the immunoelectron microscopic localisation of the antigen in thin sections of various foetal bovine tissues.

3.2. MATERIALS AND METHODS.

3.2.1. Materials.

Sephacryl S-300 and Protein-A Sepharose were bought from Pharmacia, Uppsala, Sweden; Affigel-15 from Bio-rad, Watford, London. The following chemicals were obtained from B.D.H. Ltd., Poole, England:- Acrylamide, bis-Acrylamide, Sodium Dodecyl Sulphate (specially pure), NNN'N'-tetramethylethylenediamine (TEMED), Amido Black and Coomassie Blue. L.R. White resin (Medium Grade) was obtained from Agar Aids, Stansted, Essex. Amicon filtration cell and PM-10 membranes were bought from Amicon, The Hague, Holland. Nitrocellulose membranes were obtained from Schleicher and Schull, Dassel, West Germany. The sources of all the other materials mentioned in this section have been described in 2.2.1.

3.2.2. Extraction of 35k-GP from Bovine Ligamentum Nuchae.

The extraction procedure used was essentially as described by Serafini-Fracassini et al. (1981a) with two important modifications:-

(a) A more extensive programme was undertaken in the extraction stage with 5M guanidine hydrochloride. Instead of one 24-hour extraction with 5M guanidine hydrochloride, the tissue was extracted six times over a period of 21 days. An aliquot of each extract was analysed by SDS-polyacrylamide gel electrophoresis.

(b) In order to avoid SDS in the sample, a combination of ion-exchange chromatography and gel-filtration were used to purify 35k-GP, as described by Kawaguchi (1982). The purification proceedure detailed below was undertaken twice on fresh tissue from animals of approximately the same age with the same results.

The neck ligament of a three year old cow was freed from adhering tissue, cut into thin slices and stirred in 1% w/v NaCl at 4°C for two 24 hour periods. Following washing with distilled water, the tissue was defatted and dehydrated in chloroform : methanol 1:2 (v/v) for 24 hours at 4°C followed by a second treatment with chloroform : methanol 3:1 (v/v) at 4°C. The residue was allowed to dry in the fume cupboard and the dry weight recorded. The tissue was then comminuted to a fine powder and the 100-200 mesh fraction suspended in 5M guanidine hydrochloride - 0.4% EDTA - 0.1M TRIS pH7.4 (guanidine HCl buffer) and extracted for at least 72 hours at 37°C with stirring, before centrifugation at 2000g for two hours. The supernatant was saved and the residue resuspended in fresh 5M guanidine HCl buffer and the process repeated. This extraction was repeated a total of six times over a period of 21 days.

At this stage, the residue was extracted in 5M guanidine HCl buffer containing 2% mercaptoethanol for 24 hours under nitrogen at 37°C, then centrifuged at 23,000g for 30 minutes. This final extract was called 'crude microfibrillar extract' for descriptive purposes. All extracts from the above procedures were

concentrated in an Amicon ultrafiltration cell with a PM-10 membrane and dialysed against PBS (pH 7.4) at 4°C, then exhaustively against distilled water before lyophilisation.

Initially, the purification of 35k-GP was by preparative gel electrophoresis as described by Serafini-Fracassini et al. (1981a). However, a number of problems associated with this method soon became apparent, particularly with using the resulting preparation for immunisation:-

(a) only minute quantities were obtained after every preparative gel electrophoresis run;

(b) SDS associated with the sample was difficult to remove;

(c) the resulting extract was toxic to the rabbits.

Thereafter, the 35k-GP was purified by ion-exchange chromatography and gel-filtration. A 1.9 x 12cm column was packed with DEAE cellulose and equilibrated with 6M urea Tris buffer-A (see Table 3.1.). 1g of crude microfibrillar preparation was dissolved in 20mls 6M Urea/Tris buffer-A and after centrifugation to remove any insoluble material, was applied to the equilibrated column. Samples were eluted at a flow-rate of 30ml/hour with 100ml portions of buffers A to E (see Table 3.1.). With each buffer, fractions of 10mls were collected and the absorbance at 230nm measured. Fractions containing protein were pooled and freeze-dried after dialysis against distilled water.

To remove contaminating high molecular weight components, a column 2.6 x 90cm packed with Sephacryl S-300 was equilibrated

with 6M Urea/Tris buffer-C. Samples purified by ion-exchange chromatography were applied to the column, dissolved in 5ml of the same buffer, and eluted at a flow rate of 10ml/hour with the 6M Urea/Tris buffer-C. 5ml fractions were collected and those containing protein (absorbances measured at 230nm) were dialysed against distilled water and analysed on an SDS-PAGE minigel. The 35k-GP fractions were freeze-dried and stored at -20°C at a concentration of 500ug/ml.

TABLE 3.1.

BUFFERS USED IN DEAE PURIFICATION OF 35k-GP.

Buffer A :- 6M Urea-50mM TRIS-HCl containing 0.01M NaCl.

Buffer B :- 6M urea-50mM TRIS-HCl containing 0.05M NaCl.

Buffer C :- 6M urea-50mM TRIS-HCl containing 0.3M NaCl.

Buffer D :- 6M urea-50mM TRIS-HCl containing 0.5M NaCl.

Buffer E :- 6M urea-50mM TRIS-HCl containing 1.0M NaCl.

All the buffers were at pH 7.4.

3.2.3. Polyacrylamide Gel Electrophoresis (PAGE).

Polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of SDS, using the discontinuous buffer system of Laemmli (1970). Gels were prepared from a stock solution of 30% w/v acrylamide and 0.8% w/v N-N'-methylene-bis-acrylamide and polymerised by the addition of TEMED (0.045% w/v) and ammonium persulphate (0.075% w/v) to give a final acrylamide concentration of 12% for separating gels and 3% for stacking gels. Two sizes of vertical slab gel apparatus were used; standard (150 x 140 x 1.5mm) and "mini" (100 x 90 x 1.0mm). Proteins were suspended in sample buffer at a concentration of approximately 1mg/ml, incubated in a boiling bath for two minutes and then cooled quickly. Any insoluble material was sedimented by centrifugation for five minutes at 10,000g in a Beckman microfuge. Samples were then applied to the gel and electrophoresis carried out at room temperature at a constant current of 30mA raised to 40mA when the dye front had reached the separating gel.

Gels were stained for protein with Coomassie Brilliant Blue R-250, using the method of Fairbanks et al. (1971), or for quicker and more sensitive results, by the silver staining method devised by Merril et al., (1981). Glycoproteins were stained with periodic acid/Schiffs reagent (PAS) by the method of Glossmann and Neville (1971). Mobilities of macromolecules were expressed relative to that of the bromophenol blue marker dye,

measurements being taken from the leading edge of the bands. Purified protein standards used for gels include:-

B-galactosidase	116,000 Daltons
Bovine serum albumin	66,000 Daltons
Ovalbumin	45,000 Daltons
Carbonic anhydrase	29,000 Daltons
Myoglobin	17,000 Daltons

Calibration curves of mobility versus molecular weight were drawn as described as Weber and Osborne (1969).

3.2.4. Preparation of Anti-35k-GP Antiserum.

35k-GP, prepared as described in section 3.2.2., was highly insoluble in buffers of low ionic strength and therefore some of the material injected was particulate. The immunisation schedule carried out was identical to that described in Section 2.2.4. for anti-elastin antisera.

3.2.5. Preparation of IgG Antiserum.

To purify the IgG fraction of the anti-35k-GP antisera, a column in which Staphylococcal Protein A is linked to Sepharose 4B was used. The technique was as described by Goding et al. (1976). 5ml of Protein A-Sepharose gel was equilibrated with PBS and packaged into a 10ml column (plastic syringe). Rabbit serum was passed over the column, washed extensively with PBS and eluted with 0.85% (v/v) glacial acetic acid in 0.15M NaCl. The

purified IgG was neutralised immediately with 1-2 drops of 1M Tris, then dialysed and freeze-dried.

3.2.6. Immunochemical Characterisation of Anti-35k-GP Antiserum by ELISA.

Indirect ELISA method is described in section 2.2.6. Optimal coating of the plate with 35k-GP was found to be 15ug/ml as determined by checkerboard titration techniques. All antigens were coated onto the microtitre plates at this concentration. It should be noted that 35k-GP, which is insoluble in binding buffer, was dissolved in 6M Urea/Tris buffer for plating onto wells of ELISA plates. Non-reduced extracts 1-6, bovine ligamentum nuchae elastin, acid-soluble collagen, human plasma fibronectin and bovine serum albumin were checked for contaminating antibody activity.

3.2.7. Adsorption of Non-specific Activity from Anti-35k-GP IgG.

Significant cross-reactivity of anti-35k-GP IgG was demonstrated against both human plasma fibronectin and bovine ligamentum nuchae elastin by ELISA (see section 3.3.2.). In order to render the anti-35k-GP preparation free from contaminant activity, immunoadsorption of unwanted activity was undertaken by affinity chromatography. Human fibronectin was kindly donated by Dr. C. Evans, Department of Anatomy. The method used for coupling the fibronectin to the affigel was as described by Naito

and Uita (1981). 10ml of Affigel-15 was placed in a small buchner funnel and washed with 75ml isopropanol followed by 200ml cold distilled water. The affigel was then added to 10ml of 0.15M $NaHCO_3$ (pH 8) containing 200mg fibronectin. This mixture was left on a rotator overnight at 4°C. 100ul of 1M ethanolamine (pH 8) was then added per ml of gel and mixed for one hour at room tempetarure. The affigel was returned to the buchner and the supernatant removed, washed with 10ml 7M Urea, three times with 1MNaCl and then equilibrated with PBS - the buffer used during the affinity purification. Also, a two ml column of finely powdered bovine ligamentum nuchae elastin (thoroughly purified) was prepared and equilibrated first with PBS and then pre-immune rabbit IgG in order to block binding non-specific sites.

5ml of anti-35k-GP (20mg/ml) was passed through both columns at a flow rate of 5ml/hour. Protein passing straight through was collected, reconcentrated by ultrafiltration to 20mg/ml and checked for activity by ELISA. Affinity columns were regenerated after each cycle by eluting with 0.1M citric acid pH 2.5 to remove IgG bound to the columns. The proceedure was repeated until ELISA showed no residual non-specific activity in the resulting anti-35k-GP preparation. The IgG preparation purified by affinity techniques will be referred to as **anti-35k-GP-A** in this thesis.

3.2.8. Western Blotting.

To confirm that the antisera was only detecting the 35k-Da band present in the crude microfibrillar preparation, Western blotting was undertaken (Burnette, 1981). This involves the electrophoretic transfer of proteins from SDS-PAGE to a nitrocellulose sheet. The immobilised proteins can then be reacted with antibody and subsequently visualised by binding of a second antibody conjugated to peroxidase. An SDS-PAGE slab gel was run as described in Section 2.2.3. The gel was placed onto a pre-wetted nitrocellulose sheet (0.45 pore size) and sandwiched first between two sheets of Whatman chromatography paper and then two Scotch Brite scouring pads. These are fitted into a cassette which is placed vertically into a tank so that the nitrocellulose is facing the anode. The tank is filled with six litres of electrode buffer composed of 20mM Tris, 150mM glycine and 20% methanol. Electrophoretic transfer was accomplished at 75mA for 20 hours. The slab gel was loaded with three duplicates of each extraction. Once the SDS-PAGE run was complete, one triplicate was Coomassie Blue or silver stained, and the other two were transferred onto the nitrocellulose sheet. One of the transferred triplicates was stained with 0.2% Amido Black in 40% methanol and 10% acetic acid, for two minutes and destained. The last triplicate was stained by immunological means as described on the following page:-

(a) The nitrocellulose is blocked with a solution of 20mM Tris/HCl pH7.5 containing 500mMNaCl (TBS) and 3% (w/v) radioimmunoassay grade BSA, for 60 minutes at 37°C.

(b) The nitrocellulose is washed with a fresh solution of TBS-BSA solution containing anti-35k-GP-A (1:1000) and incubated for three hours at room temperature.

(c) The nitrocellulose sheet is then washed with TBS (without BSA) for ten minutes then two changes of TBS only containing 0.05% NP-40, (ten minutes each) and then washed once more in TBS alone.

(d) The nitrocellulose sheet is then immersed in a solution of goat anti-rabbit IgG peroxidase in TBS with 3% BSA for three hours.

(e) Step (c) is repeated.

(f) 4-chloro-1-napthol (60mg) in 20ml methanol is added to 100ml TBS solution containing 60ul of 3%(w/v) H_2O_2. The nitrocellulose sheet is then immersed in this solution.

(g) The nitrocellulose sheet is placed in the dark for 30-40 minutes in the 4-chloro-1-napthol solution to allow colour to develop.

3.2.9. Preparation of Tissue for Electron Microscopy.

At St Andrews slaughterhouse, ligamentum nuchae, ear cartilage, skin and aortic tissue were dissected from foetal calves within one hour of the death of the mother. The gestational age of the calves, estimated from crown-rump

measurements (Bogart, 1959), were approximately 4- and 7-months. The procedure for embedding in epoxy resin was as described in Section 2.2.7. and Figure 2.1., with the exception that after the tissues were fixed for two hours at 4°C with 2% gluteraldehyde, no post-fixation with OsO_4 was undertaken, since this treatment was found to completely destroy 35k-GP antigenicity in both epoxy and LR White resin.

Concurrent with the embedding in epoxy resin, small blocks of the same samples were placed, after fixation and dehydration in 70% ethanol, into LR-White resin and transferred into fresh resin three times over a 24 hour period. The samples were then placed in air-tight BEEM capsules and cured in an oven at 50°C for 24 hours.

3.2.10. Immunogold Staining of Sections.

The method used for immunogold staining is described in Section 2.2.8.

3.3. RESULTS.

3.3.1. Extraction of 35k-GP.

Figure 3.1. shows a Coomassie blue-stained SDS-PAGE gel of the seven extracts obtained by sequential treatment of finely powdered (100-200 mesh) bovine ligamentum nuchae with buffered guanidine HCl only and buffered guanidine HCl-mercaptoethanol. Heavy staining at the top of the stacking gel and at the interface of stacking and separating gels was most prominent in late guanidine-HCl extractions. During the initial four 72-hr periods of extraction, (lanes 4, 5, 6 and 7), several proteins, a few of which exhibited a mobility on SDS-PAGE indicative of an apparent molecular weight lower than 40-kDa, were solubilised. During the following two extractions, the majority of the proteins in solution did not enter the stacking gel (lanes 8 and 9). The ensuing treatment of the tissue with mercaptoethanol in buffered guanidine HCl released a few more proteins shown in lanes 1 and 2. The most prominent component of this final reductive extract, which is described as the **'crude microfibrillar extract'**, is the protein with an apparent molecular weight of 35-kDa (arrow in lane 1). It should be noted that there is no evidence of either MAGP or its 60-kDa aggregate. From 1kg dry weight of ligamentum nuchae tissue, 1g of crude microfibrillar extract was obtained.

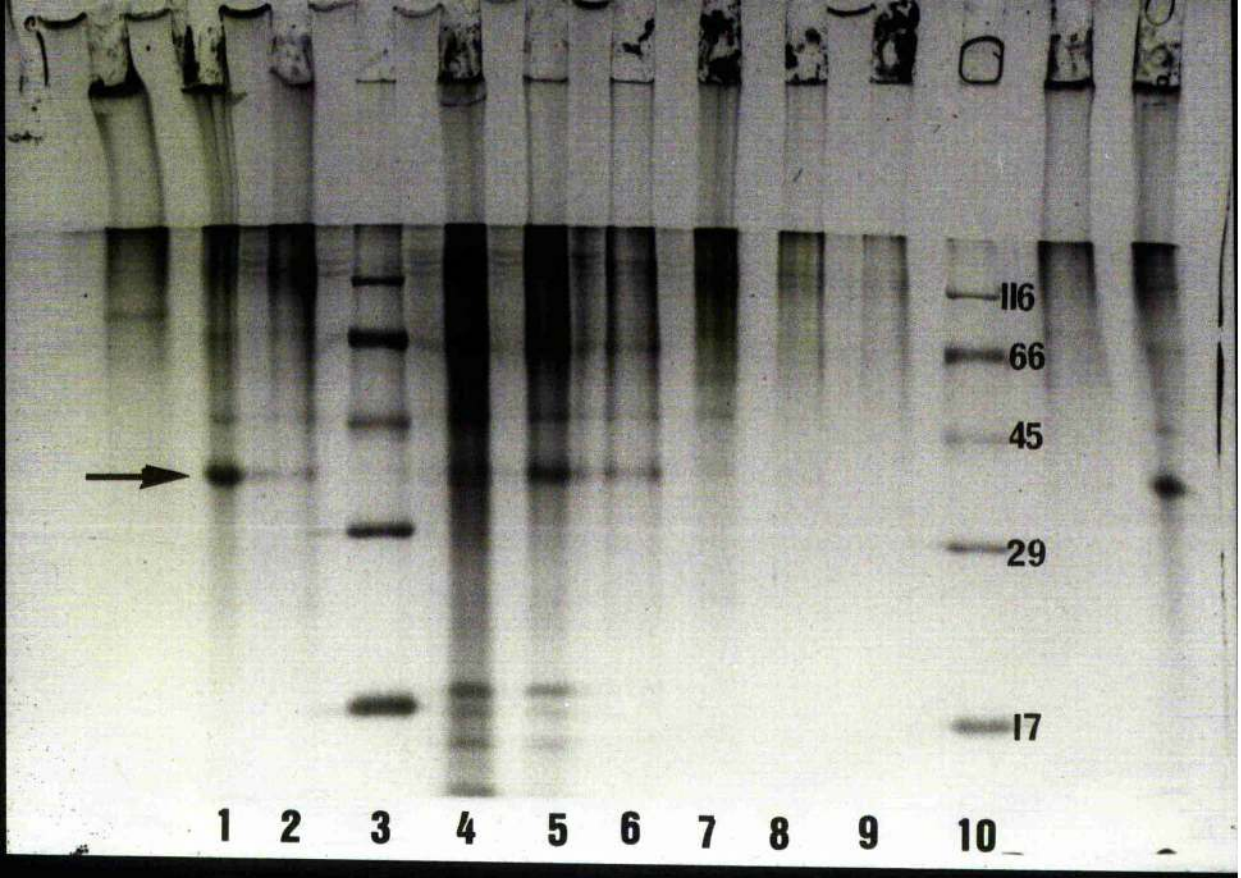
This crude microfibrillar extract could be further purified by ion-exchange chromatography, as it was discovered that 35k-GP bound strongly to DEAE-cellulose. Most of the proteins were eluted with 0.01 and 0.05 M NaCl whereas 35k-GP was displaced by 0.3 and 0.5 M NaCl (Figure 3.2), indicating that it is a highly acidic macromolecule. Some high molecular weight material (presumably proteoglycans and/or nucleic acids) which contaminated the 35k-GP fraction eluted at 0.3M was subsequently removed by gel-filtration on an S-300 column. Figure 3.3. shows the separation and apparent molecular weight of some of the contaminant proteins. After gel-filtration, 35k-GP was judged to be homogenous on analysis of a silver-stained SDS-PAGE gel (Figure 3.4.) and strongly PAS positive (Figure 3.5). Final yield of 35k-GP was approximately 10mg/kg of dry tissue.

FIGURE 3.1.

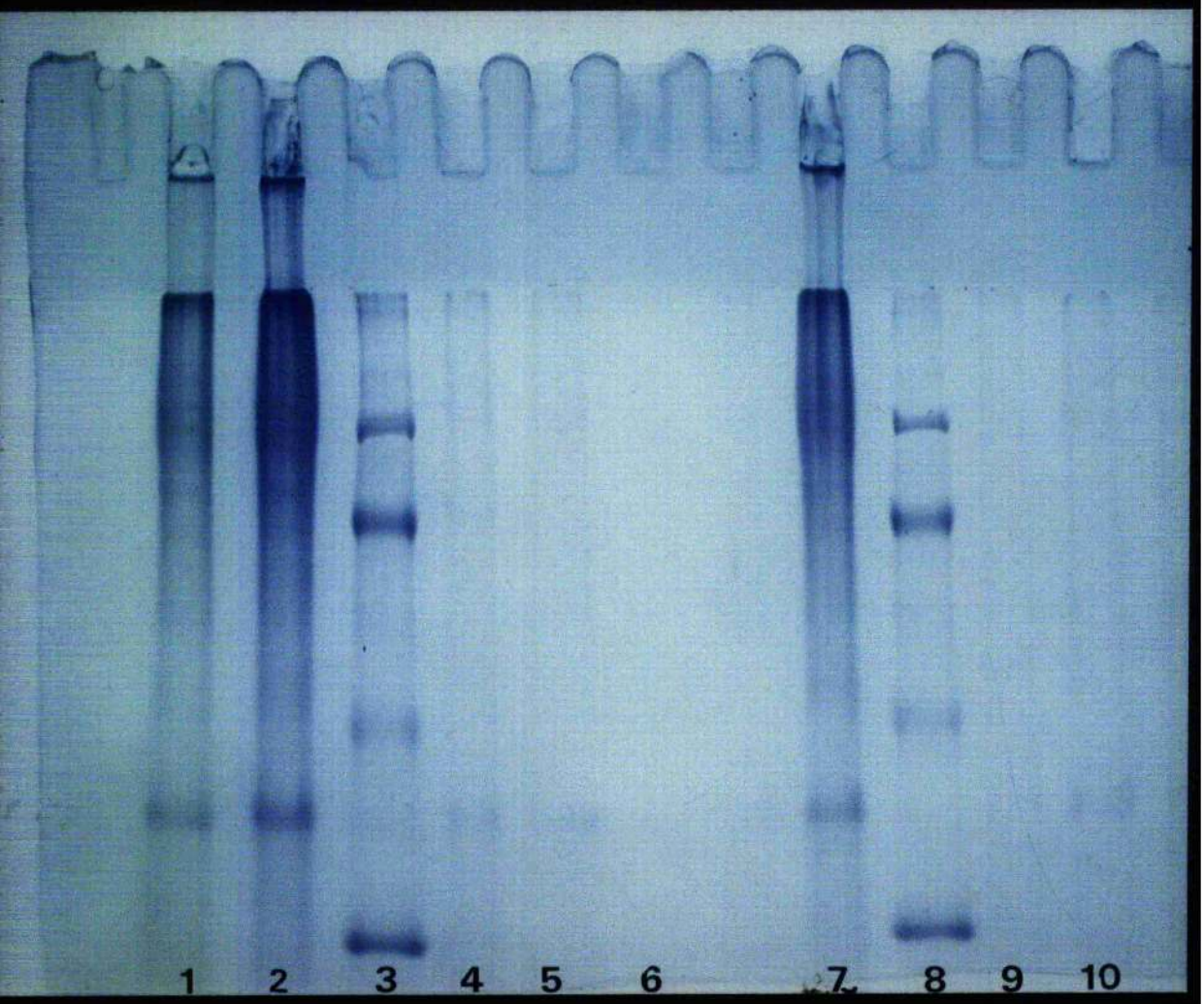
SDS-PAGE analysis of tissue extracts obtained during the isolation of 35k-GP from bovine ligamentum nuchae.
Lane 1: final tissue extraction with guanidine hydrochloride containing 2-mercaptoethanol. The extract after Amicon concentration, dialysis and freeze-drying has been redissolved in urea, prior to PAGE. The 35-kDa band is indicated by an arrow.
Lane 2: as above, but with residue redissolved in PBS.
Lanes 3 and 10: molecular weight standards - myoglobin (17-kDa); carbonic anhydrase (29-kDa); ovalbumin (45-kDa); bovine serum albumin (66-kDa); and β-galactosidase (116-kDa).
Lanes 4-9: extracts obtained during the initial sequential treatment of the tissue with guanidine hydrochloride alone for 6 x 72 h periods. The progression is from left to right. Proteins are stained with Coomassie Blue.

Figure 3.2.

SDS-PAGE analysis of fractions obtained when 1g of crude microfibrillar extract is added to a DEAE-cellulose column and eluted with buffers A to E:-
Lane 3 + 8: molecular weight standards from bottom - carbonic anhydrase (29-kDa); ovalbumin (45-kDa); bovine serum albumin (66-kDa); and β-galactosidase (116-kDa).
Lane 1 + 7: Proteins eluted with buffer containing 0.01 M NaCl.
Lane 2: Proteins eluted with buffer containing 0.05 M NaCl.
Lane 4 + 9: Proteins eluted with buffer containing 0.3 M NaCl.
Lane 5 + 10: Proteins eluted with buffer containing 0.5 M NaCl.
Lane 6: Fractions eluted with Buffer E (1.0 M NaCl) do not contain any protein. Proteins are stained with Coomassie Blue.



3.1.



3.2.

FIGURE 3.3.

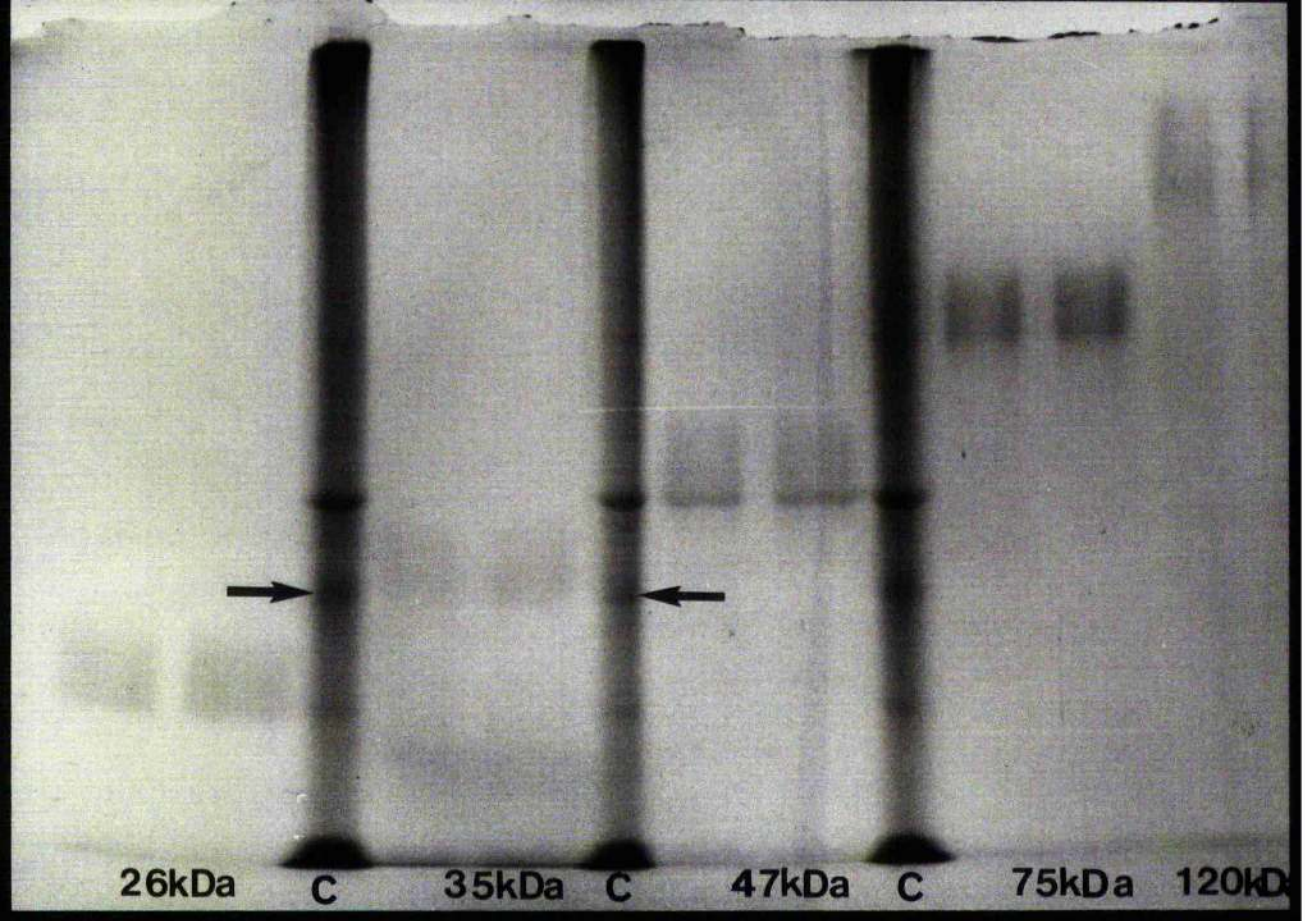
Separation (by Sephacryl S-300) and molecular weight of the components of the 0.3M NaCl extract from the DEAE column. C = crude microfibrillar extract, arrows indicate 35k-GP. Protein bands are faint because fractions from S-300 were not concentrated but analysed directly by SDS-PAGE (Coomassie Blue stained).

Figure 3.4.

SDS-PAGE gel of 35k-GP fraction from S-300 column which was silver-stained in order to detect any contaminants in the 35k-GP preparation. Standards, which were run in another lane, were too dark to be included in this photograph.

Figure 3.5.

PAS-stained SDS-PAGE gel of crude microfibrillar extract shows that 35k-GP is the only glycoprotein in this extract.



3.3.

3.4

3.5.

3.3.2. Purification of anti-35k-GP antisera.

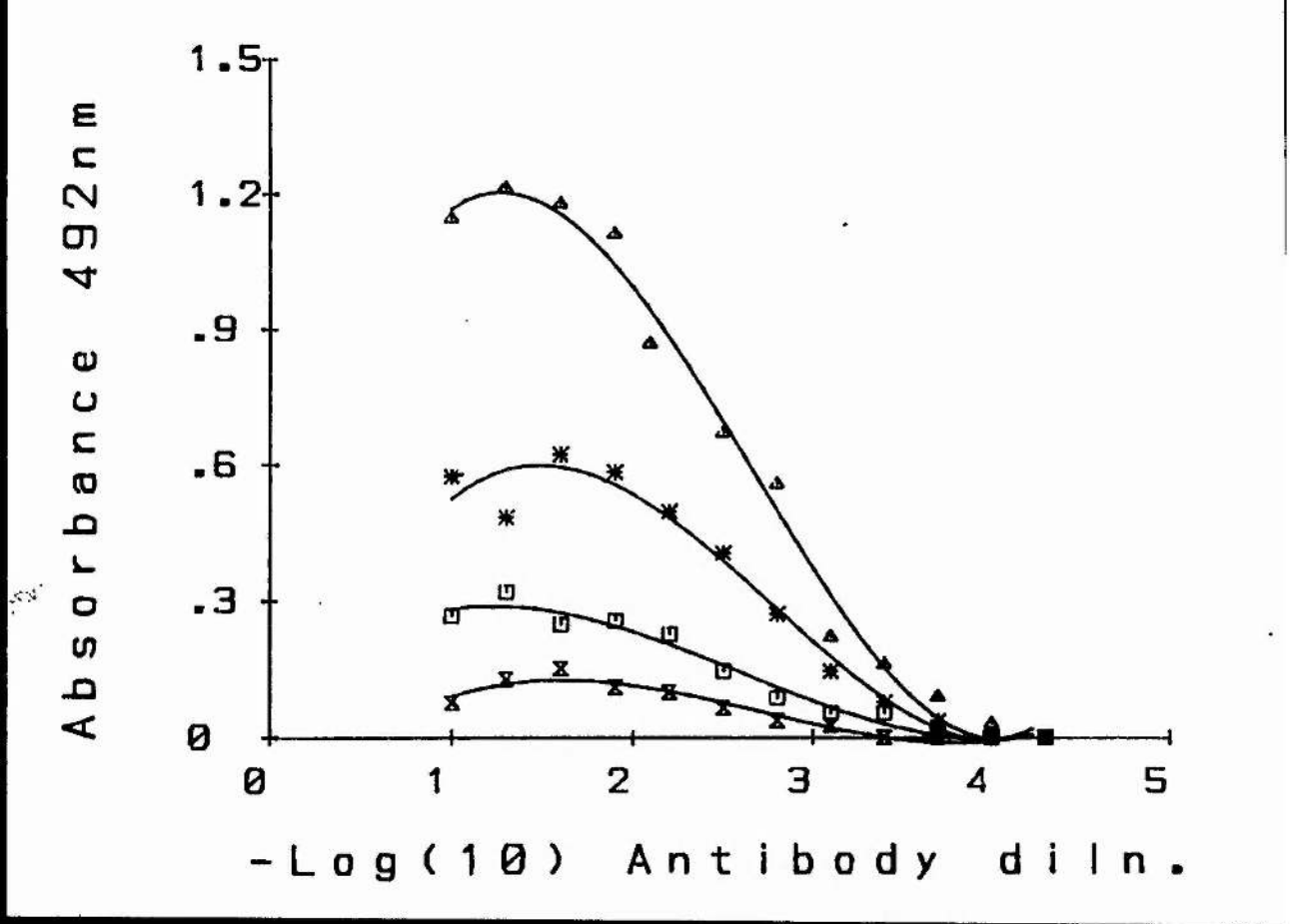
The anti-35k-GP antiserum was tested for cross-reactivity against connective tissue proteins (collagen and elastin), serum proteins (bovine serum albumin, fibrinogen and fibronectin) and the non-reduced extracts 1-6 (see 3.3.1.). Significant titres were obtained with elastin and fibronectin as shown in Figure 3.6.(a); however, these contaminating activities were removed completely by affinity chromatography, as shown in Figure 3.6.(b). The resulting antiserum was called anti-35k-GP-A. A titre of 1:5000 was routinely obtained by ELISA for anti-35k-GP-A when tested against the antigen solubilised in urea at a concentration of 15ug/ml. This antiserum was shown by Western blot to bind only to the 35-kDa band of the crude microfibrillar extract (Figure 3.7.).

FIGURE 3.6.(a).

Reactivity of anti-35k-GP antiserum to 35k-GP (△); human fibronectin (✱); bovine ligamentum nuchae elastin (⊡). Reactivity of pre-immune rabbit serum against 35k-GP (⧖). In the calculation of titre, A_{492} readings were considered positive when more than double the corresponding value for pre-immune rabbit serum. All antigens tested were at a concentration of 15ug/ml.

FIGURE 3.6.(b).

Reactivity of anti-35k-GP antiserum after removal of contaminating activity by affinity techniques. This antiserum (anti-35k-GP-A) was tested against 35k-GP (△); bovine ligamentum nuchae elastin (⊡); human plasma fibronectin (✱). Values for anti-35k-GP-A against the elastin and fibronectin were almost identical to those obtained with pre-immune rabbit serum against 35k-GP (⧖). All antigens tested were at a concentration of 15ug/ml.


Absorbance 492nm
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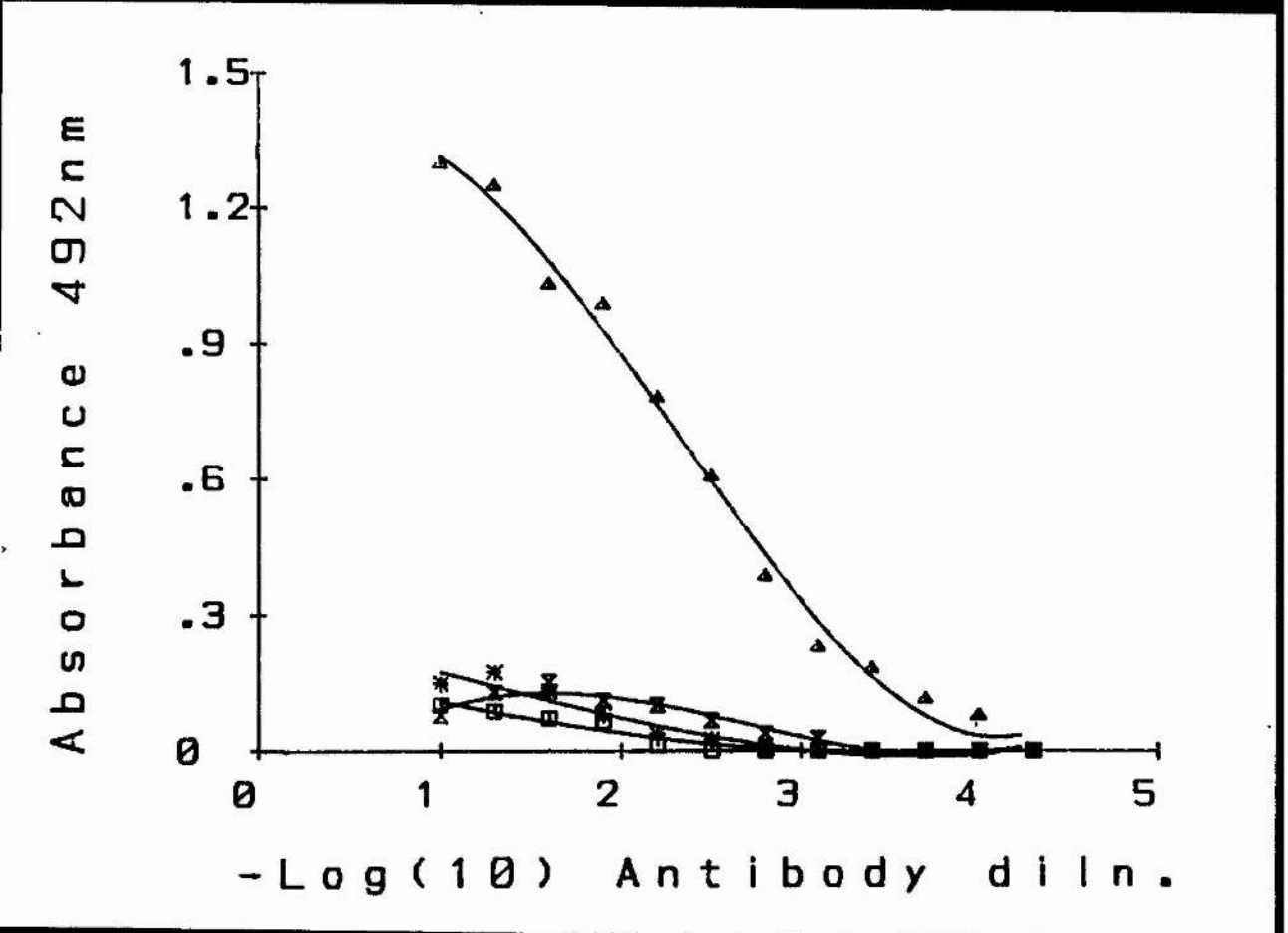
-Log(10) Antibody diln.

3.6.a


Absorbance 492nm
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1.2
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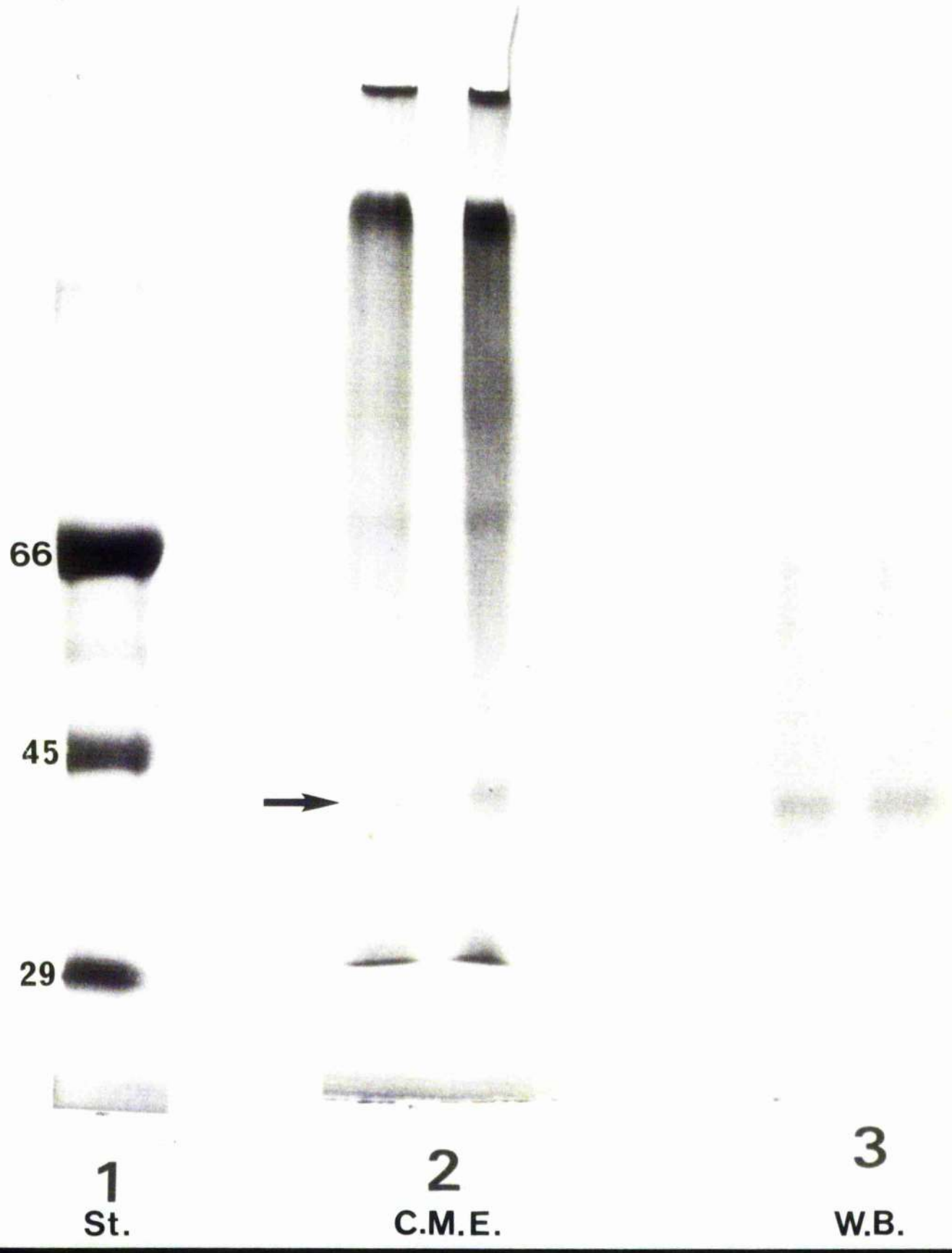
4
5
-Log(10) Antibody diln.

3.6.b

Figure 3.7.
Western blot analysis of anti-35k-GP-A.
Lane 1: molecular weight standards from bottom:- carbonic anhydrase (29-kDa); ovalbumin (45-kDa); bovine serum albumin (66-kDa).
Lane 2: Crude microfibrillar extract (CME) - final tissue extraction with guanidine hydrochloride and 2-mercaptoethanol. Stained with Coomassie Blue. Arrow indicates 35k-GP.
Lane 3: Western blot of CME showing that anti-35k-GP-A binds specifically to 35-kDa band.


66
45
29
1
St.
2
C.M.E.
3
W.B.

3.3.3. Ultrastructural Localisation of 35k-GP in Foetal Calf Ligamentum Nuchae.

As described in Chapter One (Page 41), electron-microscopic investigations have shown that microfibrils can be identified in elastogenic connective tissue matrices at a time when elastin biosynthesis has not yet been initiated. Figures 3.8. and 3.9. show longitudinal sections of loosely packed bundles of microfibrils in a four-month-old foetal ligament embedded in LR White resin. The microfibrils have a maximum diameter of about 11nm and do not yet show any association with elastin. A prominent feature in both Figures is the selective localisation of the colloidal gold particles within each microfibrillar bundle. It should be noted that preparations of the same tissue treated with epoxy resin showed negligible antibody binding.

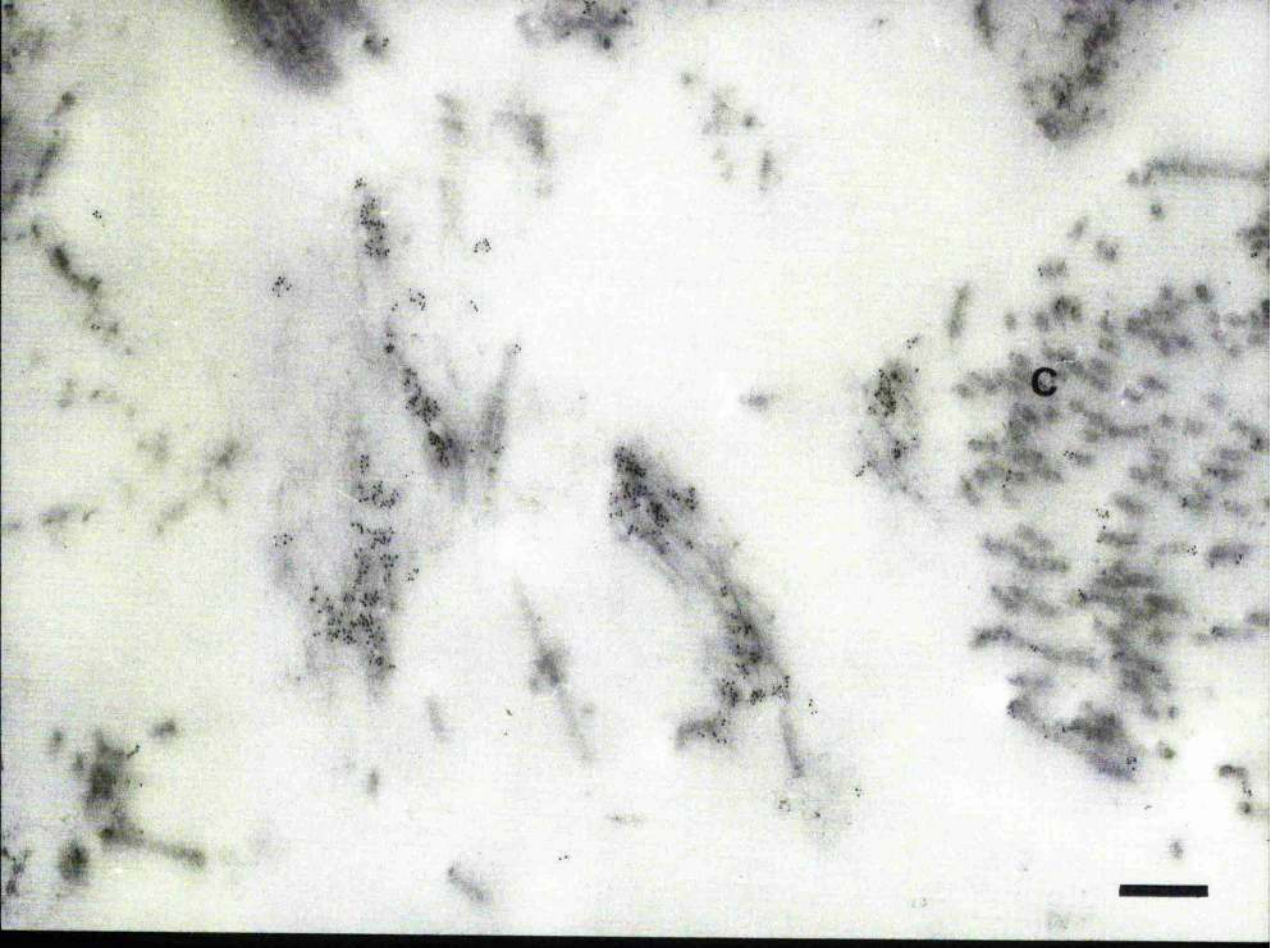
In the seventh month of foetal calf development, the rate of elastin synthesis suddenly increases and elastic fibres become detectable in appreciable amounts within the extracellular matrix. Figure 3.10. shows a section of ligament embedded in LR White resin at this stage of development. The field selected contains four immature fibres composed of microfibrils surrounding amorphous and still largely independent areas of elastin deposition. The immature nature of the elastin is clearly indicated by its high electron density (see Chapter Two). The anti-35k-GP-A antibodies are preferentially localised on the microfibrillar layer and there is no appreciable binding to either the amorphous elastin or to any other matrix component.

FIGURE 3.8.

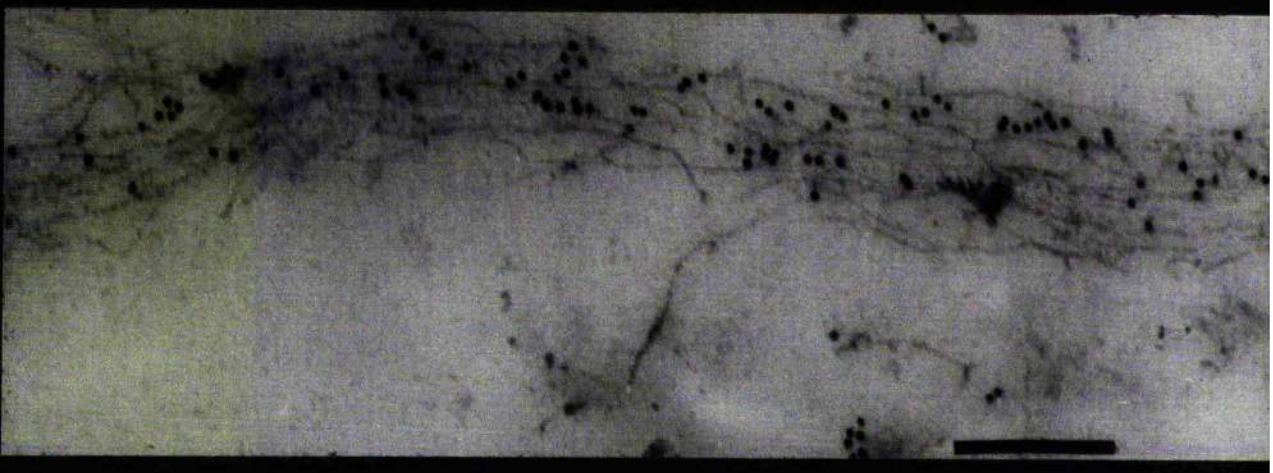
Bovine foetal ligamentum nuchae (4-month) embedded in LR White resin and treated with anti-35k-GP-A as the primary antibody. The binding of the 15nm colloidal gold is restricted to the microfibrils. Bar 0.3um; x 31,000.

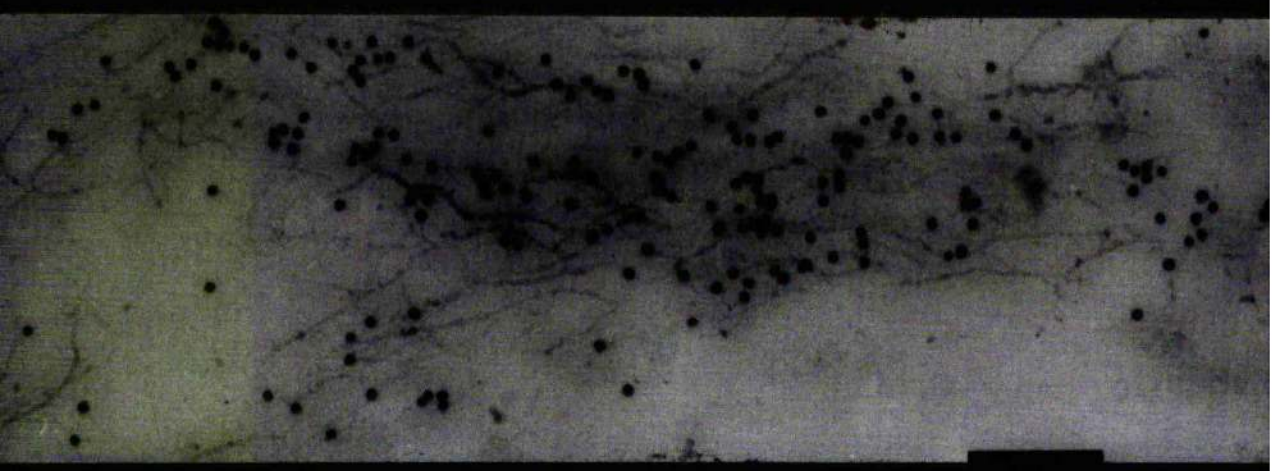
FIGURE 3.9.

Four month foetal bovine ligamentum nuchae embedded in LR White and treated with anti-35k-GP-A as the primary antibody. Two electron micrographs taken at higher magnification to show the 15nm gold particles binding to the bundles of microfibrils.
Upper photograph:- Bar 0.5um; x 34,000
Lower photograph:- Bar 0.3um; x 47,000



3.8.





3.9.

The uneven distribution of the colloidal gold over the four fibres shown in Figure 3.10. could be attributed to the different orientation of the microfibrils to the plane of the section, a factor which must critically affect the accessibility of the epitopes to antibodies, particularly in the case of structures of such small cross-sectional area. When anti-35k-GP-A, pre-adsorbed on a 35k-GP preparation, was used as primary antibody on a tissue section from the same block, no specific immunological binding was observed (Figure 3.11.).

In conformity with the results obtained with the 4-month old foetal tissues, the examination of the 7-month old preparation embedded in epoxy resin revealed lack of labelling of microfibrils associated with immature elastic fibres similar to those reported in Figure 3.10. However, in other areas of the tissue embedded in epoxy resin, more developed fibres characterised by a well-defined, electron-transparent elastin core, still lacked gold deposition on the peripheral microfibrils, but showed specific localisation of immunogold within the core (Figure 3.12.). Examination of one such fibre at higher magnification (Figure 3.13.) revealed that immunogold labelling corresponded closely to either clearly identifiable microfibrils trapped within the elastin core (arrow a) or electron-dense areas (arrow b).

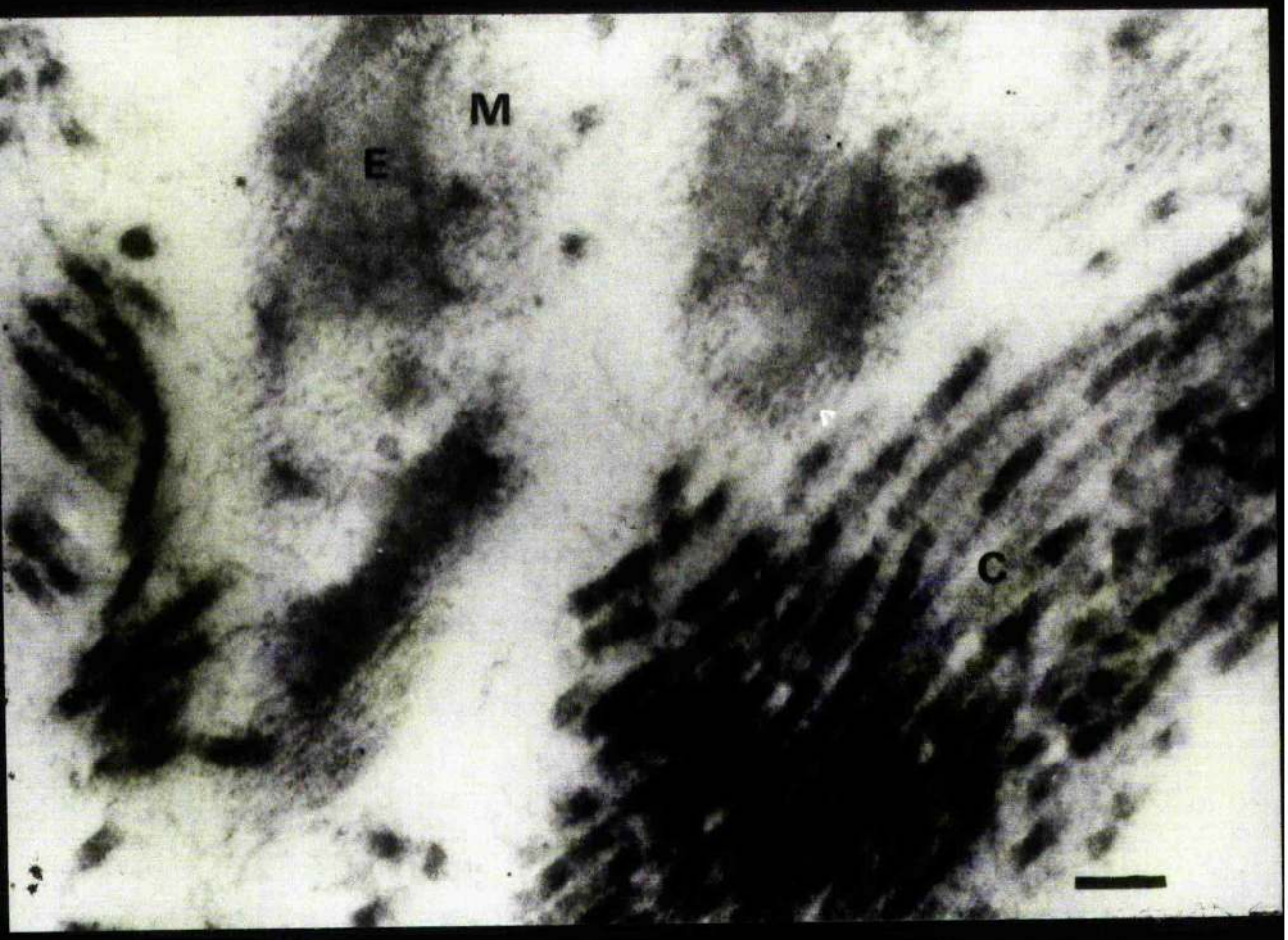
FIGURE 3.10.

Bovine foetal ligamentum nuchae (7 month) embedded in LR White resin and treated with anti-35k-GP-A as the primary antibody. Four immature elastic fibres are shown, each composed of an elastic core (E) and a surrounding layer of microfibrils (M). The 15nm colloidal gold is bound specifically to the microfibrils and there is no significant binding to collagen (C) or any other component of the matrix. Bar 0.3um; x 48,000.

FIGURE 3.11.

Bovine foetal ligamentum nuchae (7 month) embedded in LR White resin and treated with anti-35k-GP-A preadsorbed on 35k-GP. No significant binding of the 5nm colloidal gold is detectable to the microfibrillar component (M), the elastin (E), or the collagen fibres (C). Bar 0.2um; x70,000.

3. 10.



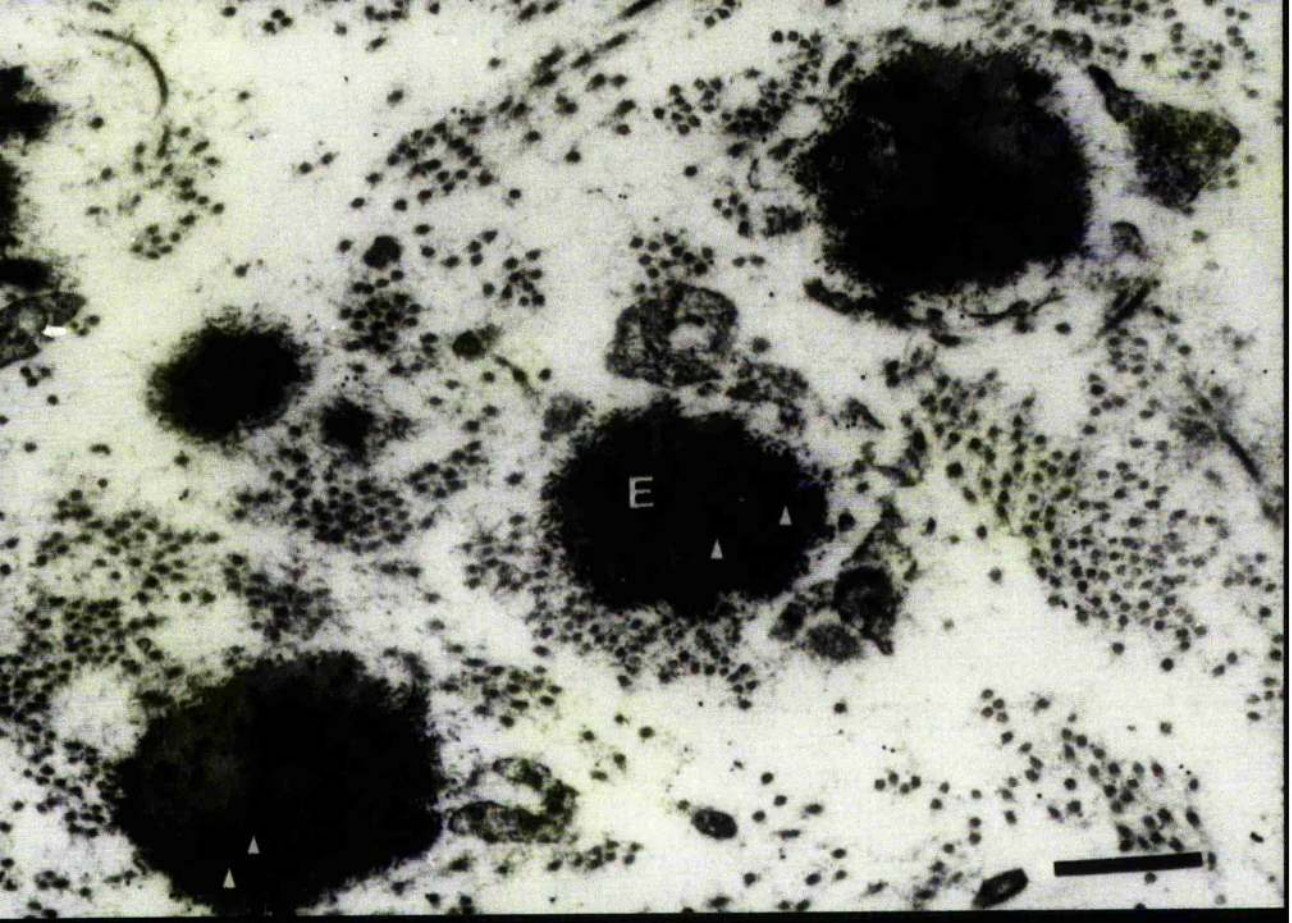
3. 11.

FIGURE 3.12.

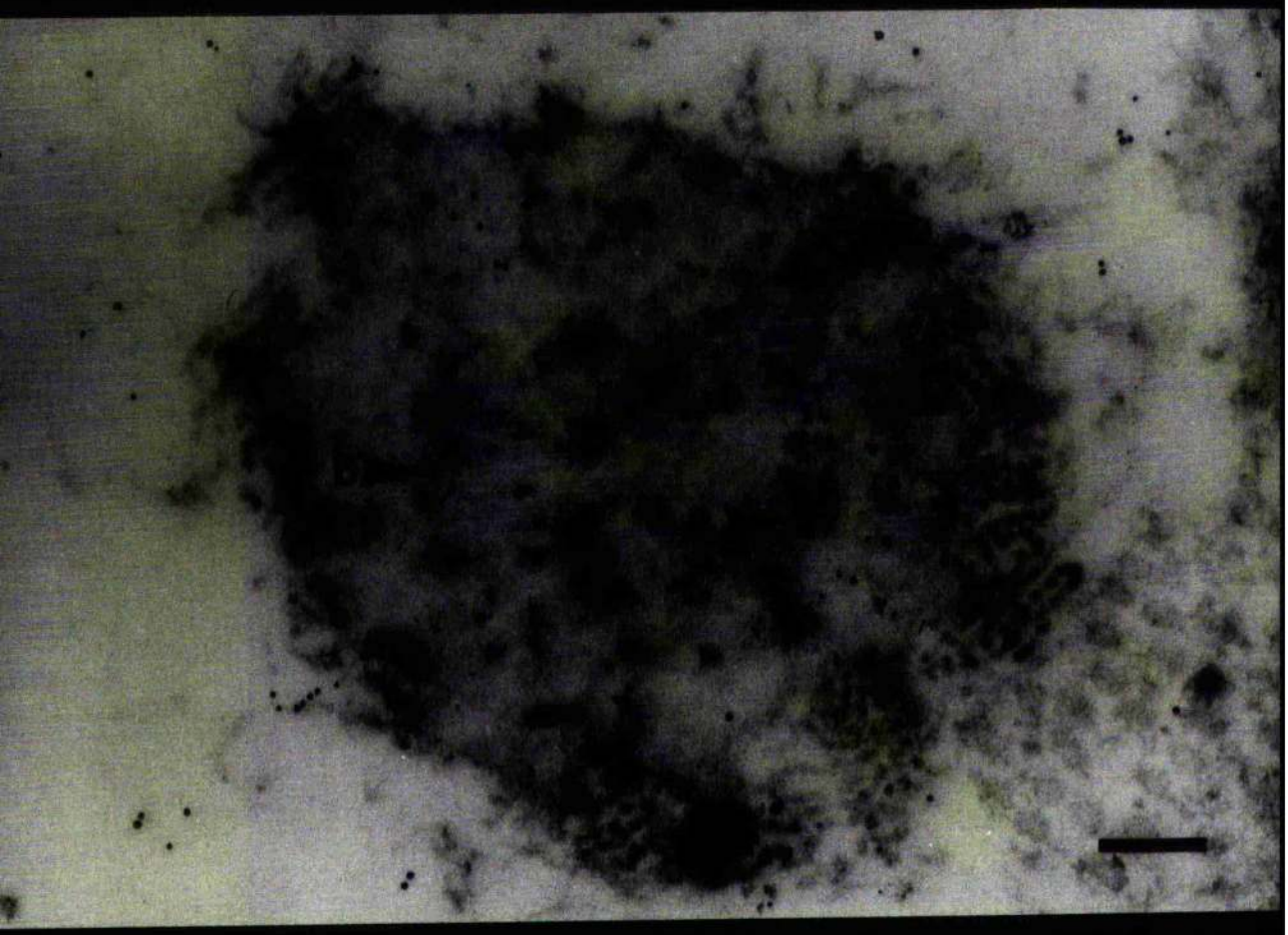
Bovine foetal ligamentum nuchae (7 month) embedded in epoxy resin and treated with anti-35k-GP-A as the primary antibody. The binding of the 15nm colloidal gold is restricted to dark patches (arrowheads) within the elastin core (E) of elastic fibres. Bar 0.5um; x 27,000.

FIGURE 3.13.

Bovine ligamentum nuchae (7 month) embedded in epoxy resin and treated with anti-35k-GP-A as the primary antibody. The 5nm colloidal gold binds predominantly to microfibrils (arrow a) and to electron-dense patches (arrow b) located within the elastic core (E). Bar 0.1um; x 107,000.



3. 12.



3. 13.

3.3.4. Ultrastructural Localisation of 35k-GP in Bovine Foetal Aorta, Skin, and Ear Cartilage.

Four Month Old Foetal Calf.

Elastic fibre formation was not detected within the extracellular matrix of the skin and ear cartilage of the four month old foetal calf. At this age however, in a few discrete areas throughout the aortic tissue, immature elastic fibres were observed. In these regions, the developing fibres consisted of bundles of microfibrils to which the anti-35k-GP-A antiserum specifically localised (Figure 3.14.).

Seven Month Old Foetal Calf.

Thoracic Aorta:-

The elastic tissue of the aorta is more mature than the ligamentum nuchae at seven months. As shown in Figure 3.15., well-defined fibrils (arrows) were found in close proximity to the elastogenic cells in a manner similar to the cell:fibre association observed in the human foetal aorta (Chapter Two). At higher resolution (Figure 3.16.), it could be seen that the 5nm colloidal gold particles were localised to the microfibrils on the perimeter, and occasionally within the core, of developing elastic fibrils situated very near to the cell membrane of the elastogenic cell (E.C.).

FIGURE 3.14.

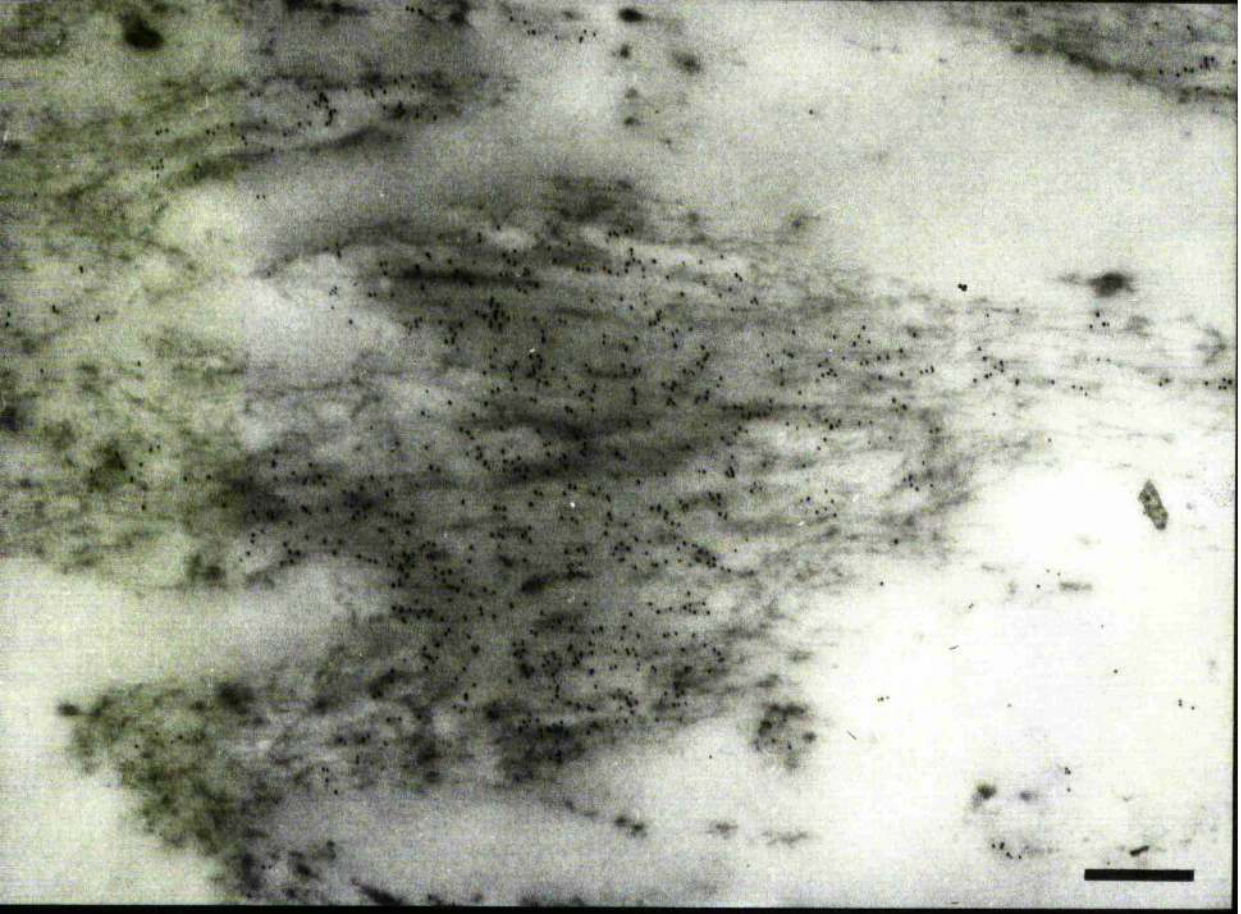
Four-month old bovine foetal aortic tissue embedded in LR White resin and treated with anti-35k-GP-A as the primary antibody. The immature elastic fibre shown in this micrograph is comprised of bundles of microfibrils to which the 5nm colloidal gold particles bind. Bar 0.3um; x 44,000.

FIGURE 3.15.

Seven-month old bovine foetal aortic tissue embedded in LR White resin and treated with anti-35k-GP-A as the primary antibody. The binding of the 5nm colloidal gold is restricted to the elastic fibrils (e) which are situated very close to the plasma membrane of the elastogenic cells (see arrows). No binding of 35k-GP-A is observed on collagen fibres (c). Bar 1um; x 11,000.

FIGURE 3.16.

Seven-month old bovine foetal aortic tissue embedded in LR White resin and treated with anti-35k-GP-A as the primary antibody. In this higher resolution electron micrograph, the elastic fibre can be seen to be composed of a central amorphous core of newly synthesised elastin (E) surrounded by 11nm microfibrils (MF) which are decorated with the 5nm colloidal gold particles. The elastic fibre is situated directly beside the elastogenic cell (E.C.). Bar 0.5um; x 32,000.



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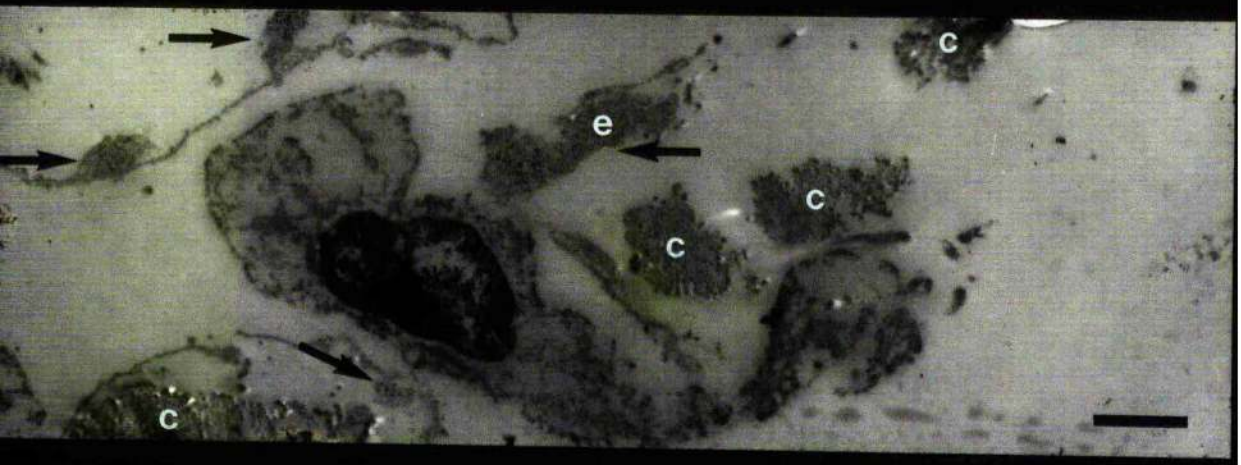
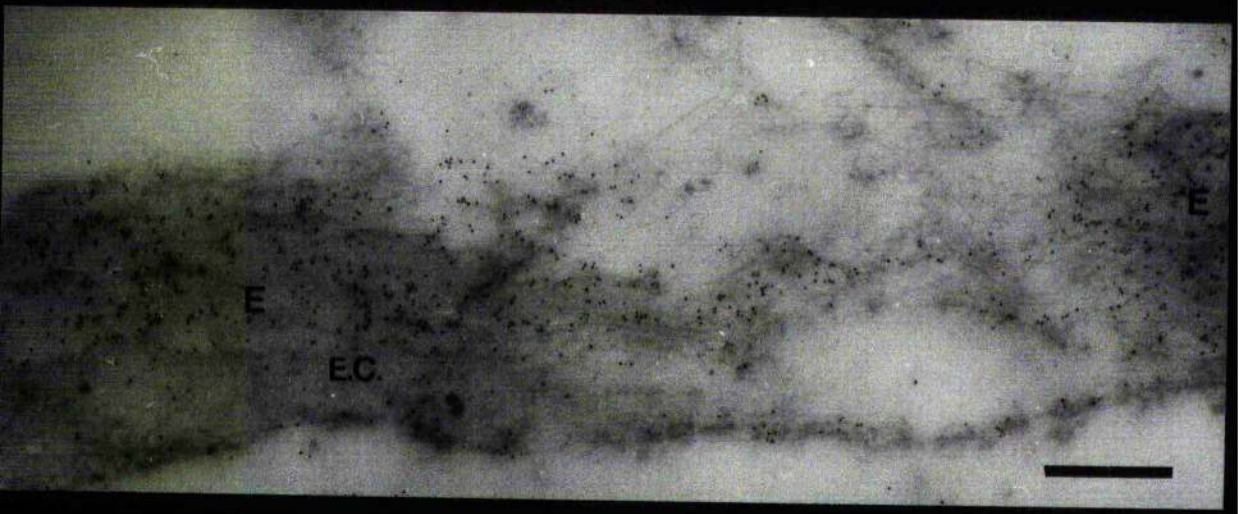


3. 15.



3. 16

Ear Cartilage:-

Large numbers of developing elastic fibres were apparent in the seven month old foetal ear cartilage. These fibres varied considerably in size and shape (the largest being of the order of 1-2um in diameter), were irregularly branched and appeared to be discontinuous. At low magnification, such fibres consisted of a central amorphous, lightly-stained core with a dark staining mantle (Figure 3.17.). At higher resolution and in cross-section, it could be seen that the elastic fibres were surrounded by a "stellate reticulum" consisting of densely packaged proteoglycans in the form of rounded or polygonal electron-dense granules of variable size and that collagen traversed the matrix in seemingly random orientations. A striking and consistent feature was the complete lack of identifiable microfibrils in association with the developing elastic fibres. Despite this, anti-35k-GP-A bound exclusively and specifically to the periphery of the immature elastic fibres (Figure 3.18.).

Skin:-

The dermis contains many cells (arrows in Figure 3.19.) but very few elastic fibres at this stage. The existing fibrils were very immature, with anti-35k-GP-A binding primarily to the microfibrils on the periphery of these developing fibres (Figure 3.20.).

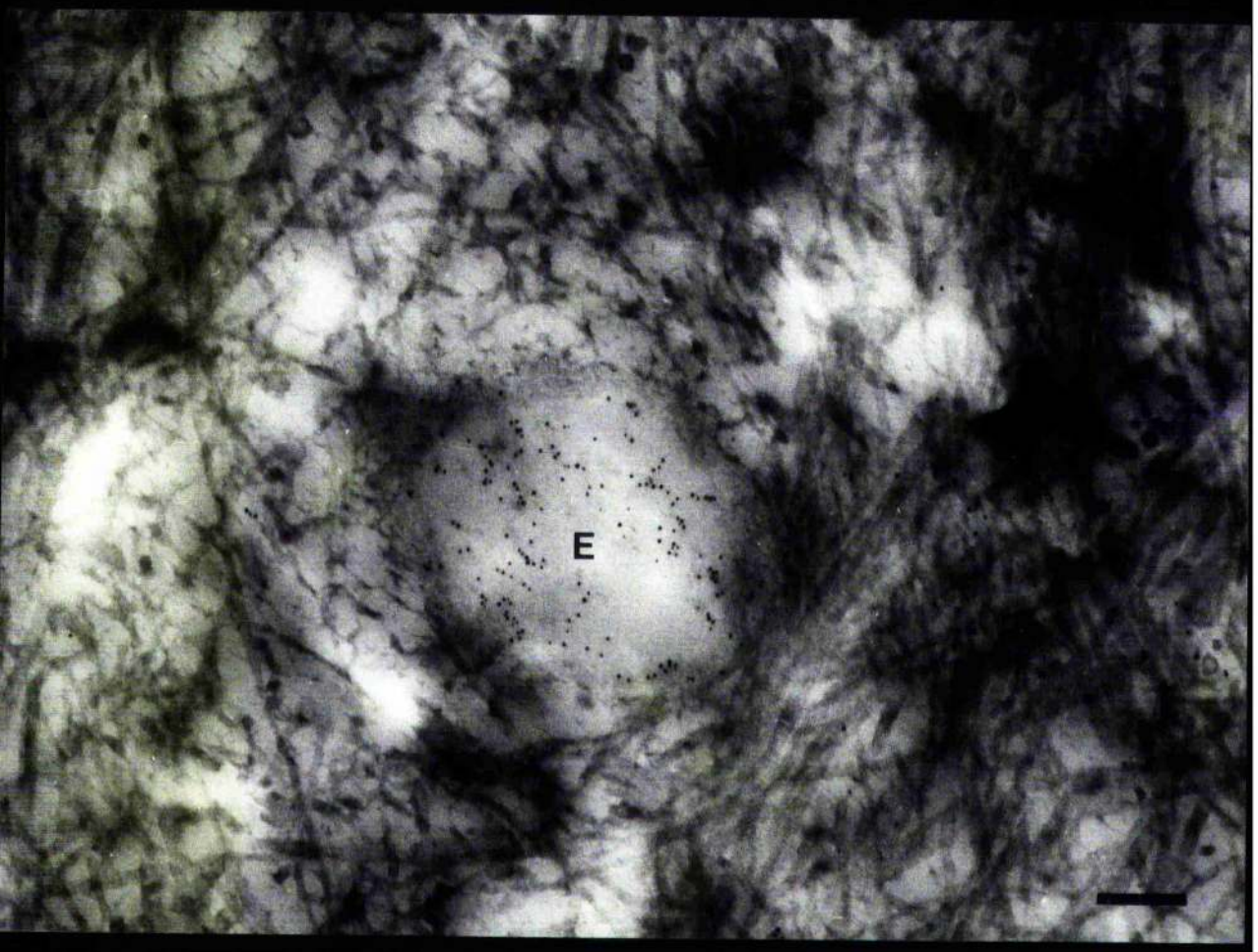
FIGURE 3.17.

Seven-month old bovine foetal ear cartilage tissue embedded in LR White resin and stained with uranyl acetate and lead citrate only. Lightly stained branched elastic fibres (E) are visible against the 'stellate reticulum' background. Bar 1um; x 16,000.

FIGURE 3.18.

Seven-month old bovine foetal ear cartilage tissue embedded in LR White resin and treated with anti-35k-GP-A as the primary antibody. In this electron micrograph the stellate reticulum can be seen to consist of rounded or polygonal electron-dense granules of variable size interconnected with collagen fibrils. Despite the absence of microfibrils in the cross-sectioned elastic fibre (E), 5nm colloidal gold particles bind to the periphery of the fibre which appears to be intimately associated with the stellate reticulum. Bar 0.2um; x 52,000.

3. 17.



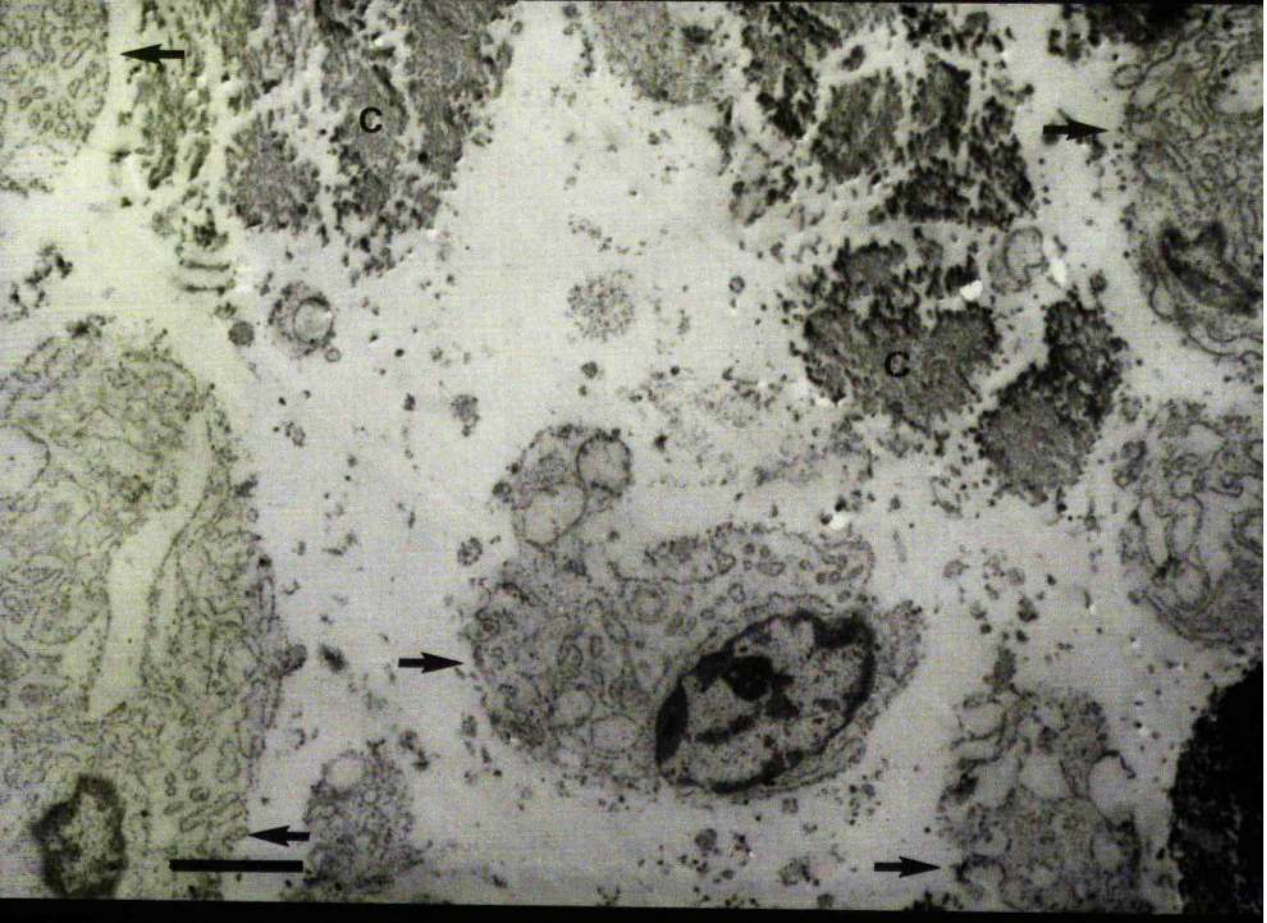
3. 18

FIGURE 3.19.

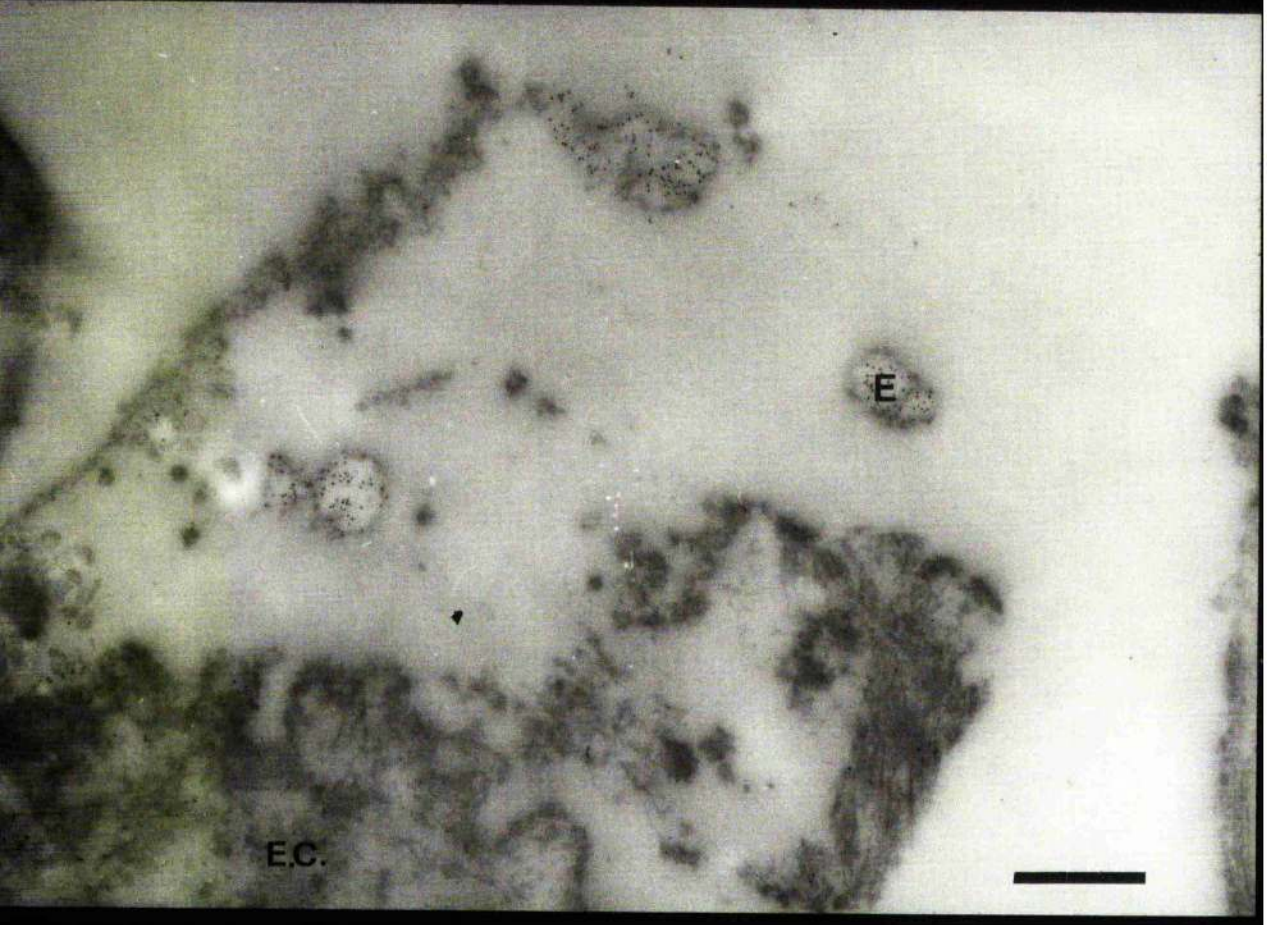
Seven-month old bovine foetal skin embedded in LR White and treated with anti-35k-GP-A as the primary antibody. The dermis at this stage consists of many cells (arrows) and has a relatively immature extracellular matrix consisting primarily of bundles of collagen fibres (C). Bar 1um; x 15,000.

FIGURE 3.20.

Seven-month old bovine foetal dermis embedded in LR White resin and treated with anti-35k-GP as primary antibody. In distinct areas spread throughout the tissue sections, small elastic fibrils (E) were found in close proximity to elastogenic cells (E.C). The 5nm colloidal gold particles bound predominantly to the periphery of the amorphous areas of newly synthesised elastin. Bar 0.5um; x 29,000.



3.19.



3.20.

3.4. DISCUSSION.

In the SDS-PAGE analysis of the 'crude microfibrillar extract' (Figure 3.1., lane 1), much of the sample failed to enter the separating gel. It can be seen that this is a heterogenous extract with 35k-GP as its major component. The 35k-GP preparation obtained by a further two step purification procedure was considered pure enough for antibody production on examination of silver stained SDS-PAGE gels (Figure 3.4.) .

Despite the apparent homogeneity of the purified 35k-GP on SDS-PAGE, antisera to the glycoprotein showed cross-reactivity to fibronectin and, unexpectedly, to elastin. This indicates the presence within the immunogen of highly antigenic peptide fragments either pre-existing in the tissue or produced during the extensive guanidine HCl treatment, despite the presence of protease inhibitors. After removal of the contaminating activity, the antiserum was shown to bind exclusively to 35k-GP by ELISA and Western blot analysis. This result (Paper in Press) and those of Gibson et al. (1986) on anti-MAGP are the first reports of polyclonal antisera, used in the immunoelectron microscopic analysis of the microfibrillar component, to appear to be directed against a single putative microfibrillar protein.

The highly selective localisation of immunogold in areas corresponding to free microfibrillar bundles and the elastin-associated microfibrillar layer of developing elastic fibrils in the skin, aorta and ligamentum nuchae is consistent

with the presence of 35k-GP in these structures. The inability to detect the microfibrillar component in the elastic tissue of the foetal calf ear cartilage is possibly due to highly-charged proteoglycan molecules, present in great numbers within the extracellular matrix, binding to sites on the microfibrils which normally bind the cationic heavy metal stains. This would result in a masking of the underlying structure of the microfibrils. If this is the case, it would appear that the 35k-GP antigen of microfibrils is still able to bind to anti-35k-GP-A antiserum, because specific binding of the 5nm colloidal gold was consistently observed at the periphery of the developing elastic fibres (Figure 3.18.).

The variability in the binding of immunogold to microfibrils in tissues embedded in different resins suggests that the epitope(s) recognised by the 35k-GP-A antibodies are temperature sensitive and presumably conformational. Epoxy resins have to be cured at 60°C whereas LR White, a cross-linked hydrophilic resin, can be cured at 50°C and this temperature difference has been shown to greatly affect the ability of some antibodies to recognise their antigens (J.A. Chandler, personal communication). LR White resin also allows the use of partially dehydrated specimens and thus tissues infiltrated with resin from 70% ethanol have an improved antigenicity over their fully dehydrated counterparts (Newman and Jasani, 1984). It is interesting to note that epitopes totally surrounded by elastin in the more mature fibres are preserved in the epoxy resin. This could be

due to additional conformational stability being imparted by the hydrophobic elastin domain.

In relation to the presence of microfibrillar epitopes within the elastin fibre core, it should be remembered that the morphogenesis of the elastic fibre involves the progressive incorporation by coalescence of finer elastic fibrils into the growing structure. In this process, some microfibrils become segregated within the core where, according to their orientation relative to the plane of section, appear as clearly identifiable fibrils or, more frequently, as small and irregular electron-dense patches (Serafini-Fracassini, 1984).

Concurrent with the establishment of the association between 35k-GP and microfibrils in bovine ligamentum nuchae, aorta, ear cartilage and skin, a 31-kDa glycoprotein called MAGP has also been shown to bind exclusively to elastin-associated microfibrils in both developing and mature tissues. The acidic MAGP with its high cysteine content is thought by Gibson et al. (1986) to bind by disulphide bonds to the tropoelastin molecule which, when recently sequenced, revealed a novel highly basic carboxy terminal region containing two cysteine residues (Cicila et al., 1985b). Other glycoproteins e.g. 'fibrillin' (Sakai et al., 1987) and the antigen to which the monoclonal antibody HB8 binds (Schmitt et al., 1986) - both discovered in human skin - may also play a role in the structure and function of elastin-associated microfibrils.

SDS-PAGE examination of the serial guanidine HCl extracts of bovine ligamentum nuchae demonstrated that a proportion of proteins with a molecular weight less than 40-kDa were solubilised in early guanidine HCl extracts in the absence of a reducing agent. Sear et al. (1981b) and Prosser et al. (1984) on electron microscopic examination of the residues at each stage of the procedure demonstrated that significant disruption of microfibrils could occur in chaotropic solutions alone. These authors inferred that this lower molecular weight material represented a proportion of the microfibrillar- associated glycoproteins which were not strongly cross-linked. This argument suggests that one could ultimately extract all the microfibrillar-associated glycoprotein very slowly by chaotropic agents alone. However, it is shown in Figure 3.2. that after an initial solubilisation of low molecular weight material during the first three extractions (3x3 days), there is a complete absence of this low molecular weight material in the following three extended extractions (3x4 days). Only when a reducing agent is added does the low molecular weight material reappear.

One question which remains to be answered, is that raised by the lack of simultaneous identification of MAGP (31-kDa) and 35k-GP in the same 'crude tissue extracts'. It is possible that MAGP is one of the components described above which can be removed from the crude extract by the chaotropic solution alone. The initial extractions in guanidine HCl were only for 48, 24 and 24 hours i.e. a total of 4 days (Prosser et al., 1984) and

therefore, even although the reducing agent was added, the tissue would still contain a lot of the initial low molecular weight material possibly including the 31-kDa MAGP.

These two glycoproteins, 35k-GP and MAGP, appear to be different in composition and function. Both glycoproteins have distinctive amino acid compositions (see Page 33) and whereas MAGP is not recognised by anti-bovine lysyl oxidase in ELISA or Western Blots, 35k-GP has been shown to catalyse the oxidative deamination of lysyl residues in ^{3}H-lysine labelled aortic elastin prepared from 15 to 17 day old chick embryos (Serafini-Fracassini et al., 1981a+b).

It appears likely that elastin-associated microfibrils are a multi-component structure, possibly composed of a core protein such as the large glycoprotein, fibrillin, onto which both 35k-GP and MAGP are perhaps transiently bound in order to play separate roles during the morphogenesis of the elastic fibre.

CHAPTER FOUR.

IN VITRO STUDIES ON THE ROLE OF 35K-GP IN THE MORPHOGENESIS OF THE ELASTIC FIBRE.

4.1. INTRODUCTION.

In the study of the morphogenesis of the elastic fibre, a central question is the nature of the signals involved in activating and regulating elastin synthesis by the elastogenic cells. As outlined in Section 1.5.3., the interaction of a cell with extracellular matrix components such as fibronectin, collagen, laminin and vitronectin can alter its adhesion, biosynthetic patterns, and capacity to migrate and proliferate.

In 1982, Knox et al. observed that 35k-GP could form a substratum in vitro for the attachment and spreading of established cell lines (BHK-21, 3T3, human osteosarcoma cell line) and primary cultures (chick embryo muscle, human skin fibroblasts, neonatal rat cells) when these cells were subcultured in polystyrene Petri dishes onto which discrete areas had been coated with a microfilm of aggregated 35k-GP.

From these results and the morphological evidence for the existence of extensive contacts between cells and the microfibrillar component of the developing elastic fibres (Chapter Two), it is postulated that the 35-kDa glycoprotein component of elastin-associated microfibrils provides the substratum necessary for the initial binding of elastogenic cells to the microfibrillar bundles and also induces the elastogenic cells to interact with the surface of the growing elastic fibre. An essential element in this model is that the interaction with 35k-GP not only stimulates the cell to adhere and spread on the

microfibrillar component but that this change in cell shape induces the expression of the gene(s) responsible for the synthesis of elastin. In this model 35k-GP would play a role similar to that undertaken by fibronectin, vitronectin and laminin in other systems.

Investigators have used various *in vitro* systems as a means of studying cell-extracellular matrix interactions and in particular, Mecham *et al* (1984a+b) have examined the role of the extracellular matrix in elastin biosynthesis. In order to assess the potential of the *in vitro* system for exploring the process of elastogenesis, foetal calf *ligamentum nuchae* fibroblasts and human foetal aortic smooth muscle cells were grown in tissue culture. Ultrastructural studies of the cell layer of such cultures were undertaken to determine the extracellular matrix characteristics using anti-35k-GP-A and anti-elastin antisera as probes.

In parallel with this, the studies of Knox *et al*. (1982) were continued. The cell adhesion experiments were repeated using foetal calf *ligamentum nuchae* fibroblasts and human aortic smooth muscle cells. In addition, studies on the ultrastructural appearance of these cells attaching and spreading on 35k-GP were undertaken in an attempt to elucidate the mechanism of cell adhesion.

4.2. MATERIALS AND METHODS.

4.2.1. Materials.

Ham's F10 and foetal calf serum were supplied by Flow laboratories, Rickmansworth, Herts. The antibiotics were bought from Sigma Chemicals Company, Poole, Dorset. Trypsin was manufactured by Difco Labs., Detroit, Michigan USA. The Falcon plastic tissue culture flasks (25 and 75 cm^2 growth area) and the Petri dishes used in the cell-adhesion experiments were from Sterilin, Teddington, Middlesex. HPMA was bought from TAAB Labs. Equipment, Reading, Berkshire, England. All other materials and reagents were obtained from sources previously detailed in Sections 2.2.1. and 3.2.1.

4.2.2. Foetal Calf Ligamentum Nuchae Fibroblasts and Human Aortic Smooth Muscle Cells in Tissue Culture.

A foetal calf of approximately 4 months gestation (Bogart, 1959) was obtained from the abbatoire. Nuchal ligament was excised within one hour of maternal death using aseptic techniques. Small blocks of ligament were rinsed with phosphate buffered saline (PBS) containing Penicillin-G (100 Units/ml) and Streptomycin sulphate (100ug/ml) and placed into Falcon flasks (25cm^2). Six pieces were placed in each flask with a few drops of foetal calf serum (FCS) and incubated for three hours at 37°C in a 5% CO_2 incubator to allow adhesion of tissue explants.

Ham's F10 supplemented with 10% FCS and antibiotics at the concentration above, was then added to each flask (6ml). Medium was subsequently changed every 3-4 days. After 48 hours, outgrowths of fibroblastic cells began to appear from the explants spreading laterally to reach confluency in 7-10 days. Confluent cultures were subcultured by trypsinisation as follows:-

The cell layers were rinsed twice in warm PBS and then incubated with 1ml of PBS containing 0.02% EDTA (w/v) and 0.05% (w/v) trypsin at 37°C, in a 5% CO_2 incubator for 5 minutes. Cells were detached from the flask by gentle tapping and 6ml Ham's F10 containing 10% (v/v) FCS was added to inhibit further activity of the trypsin. Trypsinised cells were then suspended by gentle aspiration with a sterile pipette, transferred to centrifuge tubes and collected by centrifugation at 500g for 4-5 minutes. The supernatant was aspirated and the cells resuspended in fresh Ham's F10 : 10% (v/v) FCS. Cell density was determined with a haemocytometer and cells placed into fresh flasks at a density of 1×10^5 cells per 25cm² flask and 3×10^6 cells per 75cm² flask. Subcultures were incubated and the medium changed as before, confluency being reached in 5-7 days after seeding. In order to minimise any possible effect of repeated subculture on phenotypic expression (see Page 20), experiments were performed only on cells that were three subcultures from the primary culture.

Recently, human foetal aortic smooth muscle cells were kindly obtained from Mrs E. Cochrane at the Department of Pathology, Royal Sick Childrens Hospital, Edinburgh. These cells, which were obtained in the form of growing explants approximately one week old, were maintained as described above and used in the cell adhesion studies.

4.2.3. Storage of Cell Stocks.

Cells selected for storage were obtained from confluent secondary cell cultures by trypsinisation. After washing, the cells were resuspended at a density of 1 x 10^6 cells/ml in Ham's F10 / 10% (v/v) FCS containing 10% DMSO as a cryoprotectant. Aliquots (1ml) of the cell suspension were placed in plastic ampoules and packed in a polystyrene box and stored in the -70°C freezer overnight, to ensure a slow initial cooling rate, and then transferred to liquid nitrogen.

Rehabilitation of frozen cells was achieved by rapid thawing at 37°C for 10 minutes. The contents of one ampoule were washed in 6ml Ham's F10 / 10% FCS to remove any remaining DMSO, spun at 500g for 4-5 minutes, and then transferred to a 75cm^2 plastic culture flask containing 6ml fresh HAM's F10 /10% (v/v) FCS and incubated as described above. Cultures were refed at 2-3 day intervals and subcultured by trypsinisation.

4.2.4. Preparation of Cell Layers for Light and Electron Microscopy.

Tertiary subcultures of ligamentum nuchae fibroblasts and human foetal aortic smooth muscle cells were seeded in 25cm^2 plastic culture flasks at an initial density of 1x10^6 cells/flask. Phase contrast micrographs of cultures were taken at confluency using an Olympus phase-contrast inverted microscope.

Cell layers were fixed and processed in situ by the method of Brinkley et al. (1967). The flasks were rinsed three times in warm PBS then fixed for one hour at room temperature with 2.5% (w/v) gluteraldehyde in PBS (pH 7.4). The fixed cell layers were then washed, dehydrated and embedded as summarised in Figure 4.1. After polymerisation, small pieces were cut from the flask with a Stanley knife and stuck, edge-on, to wooden stubs with cyano-acrylate cement. This enabled examination of the full thickness of the cell layer. Blocks were trimmed and sectioned, mounted on nickel 300-mesh grids, and stained with immunogold and then uranyl acetate and lead citrate, as described in Section 2.2.8.

FIGURE 4.1.

EMBEDDING OF CELL CULTURE LAYERS IN SITU FOR ELECTRON MICROSCOPIC EXAMINATION.

Gluteraldehyde fixed cell layer rinsed X4 with PBS (pH7.4)

↓

35% ethanol for 10 minutes

↓

50% ethanol for 10 minutes

↓

75% ethanol for 10 minutes

↓

90% ethanol for 10 minutes

↓

90% hydoxypropyl methacrylate (HPMA), three 5 minute changes

↓

95% HPMA for 15 minutes

↓

97% HPMA for 15 minutes

↓

2 parts HPMA:1 part epoxy resin for 15 minutes

↓

1 part HPMA:1 part epoxy resin for 15 minutes

↓

1 part HPMA:2 parts epoxy resin for 30 minutes

↓

Pure epoxy resin, three changes, 10 minutes each

↓

Resin changed, enough fresh resin being added to cover culture layer to a depth of 2-3mm

↓

Holes burnt in tops of flasks and incubated overnight at 60°C to polymerise resin

All manipulations except polymerisation were carried out at room temperature.

4.2.5. Cell Adhesion Experiments.

The methods used for these experiments were as described by Knox et al. (1982) with some modifications. Smooth muscle cells (from a 23 week old human foetal aorta) and fibroblasts (from the ligamentum nuchae of a 4 month-old foetal calf) which had undergone two passages were harvested as they reached confluency. These two cell types were cultured on separate Petri dishes in which discrete areas had been coated with 20ul of either 35k-GP or fibronectin, both at a concentration of 1mg/ml. In the case of 35k-GP, the glycoprotein was coated onto the Petri dish dissolved in 6M urea buffer pH7.4 containing 2% mercaptoethanol and was exposed to the copper ions in 10mM $CuCl_2$ which was added to the buffer. The precipitate that formed overnight was taken to dryness and washed exhaustively prior to cell plating. Cells were seeded at $4\text{-}5x10^5$ cells per Petri dish in Ham's F10 medium either in the presence or absence of any protein supplement. The Petri dishes were incubated at 37°C in a humidified 5% CO_2 incubator and at specific intervals, the dishes were examined using an inverted microscope. The number of attached and/or spreading cells were counted for six random areas of the glycoprotein substrata and the control polystyrene substrata. The criteria for spreading were that a cell exhibited polarity and that the nucleus and nucleoli were clearly visible.

In parallel with the above, Petri dishes coated in discrete areas with 35k-GP were incubated with human aortic smooth muscle

cells for an extended incubation period of 72 hours. The resulting cell layer(s) were then fixed and embedded in epoxy resin as described in Section 4.2.4.

4.3. RESULTS.

4.3.1. Macroscopic Appearance of Cell Lines in Culture.

Four hours after seeding, the ligamentum nuchae fibroblasts exhibited a varied morphology ranging from typical bipolar elongated fibroblasts to polygonal cells with short processes. Very few detached or degenerate cells were seen, mitotic activity was frequent and confluency was reached within 5-7 days after seeding a 75cm^2 culture flask with 2x10^6 cells (Figure 4.2.).

These cultures did not grow as multi-layered cell sheets, but still maintained a reasonably active metabolism as a monolayer - indicated by the decrease in medium pH between media changes - for periods up to 60 days after confluency. The cell layers eventually detached, a phenomenon typical of late subcultures (Lamberg et al., 1980) or cultures supplemented with ascorbic acid (De Clerck and Jones, 1980; Dunn and Franzblau, 1982). Different batches of media, FCS and various seeding concentrations were tried but cell layering could not be achieved.

The human foetal smooth muscle cells were polygonal with short processes and spread rapidly from explants to form multilayers of two to five cells in thickness, approximately 20 days after attaining confluency. Figure 4.3. and 4.4. show these cells growing from an explant after one week in tissue culture.

FIGURE 4.2.

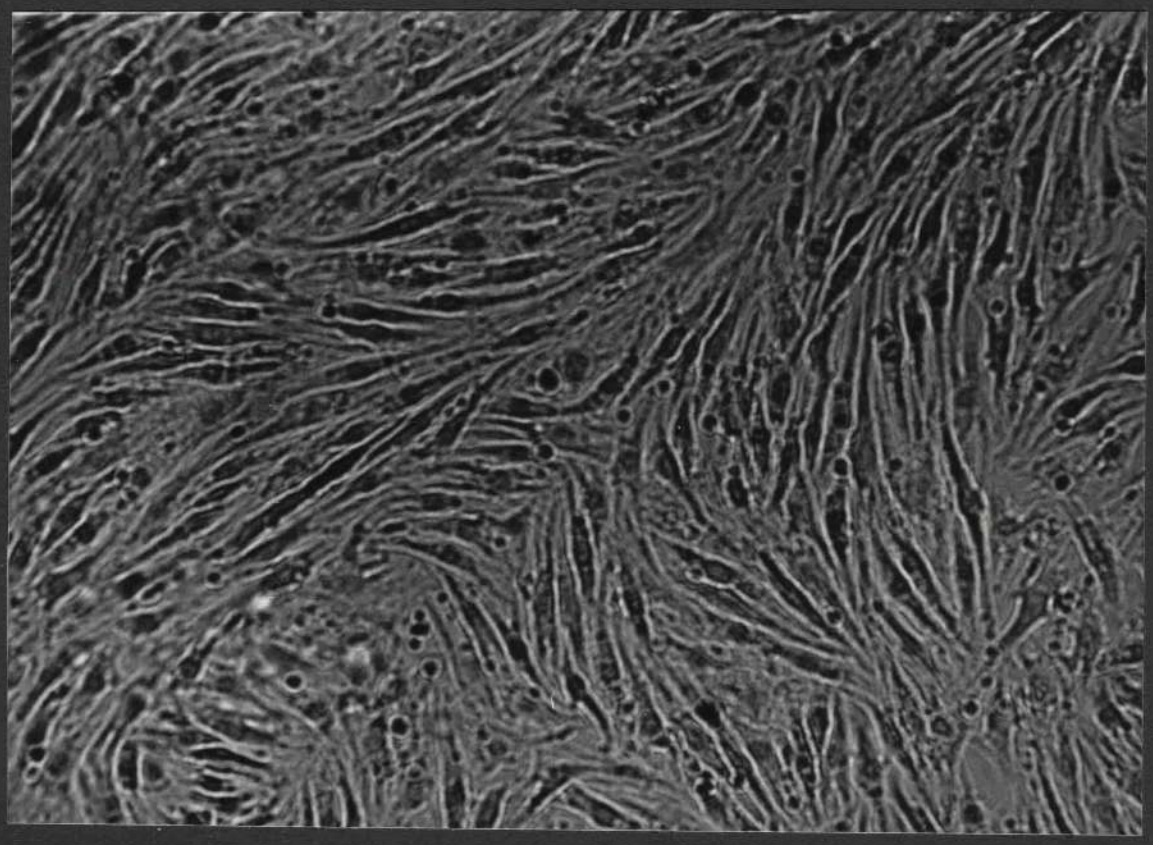
Phase-contrast photograph of a monolayer of bovine ligamentum nuchae fibroblasts 28 days post-confluency. The cells have the appearance of typical elongated fibroblasts with long dendrites.

FIGURE 4.3.

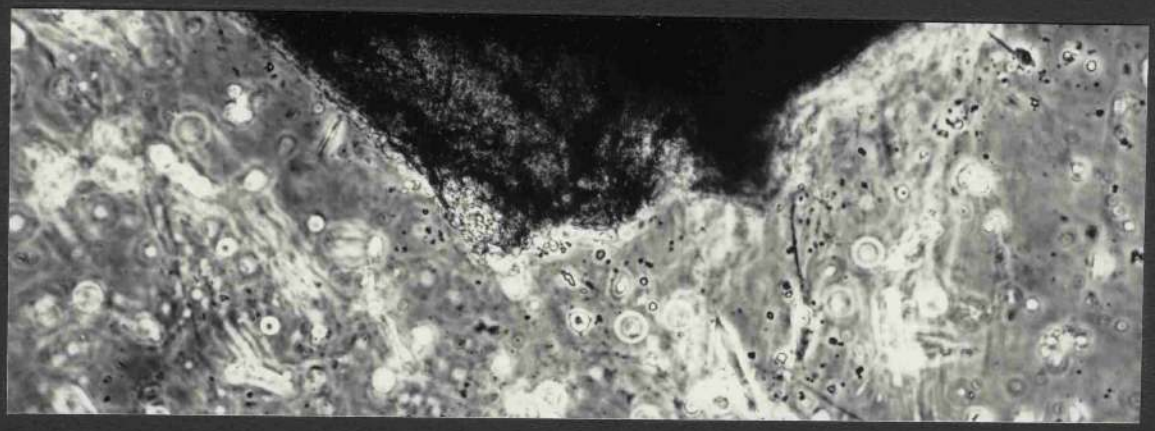
Phase contrast photograph of an explant of human foetal aortic tissue.

FIGURE 4.4.

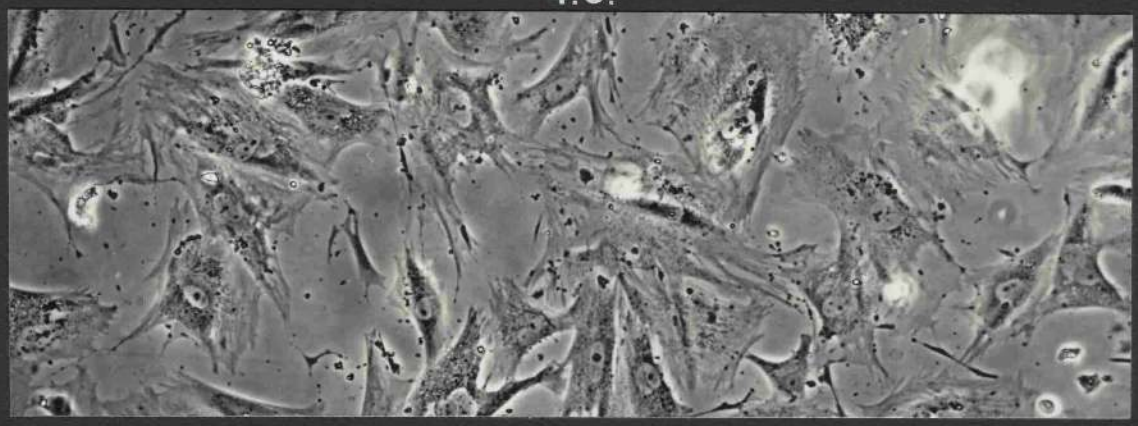
Phase contrast photograph of human foetal aortic smooth muscle cells, which are more polygonal in shape with shorter processes when compared with the fibroblasts in Figure 4.2., spreading from an explant by the seventh day in tissue culture.



4.2.



4.3.



4.4.

4.3.2. Ultrastructural Studies of Cell Cultures.

The monolayers of foetal calf ligamentum nuchae fibroblasts, examined at 14, 21, 28 and 35 days post confluency, consisted of longitudinally attenuated fibroblastic cells with elliptical nuclei containing dispersed chromatin. Figure 4.5. shows a typical area of the monolayer 28 days post-confluency which consisted of extremely elongated cells and occasional extracellular patches of amorphous electron-dense material in close apposition to the cell membrane (arrows in Figure 4.5.). The latter observation is consistent with that described by Jones et al. (1980), in their study of foetal calf ligamentum nuchae fibroblast cultures, as "fibronectin-like material". By immunogold electron microscopy, anti-35k-GP-A antibodies were detected in significant numbers in close apposition to the cell membrane nearest the plastic base of the tissue culture flask (Figure 4.6.) It could be suggested that these colloidal gold particles are trapped non-specifically within this area, however control experiments with pre-immune rabbit serum confirmed the specificity of the label produced.

The aortic smooth muscle cells were in the form of multiple layers by 21 days post-confluency (Figure 4.7.). Cells situated deep in the cell layer at this stage (Figure 4.8.) contained prominent endoplasmic reticulum (ER), Golgi apparatus (G), mitochondria (M) and discharging vesicles (V), and the extracellular matrix, which mainly consisted of bundles of

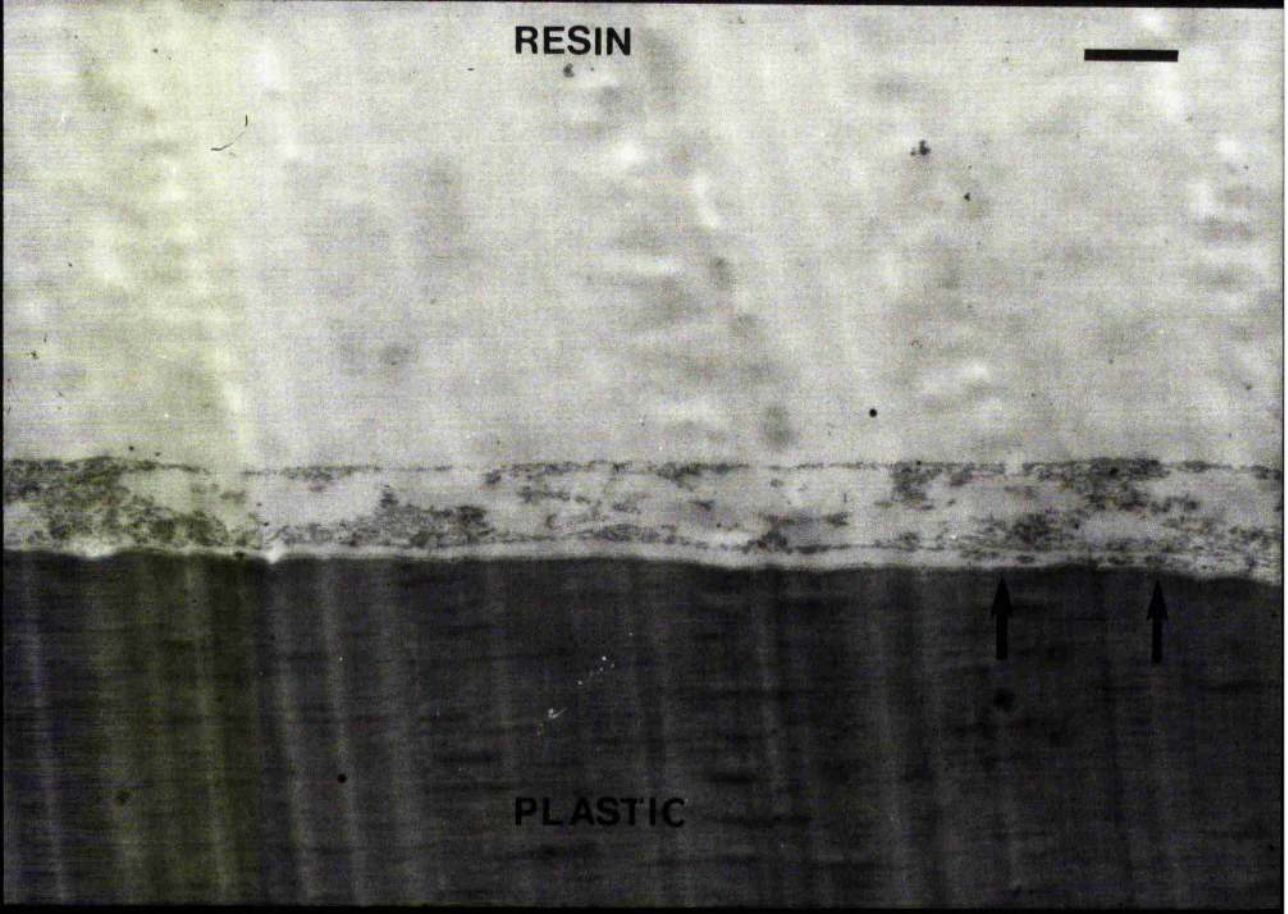
collagen fibres as shown in Figure 4.9, had begun to coalesce and form a continuous sheet between the cell layers. The superficial layers however possessed a relatively sparse intercellular matrix consisting of a few collagen fibrils and bundles of microfibrils lining extremely electron-lucent and amorphous elastin (Figure 4.10.). The anti-human elastin antisera however unable to bind to the electron-lucent material in immunogold-labelling studies, whereas the anti-35k-GP-A antiserum localised specifically to the periphery of this highly cross-linked yet immature elastic tissue (Figures 4.10. and 4.11.).

FIGURE 4.5.

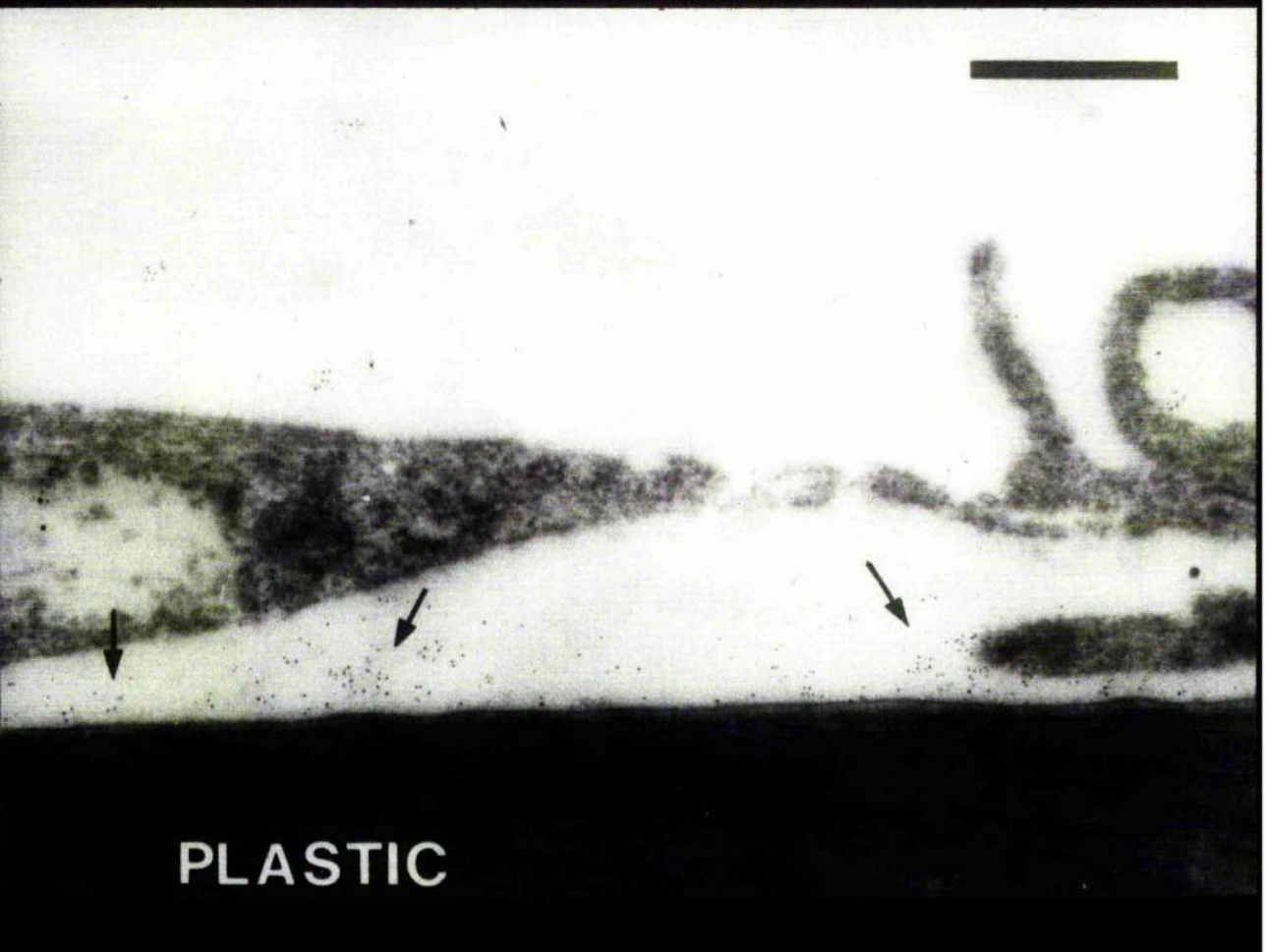
Electron micrograph showing a longitudinal section through an area of a monolayer of bovine ligamentum nuchae fibroblasts embedded in situ in a tissue culture flask 28-days post-confluency. Arrows indicate extracellular material synthesised by these cells. Bar 0.5um; x 21,000.

FIGURE 4.6.

Electron micrograph of a 28-day post-confluency cell culture of bovine foetal ligamentum nuchae fibroblasts embedded in resin. This section was treated with anti-35k-GP-A as primary antibody. The resulting distribution of 5nm colloidal gold particles at the base of the tissue culture flask is indicated with arrows. Bar 0.5um; x 50,000.



4. 5.

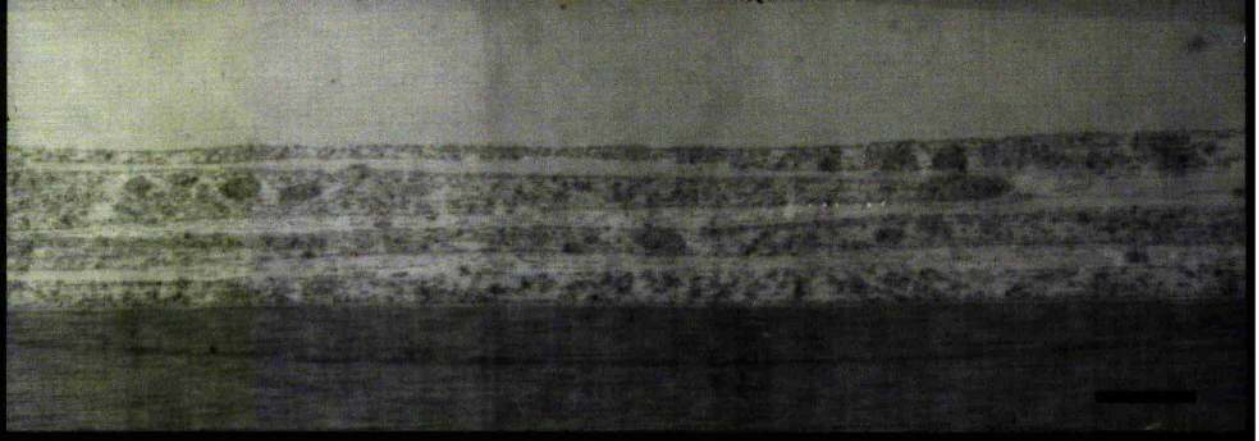


4. 6.

FIGURE 4.7.
Longitudinal section through a 21-day post-confluency cell culture of human foetal aortic smooth muscle cells embedded in resin. The section shows four layers of cells.
Bar 1um; x 12,000

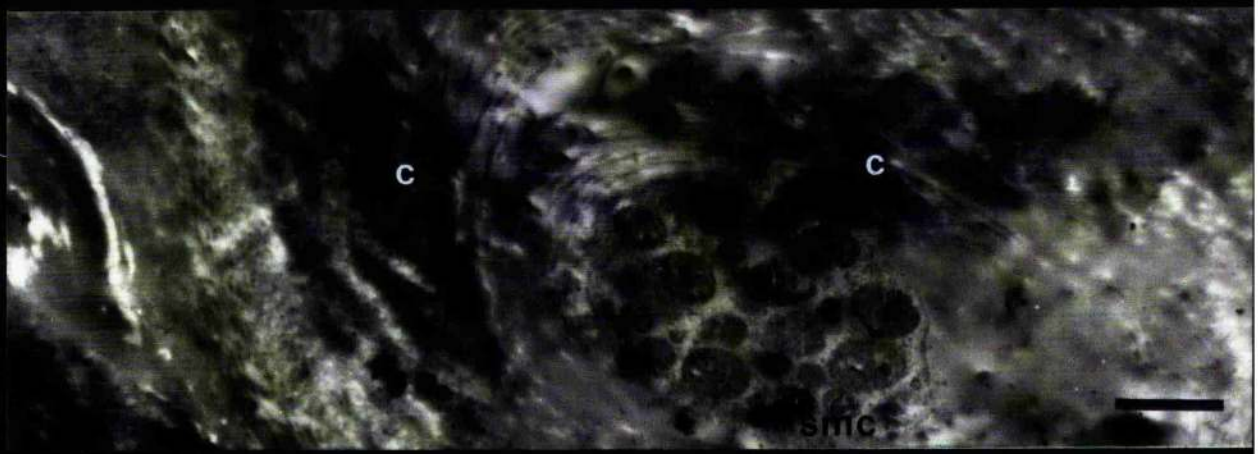
FIGURE 4.8.
A human foetal aortic smooth muscle cell situated deep in a cell layer 28-days post-confluency. This electron micrograph shows the prominent mitochondria (M), nucleus (N), endoplasmic reticulum (ER), Golgi (G) and vesicles (V) characteristic of the basal cell layers. Bar 0.2um; x 70,000.

FIGURE 4.9.
An electron micrograph of the extracellular matrix surrounding the cells in the deep layers of a human foetal aortic smooth muscle cell culture which was embedded in resin 28-days post-confluency. This transverse section shows that collagen (c) is the major component of the extracellular matrix synthesised by these smooth muscle cells (smc). Bar 0.5um; x 26,000.



4.7.

4.8.



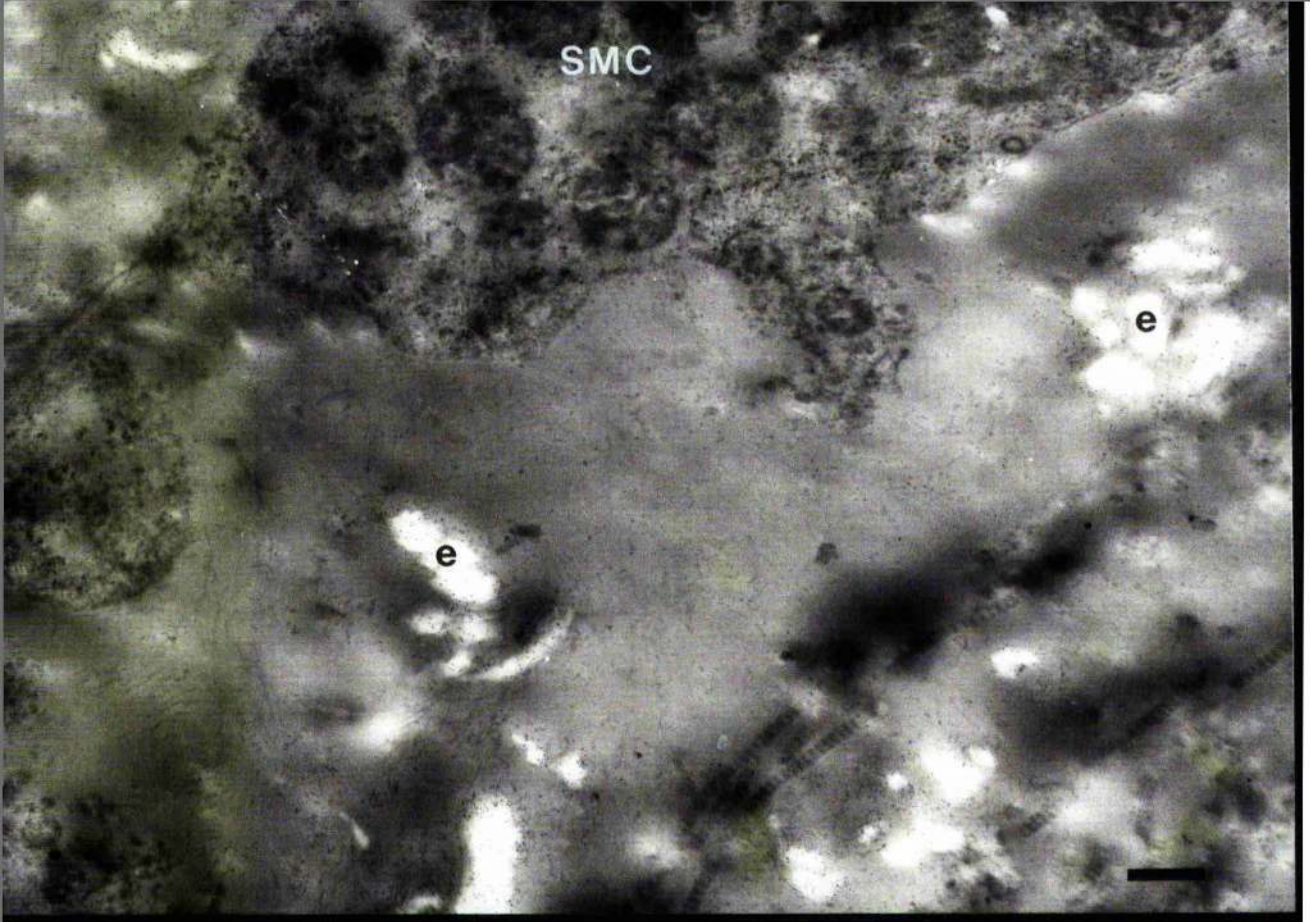
4.9.

FIGURE 4.10.

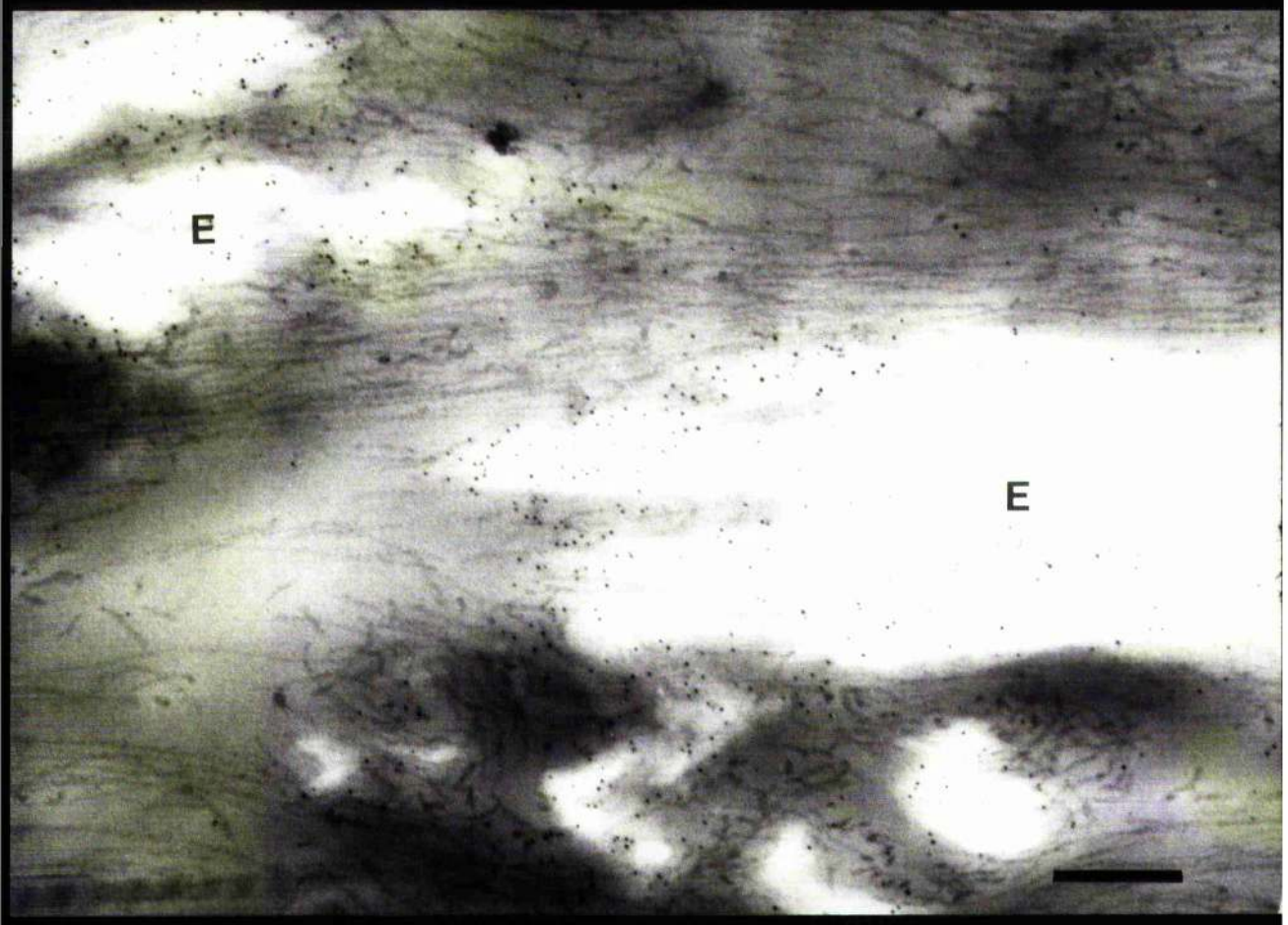
This electron micrograph shows that the extracellular matrix which surrounds the cells (SMC) in the superficial layers of the human foetal aortic smooth muscle cell culture described in Figure 4.9. (28-days post-confluency), consists of a few collagen fibres and many immature but extremely electron-lucent elastic fibres (E). This transverse section is treated with anti-35k-GP-A antiserum as primary antibody and the 5nm gold binds specifically to the periphery of the highly cross-linked elastin. Bar 1um; x 9100.

FIGURE 4.11.

As Figure 4.10. but at a higher magnification to show the amorphous electron-lucent elastin (E) surrounded by the elastin-associated microfibrils which are decorated with the 5nm colloidal gold particles. Bar 0,2um; x76,000.



4. 10.



4. 11

4.3.3. Cell Adhesion Studies.

The Petri dishes used for these studies were made of untreated polystyrene with negligible surface charge and only a limited capacity to support cell adhesion. Using a single Petri dish it was therefore possible to compare cell attachment, spreading and proliferation of cells on a 35k-GP substratum with that on fibronectin and on areas of untreated polystyrene. In these adhesion experiments, both foetal calf fibroblasts and human aortic smooth muscle cells gave identical results.

In one set of experiments, the foetal aortic cells were subcultured in Ham's F10 medium without any protein supplement (Figure 4.12.). Under these conditions, however, the fixed charges on the polystyrene were not suppressed and the cells adhered almost to the same extent to both uncoated plastic and areas coated with fibronectin and 35k-GP. However, over the 25 hour period examined, those cells which adhered to the plastic did not exhibit any sign of cellular activity, whereas the cells adhering to either of the glycoproteins showed a substantial increase in cell spreading.

Figure 4.13. shows the kinetics of attachment and spreading in another set of experiments in which the medium was supplemented with 10% foetal calf serum. In this case, the fixed charges on the polystyrene were blocked by serum components, and the cell attachment was limited to the areas coated with either fibronectin or 35K-GP. The cells showed a considerable tendency

to retain their contact with both substrata as indicated by the discrete boundary between coated and uncoated areas in Figures 4.14. and 4.15. Four hours after plating, there was a marked increase in spreading, particularly on the fibronectin substratum. By 24 hours, however, the number of cells spreading on 35k-GP had superceded the amount of cells spreading on fibronectin, the latter value having levelled off by 20 hours.

In one Petri dish the cells were left for 48 hours on the 35k-GP matrix and the resulting cell layer(s) embedded in epoxy resin. The lower surface of the smooth muscle cell layer(s) adhering to the 35k-GP substratum appeared to be fairly uniformly apposed to the substratum. However, at higher magnification, specific sites of contact between cell and substratum could be discerned (indicated with arrows in Figures 4.16.). The type of adhesions sites observed between the cell and the 35k-GP substratum conformed to those described as "focal contacts" and "close contacts" by Chen and Singer (1980, 1982). Most adhesion sites were found to exhibit the following characteristics which are typical of focal contacts:- spacings of approximately 10-20nm between the strands which bridge the substratum; the presence of an electron-dense submembranous plaque; and a membrane contour which is drawn up from the central area. In Figure 4.17. these focal contacts (arrows) can be compared with an adjacent "close contact" which occupies a larger linear dimension (width of 0.5um) and has spacings of 30-40nm between the cell surface and the substratum. These results, observed in separate Petri-dish

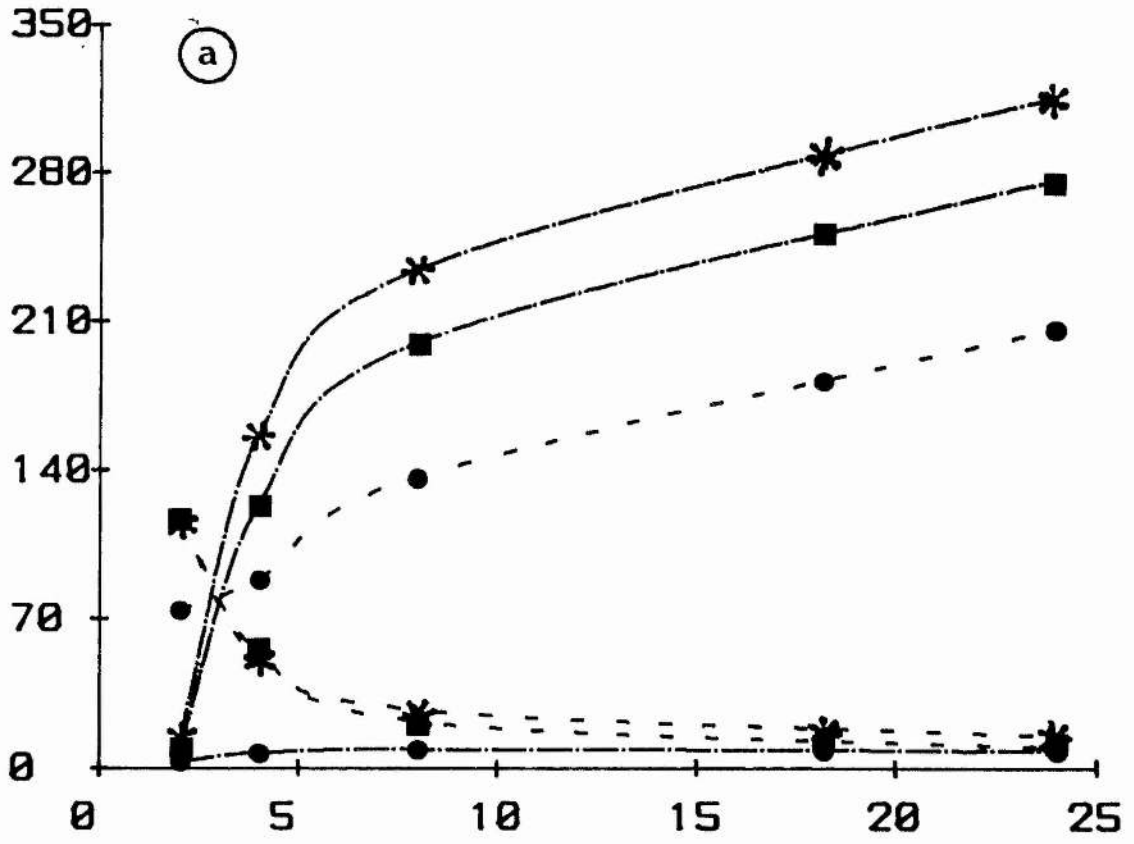
experiments, is discussed in Section 4.4. with respect to the type of adhesion occuring and its significance in the deposition of extracellular matrix components.

FIGURE 4.12.

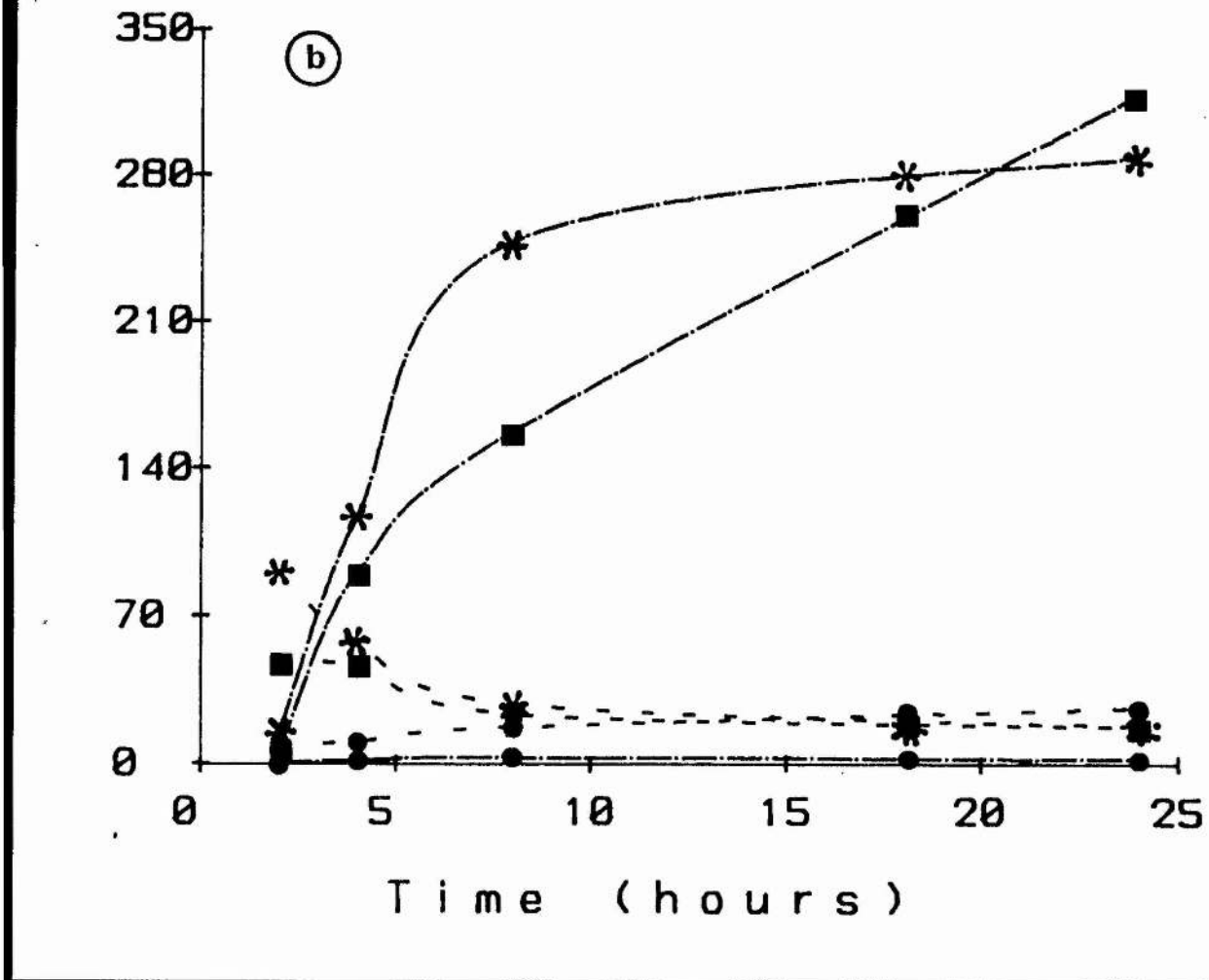
Cell attachment and spreading in Ham's F10 medium on: (●) uncoated plastic; (✱) fibronectin; and (■) 35k-GP. Values reported on the abcissa refer to number of cells attached (-----) and spreading (—·—·—) cm^{-2}.

FIGURE 4.13.

Cell attachment and spreading in Ham's F10 medium supplemented with foetal calf serum on: (●) uncoated plastic; (✱) fibronectin; and (■) 35k-GP. Values reported on the abcissa refer to the number of cells attached (-----) and spreading (—·—·—) cm^{-2}.


a
350
280
210
140
70
0
0
5
10
15
20
25

4.12.


b
350
280
210
140
70
0
0
5
10
15
20
25
Time (hours)

4.13.

FIGURE 4.14.

Phase contrast micrograph of a primary culture of smooth muscle cells from human aorta spreading in Ham's F10 medium supplemented with foetal calf serum, 10 hours after plating the petri dish. Note the discrete boundary between the cells located on the area coated with 35k-GP and the uncoated plastic.

FIGURE 4.15.

Phase contrast micrograph as above except that this petri dish was coated in discrete areas with fibronectin. Note that at this stage (10 hours) there appears to be more cells spreading on fibronectin than on 35k-GP (Figure 4.14.) and that there is a discrete boundary between the cells located in the fibronectin coated area and the surrounding plastic.

4. 14.

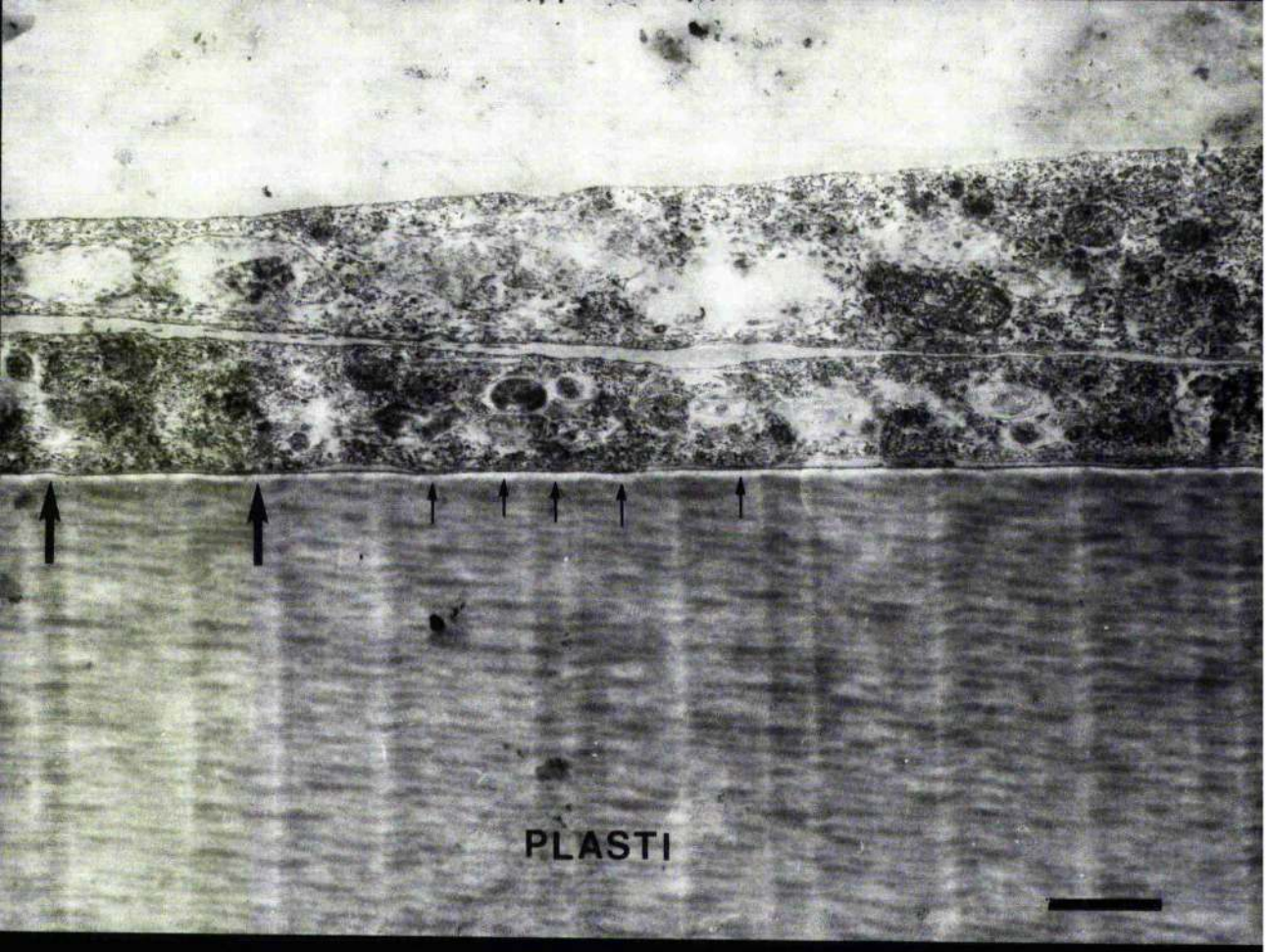
4. 15.

FIGURE 4.16.

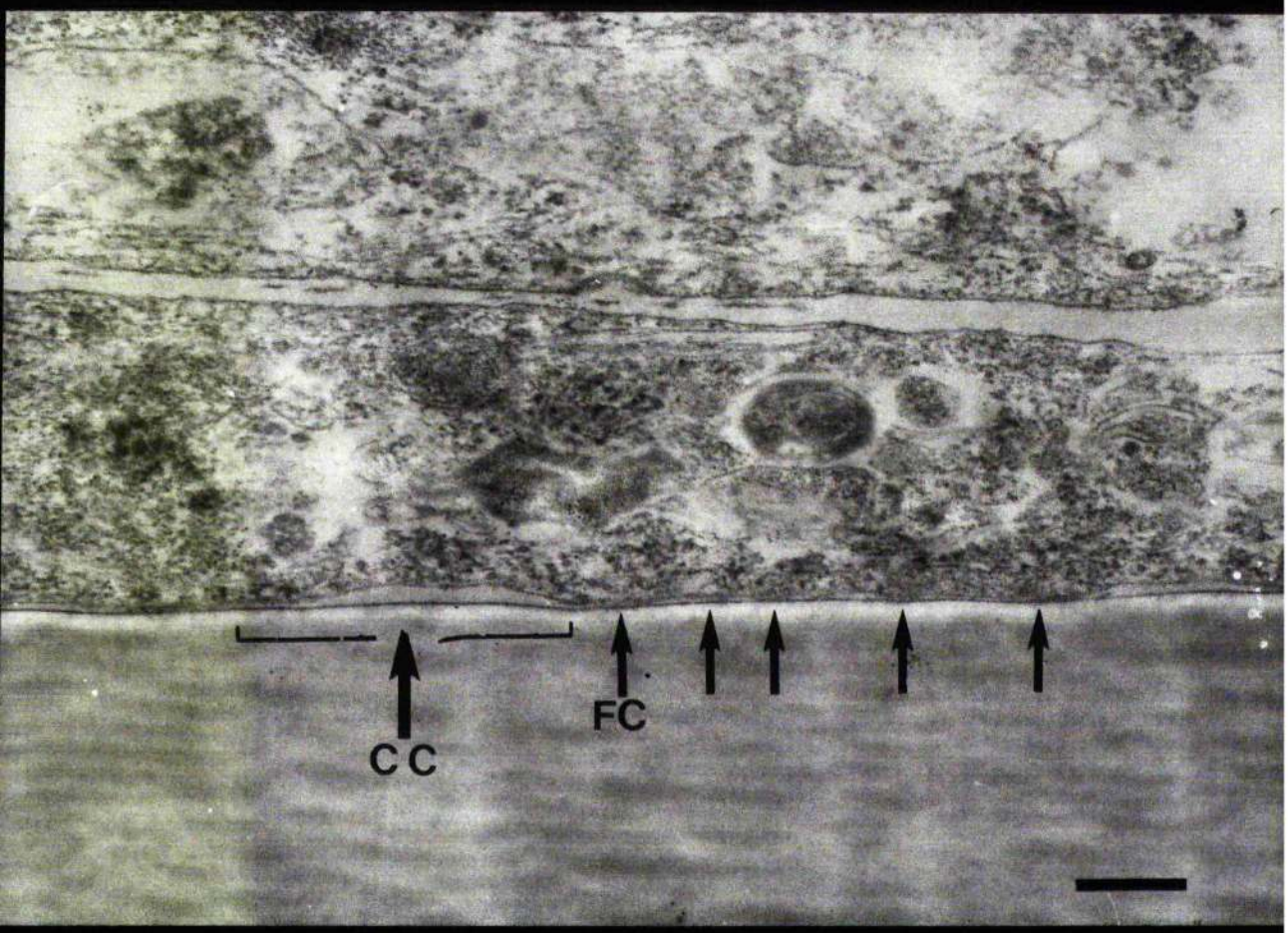
Electron micrograph of a longitudinal section of a bilayer of smooth muscle cells attached and spreading on a plastic petri-dish coated with 35k-GP as in Figure 4.14. Arrows indicate sites of contact between cell and 35k-GP substratum.
Bar 0.5um; x 26,000.

FIGURE 4.17.

Electron micrograph of the bilayer in Figure 4.16. magnified to show sites of close contact (CC) and focal contacts (FC).
Bar 0.2um; x 65,000.



4. 16.



4. 17

4.4. DISCUSSION.

Due to the age of the foetal calf, approximately 4 months gestation (120 days), the fibroblasts derived from the ligamentum nuchae were probably too immature to form multilayers and synthesise connective tissue components. This is in agreement with Lamberg et al. (1980) who similarly observed that subcultures of foetal ligamentum nuchae fibroblasts grew as monolayers only and that these layers tended to separate from the culture dish 14-21 days after passaging. It is apparent that gestational age is an important determinant in the capacity of foetal calf ligamentum nuchae fibroblasts to produce elastin. In vivo, elastin synthesis has been shown to be maximal in the third trimester of gestation (220 days) (Cleary et al., 1967) and in vitro, Mecham et al., (1981) have shown that ligamentum nuchae fibroblasts from a 110 day old foetal calf produce only 0.09ug of elastin per ug DNA as compared to 0.46ug elastin per ug DNA by fibroblasts from a 218 day old foetal calf neck ligament. These observations suggest that the fibroblastic cell lines used in the experiments described in this chapter were probably in a early non-differentiated state with respect to their elastin synthesising function(s). It was therefore decided to concentrate on in vitro synthesis by the human foetal aortic smooth muscle cells. This has the additional advantage of allowing the use of the species-specific anti-elastin antiserum as well as anti-35k-GP-A antiserum as probes. Explant cultures

of human foetal smooth muscle cells were readily established. Cellular morphology and culture characteristics were similar to those described by other workers (Burke and Ross, 1979, Kadar et al., 1981) and the ultrastructural appearance of the cultured smooth muscle cells and extracellular matrix corresponded closely to that described in intact aortic explants and in cell culture by other authors (Sandberg et al., 1979; Toselli et al., 1981).

With the recent acquisition of human foetal aortic explants our tissue culture experiments have been more successful and now the detection of 35k-GP and tropoelastin in both the media and the cell layer are being undertaken by immunoprecipitation and Western blotting analysis.

The cell adhesion experiments have provided an interesting insight into a possible role for the 35k-GP component of elastin-associated microfibrils in vivo. The extracellular matrix molecules fibronectin and laminin have already been shown to promote all three stages of fibroblast adhesion:- attachment, spreading and focal contact formation (Badley et al., 1981; Couchman et al., 1983). In these systems focal contacts have been shown to be linked to cytoskeletal reorganisation (Couchman et al., 1982) and in the case of fibronectin, the deposition of extracellular fibronectin (Woods et al., 1983). There is obviously a great deal of work that has to be undertaken to prove that a similar phenomenon is occuring in the case of the attachment and spreading of cells on 35k-GP substratum. By using interference reflection microscopy (IRM) for example, it will be

possible to discover whether substrate-specific focal contacts, which are specific for locomoting cells and last for 15-20 minutes, are occuring or if the related phenomena, present in stationary rounded cells, of focal adhesions (which are larger in area, give rise to a more dense image and persist for many hours) and close contact (which have the largest area of contact, are less dense in appearance and have a lifetime of only seconds) are in fact being observed. Future experiments are described in Chapter Five. These preliminary in vitro experiments however suggest that the microfibrillar component 35k-GP may be essential for the adhesion of elastogenic cells to the sites of future elastic fibre formation and may possibly be responsible for the induction of elastin synthesis.

CHAPTER FIVE.

SUMMARY.

During embryogenesis, the inherited information on the spatial arrangement of a particular tissue results in the deposition of extracellular matrix components. The overall tissue architecture is the responsibility of the embryonic cells which use the physico-chemical properties of the synthesised matrix components to assure local stability and form. Trelstad and Birk (1984) have postulated that embryonic cells are inherently polarised in both structure and function and that the quality, quantity and orientation of the extracellular matrix is under cellular control. They have shown that the cellular polarity and matrix patterning in embryonic chick tendon and corneal matrix is very similar to that proposed in this thesis for the morphogenesis of the elastic fibre. During matrix secretion, epithelial cells in the cornea are observed to undergo a reversal of their polarity with up to 80% of the cells relocating their Golgi to the basal cell pole. Discrete packets of collagen are then transported to the cell surface where the vacuoles are brought into register with the orientation of the underlying matrix by the alignment of ordered filaments and/or microtubules in the basal cell cytoplasm.

The fact that elastin appears in the extracellular matrix at a later stage in development than most other connective tissue macromolecules suggests that elastogenic cells require a highly structured or 'mature' extracellular matrix upon which to organise the complex elastic fibre and that only late in gestation is the matrix suitable for the fibre organisation.

Accordingly, a component of the extracellular matrix such as the microfibrils may provide the information that signals the appropriate time for elastin synthesis and secretion. This view is supported by the cellular polarity and the presence of both secretory vesicles and aligned microtubules (Chapter Two) observed when elastin-producing cells are in close proximity to the microfibrillar component of developing elastic fibres.

The belief that elastin-associated microfibrils play a crucial role in the morphogenesis of the elastic fibre has until recently been based primarily on electron microscopic studies of the development of elastic tissue. In Chapter Three, purified 35k-GP was shown by immunoblotting and immunochemical techniques to be a constituent of elastin-associated microfibrils in developing elastic tissues within the ear, skin, aorta and ligamentum nuchae of the foetal calf. No binding of anti-35k-GP was detected in adult bovine ligamentum nuchae, aorta or ear which suggests that this glycoprotein plays an important but transient role in the development of elastic tissue. It is proposed that the role of 35k-GP in the function of microfibrils is to induce the adhesion and morphogenetic movements of elastogenic cells during elastic fibre formation. The preliminary results described in Chapter Four support this view and we now plan to undertake a more detailed study with the following objectives in mind:-

1.(a)To examine by immunofluoresence the involvement of cytoskeletal structures such as microtubules and

microfilaments in the interaction of elastogenic cells with 35k-GP.

(b) To determine the type and duration of the 35k-GP induced adhesion by time-lapse video interference reflection microscopy to discover whether a substrate specific focal contact is occuring resulting in cytoskeletal reorganisation and ultimately in the synthesis and extracellular deposition of elastin.

2. A study of the ability of 35k-GP fragments to induce cell adhesion and spreading. This will allow the elucidation of a possible specific cell-binding region of the molecule.

3. An investigation into the identity of a specific cell receptor, the presence of which appears essential to the primary recognition event in the binding of 35k-GP and the possible existence of additional cell surface molecules to the cell-substratum attachment mechanism.

4. In conjuction with the above, a gene library from human foetal aortic smooth muscle cells is being created with the intention of developing cDNA probes to 35k-GP specific mRNA from this tissue.

Finally, it seems appropriate to offer some personal speculation as to the nature of elastin-associated microfibrils. It is my view that the core of microfibrils is composed of cross-linked chains of a glycoprotein such as Sakai's "fibrillin" covered in a spiral sheath, as seen in the electron microscope,

of two or more smaller glycoprotein chains which possess different specialised properties. This composite model is consistent with the observed properties of microfibrils during elastogenesis. Biosynthesis begins in developing elastic tissues with the formation of microfibrils which aggregate (by an unknown mechanism) into arrays. The ensuing deposition of tropoelastin onto the microfibrils and its subsequent cross-linking into amorphous elastin may be associated with a degradation of the glycoprotein sheath and loss of the microfibril's cationic staining properties, explaining the increased electron-lucency of mature elastin and the gradual disappearance of microfibrils. In special situations such as ocular zonules and skin, stabilisation of the glycoprotein core may be responsible for the persistence of microfibrils into adult life .

In summary, it appears that the study of the morphogenesis of the elastic fibre is entering an exciting phase. It is anticipated that within the next few years the true structure and function of the microfibrillar components will become clear allowing a more precise understanding of their role in elastic fibre formation.

CHAPTER SIX.

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