ORIGINAL ARTICLES – Laboratory science

Immunogold study of non-collagenous matrix components in normal and exfoliative iris

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Abstract
The present investigation was undertaken to determine if some of the components of exfoliation material in iris tissue were unique to exfoliation or were part of normal iris architecture. Eleven normal iris specimens and 10 exfoliative iris specimens were processed for cryoultramicrotomy and London resin white embedding. Immunogold electron microscopy was used to investigate the fine structural distribution of amyloid P component, elastin, entactin, fibronectin, gp115, and vitronectin in normal iris and their association with exfoliation material. Exfoliation material was positive for amyloid P component and possibly gp115, neither of which were present in normal iris tissue. Elastin and fibronectin were present in the normal iris stroma but were not associated with exfoliation material. The distribution of amyloid P component in the vessel lumen and wall led to the conclusion that amyloid P is a serum contaminant. The presence of gp115 in exfoliation material represents the synthesis of a component novel to the iris vascular cell synthetic repertoire.
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The exfoliation syndrome or pseudoexfoliation syndrome is considered to be a disorder in which there is an abnormal production and deposition of extracellular matrix in intra- and extraocular tissues.[1-3] Exfoliation syndrome is an age-related disease which is often complicated by glaucoma.[4 5]

By light microscopy exfoliation material is seen as eosinophilic bush-shaped accretions attached to zonular fibres, the surfaces of the ciliary processes, lens and iris, and within the trabecular meshwork.[6-8] By transmission electron microscopy, exfoliation material appears as a polydimensional accumulation of cotton-like fibres consisting of filamentous subunits, with a varied periodicity (17–56 nm) embedded in an interfibrillar matrix.[2] The biochemical nature of exfoliation material has been the subject of intensive study but the exact composition remains to be elucidated. It is not known which of the constituents are the primary products of disordered cell secretion or which are simply absorbed into the abnormal matrix.

Several extracellular matrix (ECM) components have been localised to exfoliation material using the immunogold technique on post-embedded tissue, including amyloid P component,[9-11] chondroitin sulphate,[9 10] elastin,[9 11] entactin (nidogen),[9 10] fibronectin,[10] heparan sulphate,[9 10] laminin,[10 12 13] and vitronectin.[9]

The post-embedding immunolabelling technique carries the risk of masking antigenic epitopes. It is therefore more desirable to apply the cryoultramicrotomy technique in which immunolabelling is conducted before embedding, thus allowing a greater access to antigenic epitopes. It is, however, important that both cryoultramicrotomy and plastic embedding techniques are applied when it is necessary to identify the constituents of an abnormal accumulation of a complex biochemical nature.

In terms of pathogenesis, the accumulation of exfoliation material may simply be due to an uncontrolled production of ECM components by cells which are normally engaged in their synthesis. Alternatively, the cells may be triggered to synthesise ECM components completely outside their normal repertoire. Thus it is necessary to determine the status of these components in normal tissue. We therefore studied the distribution of a select number of ECM components – namely, amyloid P component, elastin, entactin, fibronectin, and vitronectin, in the normal iris and to confirm their presence in exfoliation material. To further investigate the elastic constituents, we also used a recently commercially available antibody to elastin associated glycoprotein (gp115).

Materials and methods
Normal iris tissue was obtained from four surgical enucleations, two postmortem enucleations (within 1·5 and 3 hours of death respectively) and six iridectomies (see Table 1 for clinical details). All specimens were considered normal by histological examination of toluidine blue stained semithin (1 μm) London resin (LR) white plastic embedded sections. Ten exfoliative iridectomy specimens were obtained from patients operated on for exfoliation glaucoma (Table 2).

It is well recognised that the level of glutaraldehyde concentration can have profound effects on the degree of antigenic preservation: for this reason specimens were exposed to concentrations of glutaraldehyde varying from 0% to 1% (see Tables 1 and 2).

FIXATION
Normal iris tissue obtained from surgical and

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Table 1 *Patient and processing details of normal iris specimens*

Case	Age	Sex	Diagnosis	Operation	Technique	Fixation (% glutaraldehyde +4% PFA)
1	35	F	Orb tumour	E	Cryo	PFA only
2	38	F	CM	E	Cryo	0·5%
3	40	M	PM	E	Cryo	0·5%
4	52	M	CM	E	LRW	0·1%
5	63	M		I	LRW	PFA only
6	64	F		I	LRW	PFA only
7	67	F		I	LRW	PFA only
8	80	F	PM	E	Cryo	0·75%
9	81	F	Sec glaucoma	E	LRW	0·1%
10	85	M		I	LRW	PFA only
11	93	F		I	LRW	PFA only

CM=choroidal melanoma. PM=post mortem. E=enucleation. I=iridectomy. Cryo=cryoultramicrotomy. LRW=LR white.

Table 2 *Patient and processing details of exfoliative iris specimens*

Case	Age	Sex	Diagnosis	Operation	Technique	Fixation (% glutaraldehyde +4% PFA)
1	62	M	EXF glaucoma	I	LRW	0·1%
2	68	M	EXF glaucoma	I	LRW	0·1%
3	68	F	EXF syndrome	I	LRW	0·1%
4	71	M	EXF syndrome	I	LRW	0·1%
5	72	F	EXF syndrome	I	LRW	0·1%
6	78	F	EXF syndrome	I	LRW	0·1%
7	82	M	EXF glaucoma	I	LRW	PFA only
8		M	EXF glaucoma	I	LRW	0·4%
9					Cryo	
10					Cryo	

EXF=exfoliation. I=iridectomy. Cryo=cryoultramicrotomy. LRW=LR white.

Table 3 *Details of primary antibodies*

Antigen	Type	Dilution LRW	Dilution Cryo	Company	Specificity tests
Amyloid P	Rabbit poly	1:140	1:1200	Dako, Denmark	Crossed and rocket immunoelectrophoresis Single radial immunodiffusion
Elastin	Mouse mono	1:100	1:100	Sigma, UK	Immunoaffinity chromatography
Entactin	Mouse poly	1:100	1:50	UBI, USA	Western blot
Fibronectin	Mouse mono	1:100	1:50	Sigma, UK	Immunoblot
gp115	Mouse mono	1:150	1:800	Chemicon, USA	Immunoblotting Immunoprecipitation
Vitronectin	Mouse mono	1:100	1:50	Manheim Boehringer, Germany	ELISA, Western blot

postmortem enucleations was used as control material. This material had been fixed in 4% paraformaldehyde (PFA) and 0·1–1·0% glutaraldehyde in 0·1 M cacodylate or phosphate buffer (pH 7·4) at room temperature for 2 hours and immediately processed (see below). In the current study, control iridectomies from primary open angle glaucoma cases were fixed in 4% PFA (cacodylate or phosphate buffer pH 7·4) for 1·5–2 hours at room temperature. Six of the exfoliative iridectomies were fixed in 0·1% glutaraldehyde (cacodylate buffer) for 1·5 hours and the other four exfoliative iridectomies in 4% PFA (1·5 hours). Blocks were washed three times in buffer (5 minutes each) and allocated for cryoultramicrotomy and LR white embedding. LR white is a hydrophilic hydroxymethacrylate resin which can be used to embed tissue that has been dehydrated in 70% alcohol, thereby allowing greater preservation of antigenicity.[14]

LR WHITE PLASTIC EMBEDDING
Blocks allocated for LR white plastic embedding

were dehydrated by 15 minute changes in 20% and 50% ethanol, block stained with 2% uranyl acetate in 70% ethanol for 1 hour, washed in three 20 minute changes of 70% ethanol, and infiltrated by two, 1 hour changes of LR white resin (hard grade). The above steps were carried out at 4°C. Tissue blocks were embedded in OO size sealed gelatin capsules and the resin polymerised at 43°C for 48 hours.

Gold or silver coloured ultrathin sections were cut on an LKB Ultrotome Nova (Cambridge Instruments), mounted on 200 mesh nickel grids, allowed to dry for 2 hours and rehydrated with distilled water. The sections were quenched of free aldehyde groups with 0·5 M ammonium chloride in 50 mM Tris buffer (pH 7·2) for 30 minutes and then washed by four, 2 minute changes in 50 mM Tris buffer before overnight preincubation in 1% bovine serum albumin (BSA, essentially immunoglobulin free, Sigma) in Tris buffer at 4°C.

CRYOULTRAMICROTOMY
Blocks allocated for cryoultramicrotomy were infused overnight with 2·3 M sucrose at 4°C, mounted on silver pins, snap frozen, and stored in liquid nitrogen. Ultrathin frozen sections were cut at −83°C on a Reichert Jung FC4D cryoultramicrotome (Cambridge Instruments) and transferred on 2·3 M sucrose drops to 200 mesh carbon Formvar composite nickel grids and preincubated on 1% BSA in Tris buffer overnight at 4°C. Free aldehyde groups were quenched by two 5 minute changes in 30 mM glycine in Tris buffer before incubation in the primary antibody.

IMMUNOCYTOCHEMISTRY
The Tris buffer employed throughout the immunocytochemical procedure was 50 mM Tris pH 7·2 with the addition of 1·5 g/l of sodium azide, 0·5 M NaCl[15] and 0·05% Tween 20 (Sigma). Tween 20 was omitted from the buffer when labelling ultrathin frozen sections (cryoultramicrotomy). Primary antibody incubation was conducted for 2 hours at room temperature with the addition of 1% BSA to the Tris buffer. Unbound antibody was removed by six 5 minute changes in buffer and the grids incubated for 5 minutes on 1% BSA (in Tris) before labelling with a 1:50 dilution of 10 nm immunogold conjugate (Biocell Laboratories). Excess immunogold antibody was removed by six 5 minute changes in Tris buffer and the grids subsequently washed in two changes of distilled water. LR white ultrathin sections were stained with saturated aqueous uranyl acetate for 12–15 minutes and ultrathin frozen sections (cryoultramicrotomy) were embedded in a 10:1 mixture of 3% polyvinyl alcohol and saturated aqueous uranyl acetate. Grids were then examined in a Jeol 1200 EXII transmission electron microscope at 80 kV.
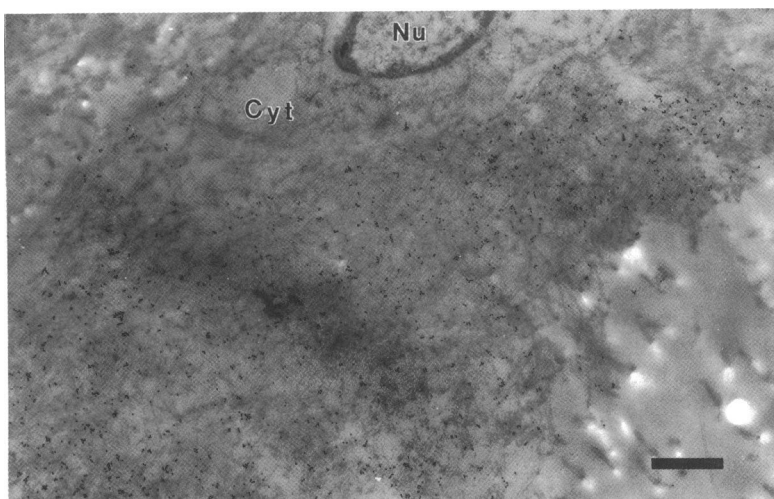
ANTIBODIES
The optimal working dilution of each antibody was determined experimentally. Higher dilu-

Figure 1A   Exfoliation aggregate (ex) labelled for amyloid P component, is associated with vascular supporting cells (V) of an exfoliative iris vessel. (L)=vessel lumen. (LR white, ×11 350, bar=1 μm).

Figure 1B   Higher power of Figure 1A. Immunogold labelling for amyloid P component. Immunogold particles are localised to exfoliation material and are absent from electron lucent spaces within the aggregate, the cell nucleus (Nu) and cytoplasm (Cyt). (LR white, ×18 600, bar=0·5 μm).

CONTROLS

Two negative controls were incorporated in the immunocytochemical procedure: (1) omission of the primary antibody, and (2) substitution of the primary antibody with non-immune serum from the same species in which the primary antibody was raised (Sigma). Non-immune sera dilutions were identical to those of the primary antibodies.

Immunogold labelling of the extracellular matrix was considered specific only when immunogold particles were absent from red blood cells, cell nuclei, mitochondria, and pigment granules. Non-specific labelling was best detected by examination of these structures.

Results

Exfoliative material was readily recognised in LR white embedded tissue but was not as easily differentiated from other structures in ultrathin frozen sections. Reliable identification required examination at low magnifications. Individual exfoliation fibres were thinner and more prominent in LR white embedded tissue, giving the impression of being shrunken or collapsed (Figs 1, 2). In ultrathin frozen sections exfoliation fibres were often obscured by the abundance of an associated matrix of similar electron density (Fig 3).

AMYLOID P COMPONENT

Normal iris

Amyloid P component was localised, in LR white embedded sections of normal iris, specifically to fine granular deposits of plasma within the lumen of blood vessels. Vessels which did not contain plasma were free of immunogold particles. Labelling intensity of the plasma did not vary with the type of fixative used. Antibodies against amyloid P component did not label basement membranes associated either with blood vessels, the posterior pigment epithelium, or the dilator and sphincter muscle. Labelling for amyloid P component was absent from the stroma.

Amyloid P component was localised to the lumen of blood vessels in ultrathin frozen sections (cryoultramicrotomy) of normal iris. As in LR white embedded iris tissue, labelling was absent from basement membranes of blood vessels, the pigment epithelium, and sphincter muscle.

Exfoliative iris

Antibodies against amyloid P component specifically labelled exfoliation material both in LR white embedded tissue sections (Figs 1, 2) and in ultrathin frozen sections (Fig 3). More specifically, immunogold particles were present over both fibres and the granular matrix and labelling intensity for amyloid P component increased with increasing density of the exfoliation deposit. The degree of labelling was greater in ultrathin frozen sections than in LR white embedded tissue (compare Fig 3 with Figs 1, 2). Labelling of LR white embedded exfoliative fibres varied with the precise fixation regime used. The signal was strong when either 4% PFA
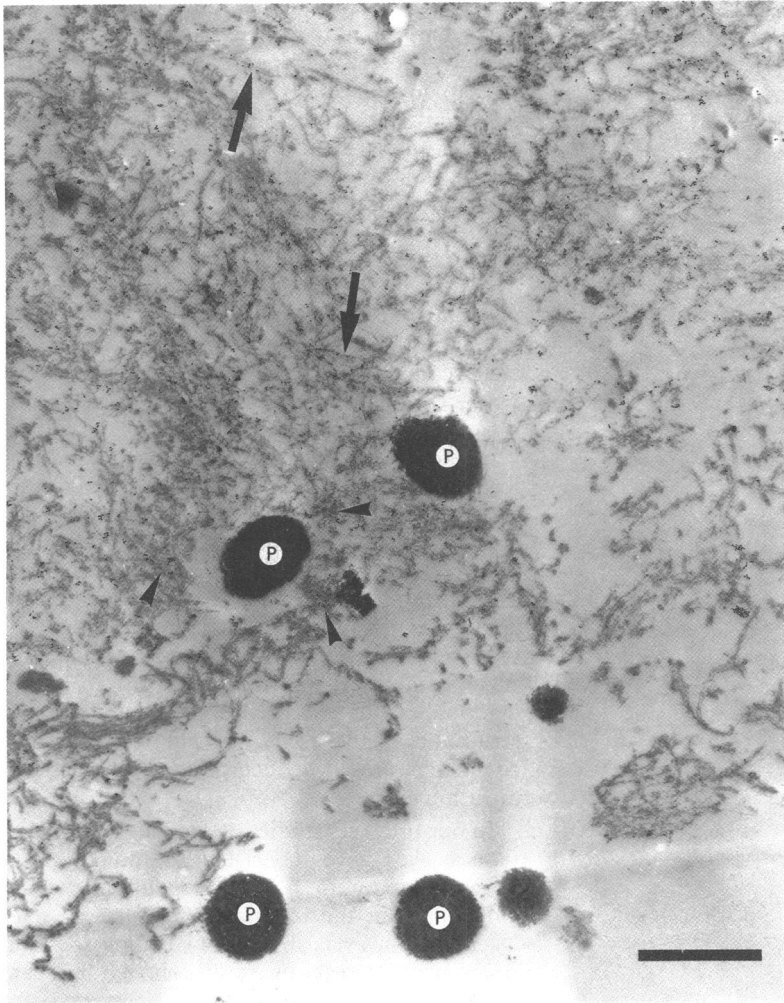
tions were required for optimal labelling in ultrathin frozen sections (Table 3). Details of the primary antibodies, including specificity tests conducted by the suppliers, are given in Table 3. Rabbit primary antibodies (amyloid P component, vitronectin, and entactin) were visualised by 10 nm goat anti-rabbit immunogold conjugate (Biocell Laboratories) and mouse primary antibodies (elastin, fibronectin, and elastin associated glycoprotein) by 10 nm goat anti-mouse immunogold (Biocell Laboratories).

Figure 2 Exfoliation aggregate attached to posterior iris pigment epithelium. Labelling for amyloid P component is present both over exfoliative fibres (arrows) and the granular matrix (arrowheads). Pigment granules (P) are present within the exfoliation deposit. (LR white, ×15 500, bar=1 μm).

or 0·1% glutaraldehyde was employed and the use of paraglutaraldehyde (4% PFA plus 0·1–0·5% glutaraldehyde), although significantly improving ultrastructural preservation, resulted in minimal and sometimes complete absence of labelling.

Immunogold labelling for amyloid P component was absent from areas free of exfoliation material in LR white embedded tissue. However, in ultrathin frozen sections, labelling was also present in the iris stroma at intensities comparable with those of the normal iris specimens. The precise distribution of immunogold particles was also similar, being present predominantly in the electron lucent regions of the stroma.

ELASTIN

Immunogold labelling for elastin was not observed in either normal or exfoliative iris embedded in LR white resin. Labelling for elastin was present in ultrathin frozen sections of both normal and exfoliation specimens. Immunogold particles were found in regions devoid of other identifiable matrix constituents (Fig 4) and occasionally the distribution of immunogold particles did not appear random but suggested the labelling of very thin filaments (Fig 4). Cellular elements, exfoliation fibres and their associated granular matrix were free of label as were areas of the carbon Formvar composite film from which tissue was absent.

ENTACTIN

Immunolabelling of both normal and exfoliative LR white embedded iris specimens failed to demonstrate the presence of entactin, regardless of the fixation regime employed. Similar negative results were obtained when labelling ultrathin frozen sections.

FIBRONECTIN

Fibronectin was not detected in any anatomical subcompartment in either normal or exfoliative iris tissue embedded in LR white plastic. However, some immunogold labelling was present in ultrathin frozen sections in both normal and exfoliative specimens to electron lucent regions in the iris stroma (Fig 5). Anti-fibronectin antibodies were not associated with either thick or thin collagen fibrils (Fig 5). Examination of the stroma at high magnification could not demonstrate the presence of fine filaments or any other discrete structural entities with which immunogold particles were associated. No labelling for fibronectin was observed over basement membranes. Cellular profiles and areas of the carbon Formvar composite film from which tissue was absent were devoid of immunogold particles. A similar pattern of labelling for fibronectin was also observed in ultrathin frozen sections of exfoliative iris tissue, but was absent from exfoliative deposits.

ELASTIN ASSOCIATED GLYCOPROTEIN (gp115)

Immunolabelling for elastic associated glycoprotein (gp115) was absent from sections of normal iris prepared either by LR white embedding or by cryoultramicrotomy.

Labelling for gp115 was present in the stroma and over exfoliation deposits of exfoliative iris sections prepared by cryoultramicrotomy (Fig 6). Some association was observed between gp115 and striated collagen fibril bundles within the iris stroma. Immunogold particles were also randomly distributed over electron lucent areas of the stromal matrix and over aggregates of exfoliation material. The weak labelling of exfoliation deposits was in contrast with that observed for amyloid P component. Labelling observed in ultrathin frozen sections of exfoliative iris was not duplicated in sections of exfoliative specimens embedded in LR white plastic.

VITRONECTIN

Antibodies against vitronectin were not specifically localised to any of the anatomical subcompartments examined in either normal or exfoliative iris embedded in LR white plastic. Even light fixation (4% PFA) did not permit positive identification of vitronectin. None of the exfoliation aggregates present in exfoliation specimens exhibited labelling for vitronectin. Increasing the concentration of primary antibody beyond a 1:50 dilution resulted in completely non-specific labelling of cells and extracellular matrix. The positive localisation of vitronectin was not achieved with ultrathin frozen sections of either normal or exfoliative iris.
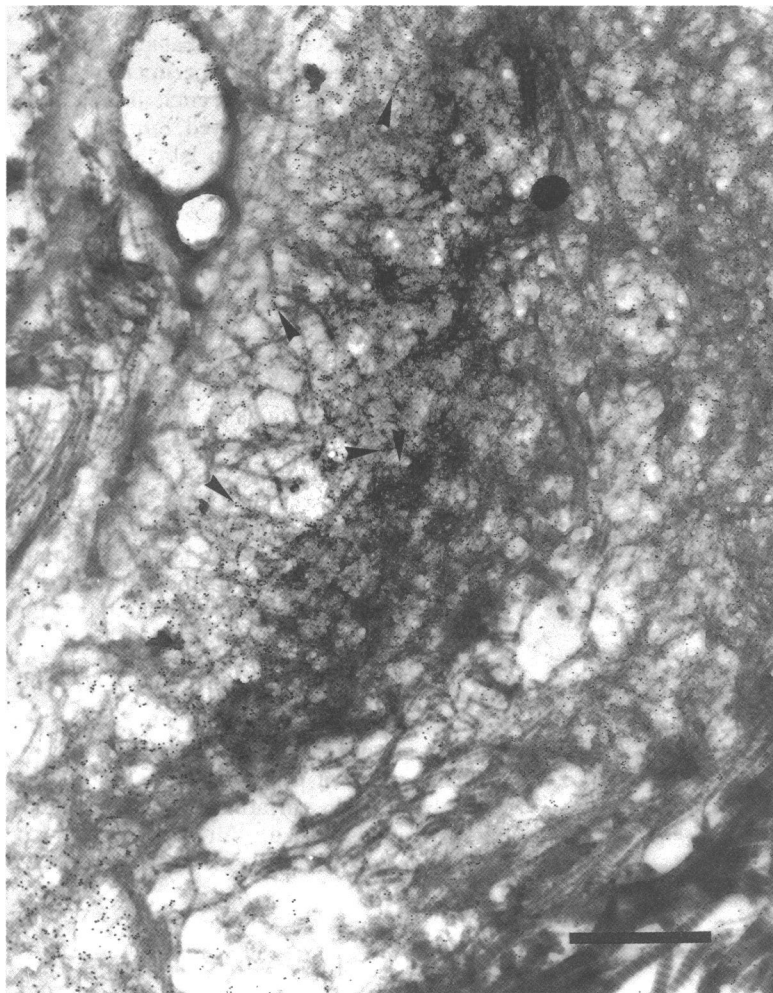
Figure 3 Ultrathin frozen section of exfoliation material labelled for amyloid P component. Exfoliation fibres cannot be identified within the large amounts of amorphous matrix. Immunogold particles frequently lie in short rows (arrowheads) (cryo, ×18 600, bar= 1 μm).

NEGATIVE CONTROLS

Immunogold particles were a rarity in rabbit anti-mouse and goat anti-rabbit immunogold negative control LR white or ultrathin frozen sections (omission of primary antibody). Immunogold labelling was not observed in the negative normal goat and rabbit serum control sections (substitution of the primary antibody with non-immune serum from the same species in which the primary antibody was raised).

Discussion

For more than seven decades a failure to identify the constituents of exfoliation material has led to controversies concerning its origin, nature, and pathogenesis. Streeten and co-workers[1-3 16-18] postulated that exfoliation material is derived from abnormal polymerisation of glycoproteins associated with the zonular elastic microfibrillar system in a type of elastosis. This postulate is based on histochemical and immunohistochemical similarities between exfoliation material, the ocular zonules,[18 19] and elastic components.[1]

An alternative theory is that exfoliation material is 'an abnormal basement membrane synthesised at multiple sites by abnormal (aging) cells'.[20] This theory was based on the close proximity of exfoliation material with basement membranes, and the extensive basement membrane changes associated with exfoliative deposition. These changes are particularly prominent in basement membranes of the iris vasculature

and a putative evolution of the vasculopathy has been constructed from extensive examination of iris blood vessels.[21] Documentation of the distribution of collagen types I-V and laminin in normal iris vessels[12 22] provided a baseline for studying abnormalities in their distribution in exfoliative vasculopathy. Exfoliation deposits appeared to stimulate the deposition of collagen types I and IV (basement membrane collagen) by vascular supporting cells at an early stage of the disease, whereas advanced exfoliation vasculopathy was accompanied by a reduction in the amount of collagen types I and IV.[13] An associated reduction in the laminin content of iris vessel walls was also noted.[13]

Considering the theories concerning the pathogenesis of the exfoliation syndrome it has been logical to attempt to identify components of the elastic system (amyloid P component, elastin and gp115) and the various glycoproteins which are known to be present in basement membranes (entactin and fibronectin). It is also reasonable to attempt to identify other components associated with the extracellular matrix such as vitronectin. A fundamental problem in this approach to determining the pathogenesis of the exfoliation syndrome is that some, if not all, of these components could be incorporated by a passive diffusion process.

In our previous studies of aged normal ocular tissues we have consistently found cryo-ultramicrotomy to be superior to LR white embedding in the localisation of type VI collagen.[23-25] However, absence of labelling does not necessarily mean absence of the antigen, but can be attributed to failure of antigenic preservation, or lack of antibody specificity. The advantage of cryoultramicrotomy is that loss of antigenic preservation can only be due to fixation since subsequent steps in processing have no adverse effects on antigenicity.

An intrinsic disadvantage of the post-embedding technique is that absence of labelling in LR white sections may result from inaccessibility of the antigenic epitope. LR white is a post-embedding technique in which only epitopes at the plastic section surface are available for antibody binding.[14] Since embedding occurs after antibody labelling in the ultrathin frozen section technique, cryoultramicrotomy avoids this limitation.[26]

ELASTOTIC COMPONENTS

Amyloid P component

Using the two techniques of cryoultramicrotomy and LR white embedding we were able to confirm previous identification of amyloid P component within exfoliation deposits.[9 11 17] However, it cannot be inferred from this that exfoliation material is an amyloid-like substance, because amyloid P is only a minor component of primary amyloid, constituting about 5–10% dry weight.[27] The mandatory amyloid components (amyloid A, prealbumin, and immunoglobulin light chains) are absent from exfoliation substance.[17]

Amyloid P component was localised, in the present study, to plasma within blood vessels of
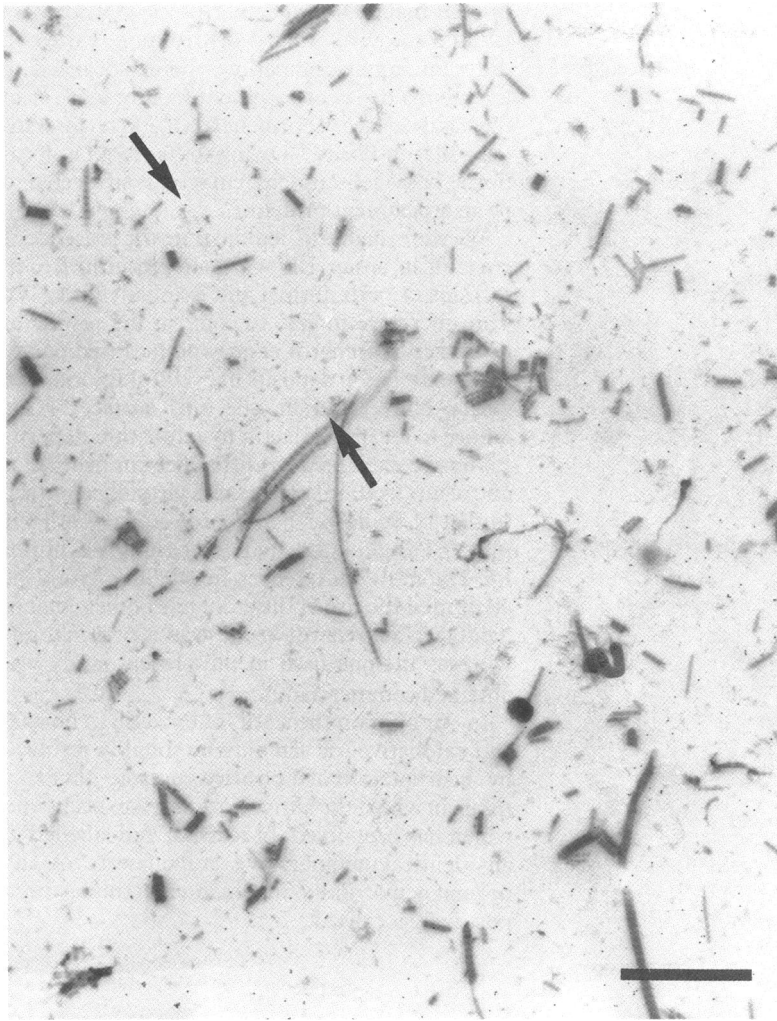
Figure 4   Elastin labelling in an ultrathin section of normal iris stroma reveals a linear distribution of immunogold particles (between arrows) suggesting the labelling of very fine filaments. No morphological detail of such putative filaments is evident (cryo, ×16 750, bar=1 µm).

normal and exfoliative iris, but was absent from both vascular and epithelial basement membranes. Stromal labelling in control and exfoliative tissue may have arisen from selective binding of serum amyloid P to some stromal component, probably fibronectin as seen by cryoultramicrotomy. De Beer *et al*[28] demonstrated a calcium dependent selective binding of serum amyloid P to fibronectin and C4-binding protein. The possibility of selective binding to fibronectin is substantiated by the fact that fibronectin was localised to the iris stroma but not to the vascular basement membranes which did not contain amyloid P. To date, amyloid P component has been shown to be an intrinsic component only of glomerular basement membranes and may be covalently linked to one of the normal basement membrane glycoproteins.[29] Whether amyloid P is synthesised by the glomerular basement membrane producing cells or is simply serum amyloid P that is incorporated into nascent basement membranes is unknown.[29]

We would concur with Li *et al*[17] that amyloid P is not likely to be an intrinsic component of exfoliation material, but is the result of a ligand binding reaction, probably calcium dependent, of serum amyloid P. Although iris vessels maintain a blood-aqueous barrier, exfoliative iris vasculopathy is associated with increased vascular permeability.[30][31] This increase would facilitate leakage of serum amyloid P component into

the iris stroma and its subsequent binding to exfoliation material.

Elastin

The putative labelling of elastin in the normal stroma and the uninvolved stroma of exfoliative iris is, to the best of our knowledge, an original finding. The presence of small amounts of elastin in the iris stroma can be correlated with tissue function. Iris tissue undergoes both constant and rapid contraction and expansion with changes in pupil diameter and it would therefore be predictable that the iris should contain an elastic element. However, we wish to express reservations on the validity of our results, for although non-specific labelling over cellular elements was absent, the failure to associate elastin labelling with any visible structure is somewhat disconcerting.

With regard to elastin in exfoliation material, our results may initially seem contradictory to those previously published. Elastin and tropoelastin have been localised to the deposits by Li *et al* using two of three antibodies: labelling of the deposits with antibody BA-4 – a monoclonal antibody against bovine elastin – was considerably weaker.[1] The same group[11] found that exfoliation aggregates from a variety of organs (heart, lung, liver, gall bladder, and eye) stained positively for elastin. Schlotzer-Schrehardt *et al*,[9] using the BA-4 monoclonal antibody, described moderate binding to exfoliation material in eyelid skin biopsies but only weak labelling of exfoliation material in the iris and ciliary body. In the present study using the BA-4 anti-bovine elastin monoclonal antibody we were unable to localise elastin to exfoliation substance in either LR white or ultrathin frozen sections. Using the same antibody we have successfully localised elastin to elastic fibres in the sclera (unpublished observations).

Elastin associated glycoprotein (gp115)

First isolated and characterised by Bressan *et al*[32] it is not to be confused with a sialoglycoprotein of the same name (gp115).[33] The latter glycoprotein (also known as CD43, sialophorin, and leukosialin) is expressed on the surface of T lymphocytes, monocytes, neutrophils, platelets, and some B lymphocytes.[34] This confusion in nomenclature between two unrelated glycoproteins is the result of a historical artefact. Both glycoproteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis with an apparent molecular weight of 115 000 and were termed gp115 in different journals at the same time.[32][33]

Light microscopic immunohistochemistry of 2-day-old chicks has shown that elastin associated glycoprotein (gp115) is present in connective tissue extracellular matrix of several organs,[35] where it exhibits co-distribution with tropoelastin (in liver, heart, skin, large vessels, striated muscle, and lymphoid organs).[36][37] Both components are present in the chick eye in Descemet's membrane, the corneal stroma, and the elastic layer of Bruch's membrane.[36] Colombatti *et al*,[35] using immunofluorescence microscopy, noted

Figure 5 Labelling for
fibronectin in stroma of
normal iris appears to be
randomly distributed in the
electron lucent matrix and
cannot be associated with
any morphological structure
(cryo, ×15 500,
bar=1 µm).

the presence of gp115 in the region of some, but
not all basement membranes. Owing to the low
resolution of the immunofluorescence technique,
they were not able to establish if gp115 is a true
component of basement membranes.[35]

In the present study gp115 was absent from
basement membranes in both the normal and
exfoliative iris but weakly labelled exfoliation
material and regions of exfoliative iris stroma
devoid of exfoliation aggregates. We regard these
observations with some caution for, as with
elastin, labelling could not be localised to specific
structures in the iris stroma. The putative pres-
ence of gp115 in exfoliation material would
represent the synthesis of a component novel to
the iris vascular cell synthetic repertoire. This
would lend further support to the suggestion of
Streeten and co-workers[1-3 16-18] that exfoliation
material is derived from abnormal polymerisa-
tion of glycoproteins associated with the zonular
elastic microfibrillar system in a type of elastosis.
However, it should not be inferred that the
synthesis of gp115 is inevitably linked with the
secretion of tropoelastin as cell culture experi-
ments indicate that gp115 has different secretion
kinetics to tropoelastin and a different fate after
secretion.[18]

BASEMENT MEMBRANE COMPONENTS

Entactin
This is a sulphated basement membrane glyco-

protein that may associate with the glycoprotein
laminin in vivo.[39] The distribution varies in
different organs, sometimes being localised to
the lamina densa of basement membranes, or to
the basal surface of epithelial cells and on intersti-
tial collagen fibrils.[39] Owing to its frequent loca-
tion at basal cell surfaces, entactin is suspected to
be an attachment protein.[39]

We were unable to demonstrate the presence of
entactin in either LR white or ultrathin frozen
sections of both normal and exfoliative iris. We
were therefore unable to confirm the results of
Schlotzer-Schrehardt et al, who localised entac-
tin to exfoliation deposits in eyelid skin, iris, and
ciliary body,[9] and on the lens surface.[10] Our
failure to localise entactin to exfoliation deposits
cannot be explained by differences in fixation as
one of our fixation regimes was virtually identical
to that of Schlotzer-Schrehardt et al,[9] (4% PFA
plus 0·1% glutaraldehyde in cacodylate buffer
for 2 hours). Differences in labelling could be
attributed the use of different antibodies (that of
Schlotzer-Schrehardt et al[9] was a personal gift
whereas the one used in the present study was
obtained commercially).

In our opinion the status of entactin in normal
and exfoliative iris can only be suitably resolved
by incorporation of a positive control – that is, a
tissue in which the antigen is known to occur and
which has been fixed, processed, and labelled in
an identical manner. We are unaware of any
normal ocular tissue in which entactin has been
positively localised.

Fibronectin
This is a glycoprotein that exists in two forms;
soluble (plasma) and insoluble (tissue). Since it
binds to a number of biological macromolecules
including fibrin, collagen, and heparin, and to a
wide variety of cultured cells its main function is
believed to be a cell matrix ligand.[39] Fibronectin
is present in both loose connective tissue and
basement membranes but there is some debate as
to whether it should be considered an intrinsic
constituent or simply arising from plasma.[39] It is
abundant in developing tissue but is either absent
or present in low amounts in mature tissues.[40]

We did observe labelling for cellular fibronec-
tin in the iris stroma on ultrathin frozen sections
of both normal and exfoliative iris. There was no
direct association of fibronectin antibodies with
the stromal collagen fibrils and basement mem-
branes were free of label. Exfoliative fibres did
not exhibit labelling for fibronectin and we would
therefore agree with Schlotzer-Schrehardt et al[9]
that fibronectin is not a component of exfoliation
material.

EXTRACELLULAR MATRIX COMPONENTS

Vitronectin
This is a multifunctional glycoprotein that is
present in plasma (S-protein) and the extra-
cellular matrix of many tissues.[41 42] During
adolescence, vitronectin together with amyloid P
component, is localised with amorphous elastin
at the periphery of dermal elastic fibres.[43]
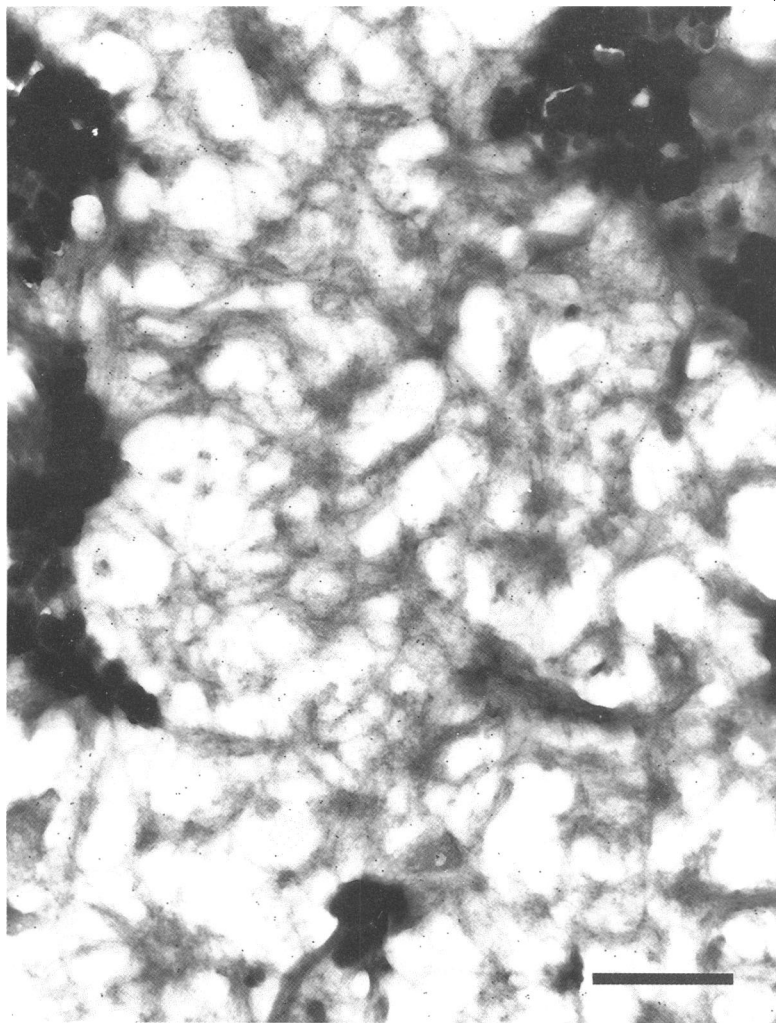It is absent from the skin of children before the

Figure 6 *Exfoliation fibres are labelled with antibodies against elastin associated glycoprotein (cryo, ×18 600, bar=1 μm).*

technique which at this stage has the numerous pitfalls that we have already outlined. The logical extension from the data already accumulated is to determine which of the candidate cells are involved in the synthesis of these components. This can be achieved by the application of in situ hybridisation which will identify messenger RNA for the end product proteins.

age of 6 and present in persons older than 13 years,[44] where it is thought to serve as a molecular link between elastic fibres and the surrounding collagen/proteoglycan scaffold.[43] Unlike fibronectin, vitronectin appears to be absent from basement membranes.[45] Experimental evidence suggests that vitronectin provides a unique regulatory link between cell adhesion, humoral defence mechanisms, and cell invasion.[43]

In spite of employing a number of fixation regimes and two tissue preparation techniques we were unable to localise vitronectin to any anatomical subcompartment in either normal or exfoliative iris. We were therefore unable to confirm the results of Schlotzer-Schrehardt *et al*,[9] who localised vitronectin to exfoliation deposits in eyelid skin, iris, and ciliary body. This discrepancy cannot be explained by differences in fixation – one of our fixation regimes was virtually identical to theirs – nor by differences in antibody specificity, as the vitronectin antibody was supplied by the same company (Boehringer, Mannheim). As with entactin, the status of vitronectin in normal and exfoliative iris can only be suitably resolved by incorporation of a suitable positive control. We are unaware of any normal ocular tissue in which vitronectin has been positively localised.

It is not surprising that contradictions have arisen in the early stages of application of a novel

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