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ULTRASTRUCTURAL CYTOCHEMISTRY OF ANCHORING FILAMENTS OF HUMAN LYMPHATIC CAPILLARIES AND THEIR RELATION TO ELASTIC FIBERS

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ABSTRACT

In previous studies "anchoring filaments" of human lymphatic capillaries have been shown to consist of microfibrils having histochemical and ultrastructural characteristics similar to elastin-associated microfibrils. When not associated with an elastin component, these microfibrils are referred to as "oxytalan microfibrils." In this study, α-glycol-containing carbohydrates and glycoconjugated sulfate groups, originating from sulphydryls and/or disulfide bridges, have been detected in anchoring filament microfibrils of human lymphatic capillaries by Thiery reaction (PA-TCH-SP) and "Hight Iron Diamine" cytochemical method (HID), respectively. Both of these chemical groups belong to the putative glycoprotein of which the microfibrils are constituted. Similar molecular characteristics have been demonstrated in elastic fiber microfibrils and oxytalan microfibrils of connective tissue.

These findings suggest a close molecular similarity among these different types of microfibrils. Thus, whatever their individual location or denomination (anchoring filaments, oxytalan fibers, or elastin-associated microfibrils), these microfibrils form a uniform population of fibrous elements. The findings further support a structural (and functional) continuity between the lymphatic capillary wall and the elastic network of adjacent connective tissues previously described and termed "Fibrillar Elastic Apparatus" (FEA). Of interest, endothelial cells also selectively react positively to the PA-TCH-SP and HID methods.

In previous studies, we have shown that the "anchoring filaments" of the lymphatic capillaries in different organs in man, consist of microfibril bundles having histochemical and ultrastructural characteristics similar to elastin-associated microfibrils (1). When unassociated with the amorphous elastin component, these microfibrils are referred to as "oxytalan microfibrils." Bundles of oxytalan microfibrils form filamentous structures with diameters up to 0.5μ in the connective tissue and are termed "oxytalan fibers" (2).

A third type of fiber, 0.5-2μ in diameter and intermediate between elastic and oxytalan fibers, has also been identified and designated as "elaunin fibers" (3). Elaunin fibers are characterized by approximately equal proportions of fibrillar and elastin components, and according to some investigators, represent developing or "young" elastic fibers (4).

From these histochemical and ultrastructural data, we have drawn two conclusions which bring together the multiple structural features into an organizational whole. On the one hand, the microfibrils of the anchoring filaments may be regarded as oxytalan microfibrils and, on the other hand, the anchoring filaments (or bundles of oxytalan microfibrils) may be considered in structural (and functional) continuity, via the elaunin fibers, with the elastic fibers. As shown previously, elastic fibers consistently surround lymphatic but not blood capillaries (5). Thus, the vessel wall of lymphatic capillaries is in close continuity with the elastic network of the connective tissue. This finding has led us (1)

to propose the existence of a perilymphatic "Fibrillar Elastic Apparatus" (FEA). This FEA consists of oxytalan fibers (or anchoring filaments) at the endothelial wall, then elaunin fibers, and externally, elastic fibers (1).

In the present study, using ultrastructural cytochemical methods, we have investigated, besides endothelial cells, the biochemical affinities between the microfibrils of the anchoring filaments, oxytalan fibers and elastic fibers. Our aim was to determine whether these microfibrils can be rightfully regarded as a single molecular and organizational entity.

MATERIALS AND METHODS

The study was performed on skin biopsy specimens taken from the lateral surface of the arm of 7 human subjects of both sexes in the 18-40 year age range. The skin samples were sliced into small fragments, some of which were fixed and treated by standard techniques for electron microscopy. Other fragments were fixed in 2% glutaraldehyde in 0.1M pH 7.2 cacodylate buffer for 2h at room temperature in preparation for "Thiery" method (PA-TCH-SP) staining of α-glycol-containing carbohydrates (6). Other fragments were fixed by the modified method of Karnovsky (7) (4% W/V paraformaldehyde, 0.5% V/V glutaraldehyde) in pH 7.3 cacodylate buffer for 2h at 4°C, in preparation for "high iron diamine" (HID) staining of glycoconjugated sulphate groups (8).

Thiery method

The fixed fragments were washed with cacodylate containing 7% sucrose, dried and embedded in Epon Araldite resin. Thin sections were placed on nickel grids and oxidized with 1% HIO_4 for 30 min. They were then washed with distilled water, treated with 2% thiocarbohydrazide for 40 min, and thoroughly washed with acetic acid at decreasing concentrations down to distilled water. The sections were then treated with 1% silver proteinate for 30 min in the dark and subsequently washed in distilled water without contrasting. Controls consisted of sections which had not been oxidized with HIO_4.

HID method

Some fixed fragments were washed with cacodylate and oxidized with 10% (w/v) oxone (a monopersulphate compound, $2KHSO_5 \cdot KHSO_4 \cdot K2SO_4$) for 60 min. Other fragments were not oxidized. All these fragments were then washed with water and treated for 18h at room temperature with HID solution prepared by adding 1.4ml 40% $FeCl_3$ to a fresh diamine solution containing 120mg N, N-dimethyl-m-phenylenediamine dihydrochloride and 20mg N, N-dimethyl-p-phenylenediamine monohydrochloride in 50ml distilled water. Some of these fragments were post-fixed with OsO_4. Subsequently the non post-fixed and post-fixed fragments were dried and embedded in London-White resin. Before observations, some thin sections were treated with 2% thiocarbohydrazide and then 1% silver proteinate for 30 min in the dark (TCH-SP) to enhance further the HID reaction products (9). Controls consisted of fragments incubated for 18h with $MgCl_2$.

The thin sections of both methods were observed with a Philips EM400 electron microscope. The observations pertain to the lymphatic capillaries of the papillary and subpapillary layers of the dermis.

RESULTS

Electron microscopy

The dermal lymphatic capillaries revealed characteristic ultrastructural features. The bundles of microfibrils of the anchoring filaments of the lymphatic capillaries projected from the abluminal membrane of the endothelial cells into the pericapillary connective tissue, as previously described (1,10,11). These microfibrils were 12-14nm in diameter and had transverse striations with a periodicity of 15-17nm. In transverse section they showed a central electron-transparent zone and a peripheral portion with 3-5 electron-dense subunits.

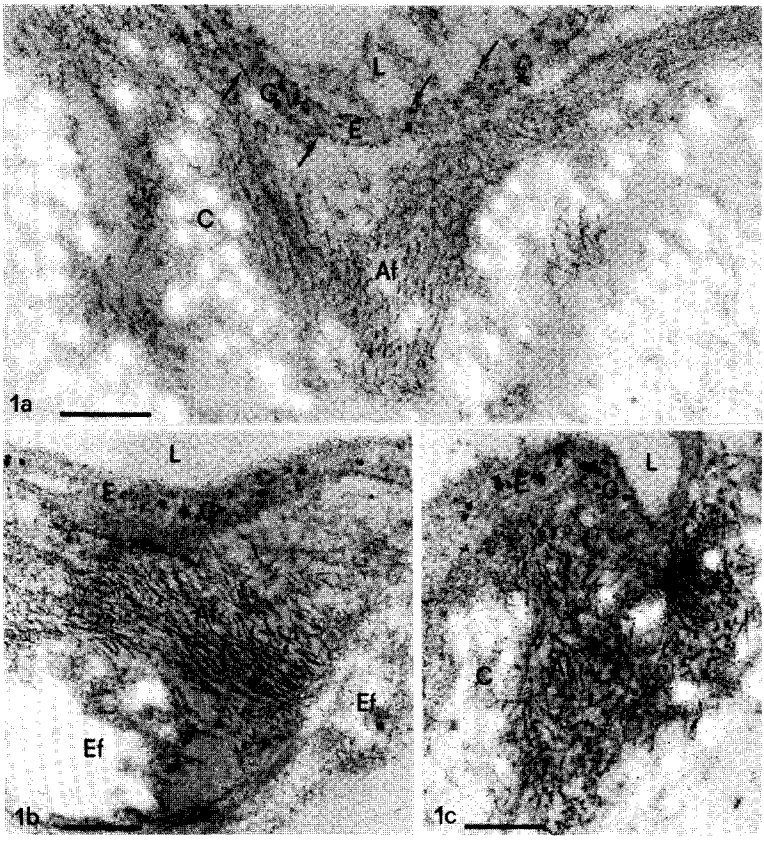

Fig. 1. PA-TCH-SP-stained lymphatic capillary. Unosmicated specimen without further counterstaining. ***a:*** *the PA-TCH-SP stains anchoring filament microfibrils (Af), endothelial membranes (arrows), cytoplasmic vesicles (*) and, intensely, glycogen granules (G). The extracellular matrix near collagen fibrils (C) appear weakly reactive.* ***b:*** *the anchoring filament microfibrils (Af), positive to reaction, converge on elastic fibers (Ef) from abluminal side of endothelium (E). The elastin component of elastic fibers appears sparsely PA-TCH-SP- stained.* ***c:*** *the anchoring filament microfibrils (Af), positive to reaction, are clearly distinguished from weakly positive material of extracellular matrix near collagen fibrils (c). E=endothelium; G=glycogen granules; L=lumen; Calibration bar=0.3μ.*

These ultrastructural features were similar to those of oxytalan, elaunin and elastic fiber microfibrils of the dermis.

Thiery method

The Thiery reaction (for α-glycol-containing carbohydrates) was strongly positive in the microfibrils of the anchoring filaments of lymphatic capillaries (*Fig. 1**a-c***). The reactivity was similar to that of oxytalan microfibrils located near fibroblasts (*Fig. 2*), of smooth muscle cells of small blood vessels in the dermis, and of oxytalan microfibrils at the dermo-epidermal junction. The same reaction was also observed in elastin-associated microfibrils (*Fig. 3*). Small electron-dense deposits along the microfibrils clearly distin-

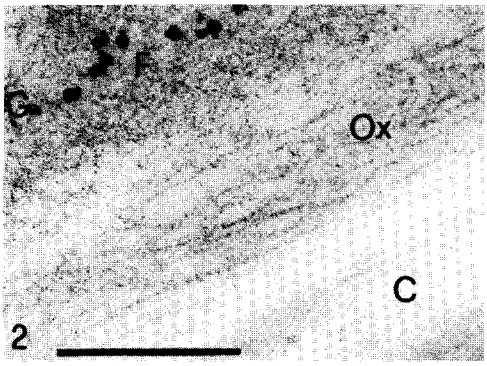

Fig. 2. PA-TCH-SP-stained fibroblast. Unosmicated specimen without further counterstaining. The PA-TCH-SP stains oxytalan microfibrils (Ox) close to a fibroblast (F). Glycogen granules(G) appear intensely stained. Longitudinally sectioned collagen fibrils (C) are stained very weakly. Calibration bar=0.3μ.

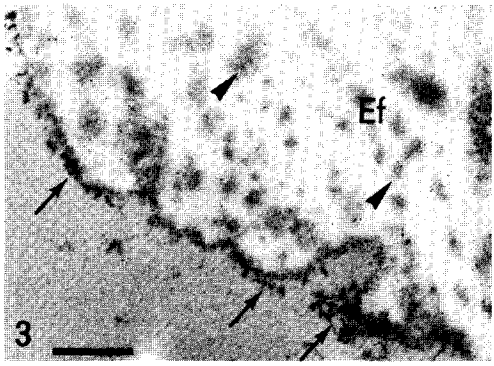

Fig. 3. PA-TCH-SP-stained elastic fiber. Unosmicated specimen without further counterstaining. In the transversely sectioned elastic fiber (EF), intense PA-TCH-SP staining on numerous peripheral microfibrils (arrows) and punctate PA-TCH-SP staining in interstices (arrowheads) of the lucent amorphous elastin are discernible. Calibration bar=0.3μ.

guished these fibrillar elements from the rest of the amorphous matrix. These deposits were also observed in the material of the extracellular matrix near collagen fibrils (*Fig. 1**a,c***). In the amorphous elastin of the elastic fibers, few granulations were observed (*Figs. 1**b**,3*).

The glycogen granules in the endothelial cells of the lymphatic capillaries showed very strong reactivity (*Fig. 1**a-c***). The reaction was also positive in the luminal membrane of the endothelial cells, and to a lesser extent in the abluminal membrane where the anchoring filaments were lacking. These membranes consisted of a succession of small, irregular, electron-dense granulations interposed between the cytoplasm and the extracellular

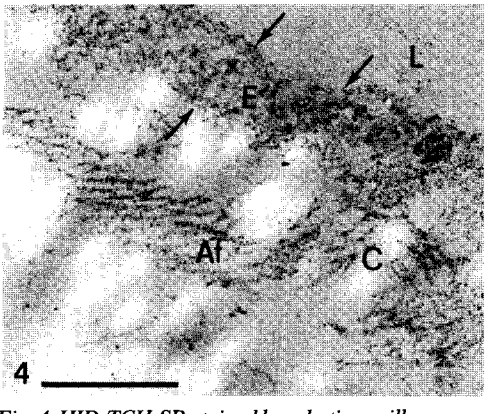

Fig. 4. HID-TCH-SP-stained lymphatic capillary. Oxone-oxidized, with postosmication specimen. The anchoring filament microfibrils (Af) and, sparsely, the endothelial membranes (arrows) appear positive to reaction. The extracellular matrix close to collagen fibrils (C) are weakly reactive. E=endothelium; L=lumen. Calibration bar=0.3μ.

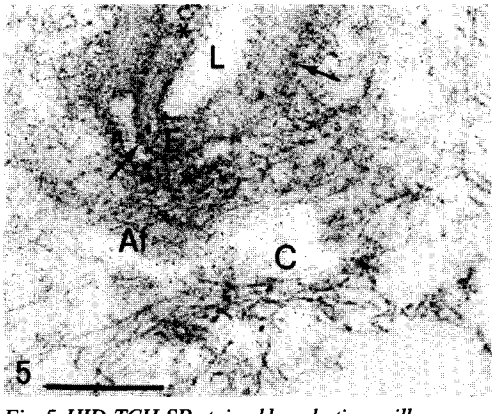

Fig. 5. HID-TCH-SP-stained lymphatic capillary ozone-oxidized, without postosmication specimen. Anchoring filament microfibrils (Af), positive to reaction, are in proximity to collagen fibrils (C) surrounded by material of the extracellular matrix with small stain deposits. Endothelial membranes (arrows) react irregularly, similarly to cytoplasmic vesicles (*). E=endothelium; L=lumen. Calibration bar=0.3μ.

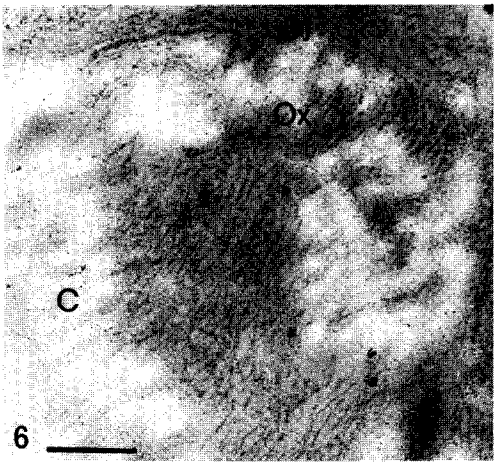

Fig. 6. HID-TCH-SP-stained oxytalan microfibrils. Oxone-oxidized, with postosmication specimen. Oxytalan fiber microfibrils (Ox) close to the dermo-epidermal junction (DJ) appear positive to reaction. Reactivity is also observed in the extracellular matrix closely associated with collagen fibrils (C). Calibration bar=0.3μ.

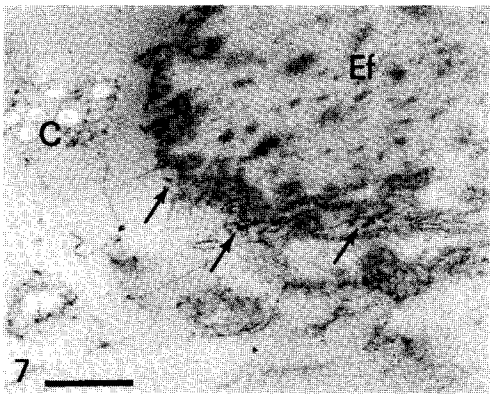

Fig. 7. HID-TCH-SP-stained elastic fiber. Oxone-oxidized, with postosmication specimen. Microfibrils (arrows) associated with elastic fiber (EF) appear positive to reaction, while the lucent amorphous elastin is sparsely reactive. Stain deposits are discernible close to collagen fibrils (C). Calibration bar=0.3μ.

environment (*Fig. 1a*). The membrane and content of certain cytoplasmic vesicles also reacted positively. The stain was more evident where the anchoring filaments joined the endothelial membrane. The intercellular junctions were particularly prominent due to the double row of reaction products of the contiguous plasmalemmal membranes.

In the unoxidized control sections, the cellular and extracellular structures did not show any significant reaction.

HID method

The HID method (for sulphate groups) stained the anchoring filaments of the lymphatic capillaries and the oxytalan fibers of the pericapillary connective tissue. The microfibrils of these elements showed a pattern of fine electron-dense granulations which stood out sharply against the amorphous extracellular matrix; the latter appeared as a uniform, weakly electron-dense background. The microfibrils of the elastic fibers also stained positively, and were seen to lie at the periphery and, to a lesser degree, within the amorphous elastin mass. The latter showed a weakly positive and sparse reaction.

A weak but diffuse reaction, in the form of dotted granulations, was observed in the extracellular matrix closely associated with collagen fibrils. In HID-TCH-SP stained preparations, the anchoring filaments of the lymphatic capillaries were strongly positive. Small regular electron-dense deposits were observed along the microfibrils (*Figs. 4,6,7*). Like the microfibrils associated with the elastic fibers, the oxytalan fibers stained in the same way as the anchoring filaments (*Figs. 6,7*). The elastin stained poorly. Electron-dense deposits were again observed close to collagen fibers (*Fig. 4*). In the preparations not post-fixed with OsO_4, the reaction products were less accentuated but more precise. The endothelial cell membranes reacted irregularly, similarly to cytoplasmic vesicles (*Fig. 5*). HID-TCH-SP stain deposits were often located at the abluminal side of endothelial cells close to anchoring filaments.

In the preparations not oxidized with oxone, the anchoring filaments, oxytalan fibers, microfibrils associated with elastic

fibers, and endothelial cell membranes did not significantly react. The elastin reacted negatively to the stain. The material of the extracellular matrix stained weakly near the collagen fibers. Control specimens incubated in $MgCl_2$, whether oxidized or not, lacked the staining in the sites described above.

DISCUSSION AND CONCLUSIONS

The present findings demonstrate that the microfibrils of the anchoring filaments have a similar cytochemical behavior to oxytalan microfibrils and the microfibrils of the elastic fibers in the dermis.

The Thiery method detects groups of polysaccharides which carry free -glycol and which are mainly linked in the carbon positions 1 and 4. The regular unbranched polysaccharide chain react strongly to oxidation, whereas the presence of branches reduces the number of α-glycol groups available. This method enables detection of glycoproteins and some proteoglycans (6). The Thiery reaction is strongly positive in the microfibrils of the anchoring filaments, in the oxytalan microfibrils near fibroblasts and near muscle cells of dermal vessels, in the dermoepidermal junction, and in microfibrils associated with elastic fibers.

These microfibrillar structures thus possess a similar and common reactivity which is of saccharide originmore precisely, of glycoprotein origin (6,12,13). Moreover, as indicated by the reaction mechanisms, the glycoprotein molecules probably contain regular and unbranched polysaccharide chains. The reactivity of the luminal and abluminal endothelial membranes originates from glycolipid and glycoprotein molecules in the lipid bilayer. On the basis of the chemical composition, the smallest granules seem to arise from the reactivity of glycolipid glucides, whereas the larger granules may arise from the more abundant glycoprotein glucides. The images indjcate that glycoproteins are more abundant in the luminal than the abluminal membrane. The punctate staining of elastin is presumably due to the embedded microfibrils. The more prominent reactivity of the sites where the anchoring microfibrils connect to the endothelial membrane suggests the presence of glycoproteins (fibronectin), presumably involved in the attachment of the proximal microfibrils of the FEA to the lymphatic capillary wall.

The reactivity observed between the collagen fibrils may, as previously suggested (14), be due to sugars linked to fibrils or/and to molecules of fibronectin. On the other hand, other findings suggest (15), that fibronectin molecules contribute in part to the reactivity of the oxytalan microfibrils and therefore of the microfibrils of FEA.

As the reaction mechanisms suggest, the HID and HID-TCH-SP methods reveal glycoconjugated sulphate groups. In the microfibrils, however, these groups are only seen after oxidation of the sections; in the unoxidized preparations, the reaction is negative.

Together the data suggest that in the molecular network of the microfibrils, the oxidative generation of sulphate groups arises largely from sulphydryl and/or disulfide groups, rather than from glycoconjugated sulphate groups existing as such and available for reaction. The endothelial cell membranes also behave in the same way.

The results confirm previous studies in which histochemical techniques based on the oxidation of sulphydryl or disulfide groups yield a positive reaction in the anchoring filaments of human and animal lymphatic capillaries (1,16). This reactivity has also been confirmed in the oxytalan fibers and in the microfibrillar component of elastic fibers. These findings are in line with the results of previous studies of oxytalan fibers of the monkey periodontal ligament (17) and microfibrils associated with the elastin of the perichondrium of rabbit elastic cartilage (18). Our results, moreover, are supported by other findings which show that the biochemical composition of the microfibrils associated with the elastic fibers contains a large proportion of cystein (48.2 residues/1000) (19,20). Furthermore, recent biochemical and immunological research has shown that these microfibrils con-

sist of glycoprotein subunits having a molecular weight of 340-350 KDa, rich in intrachain disulfide bridges but lacking sulphate groups (12,13).

The sparse staining of the elastin, as in the previous method, seems to depend on the reactivity of the embedded microfibrils. Endogenous positivity of elastin can be excluded since cytochemical studies (14) have shown that elastin lacks sulphate groups and is too poor in sulphydryl groups or disulfide bridges (4.1 residues/1000) to react (19,20).

The cytochemical reactions, in short, reveal that these microfibrils have glycoprotein-type organization and are rich in sulphydryl groups, disulfide bridges, or both. Such microfibrils, therefore, whatever their individual location or denomination (anchoring filaments, oxytalan fibers, or elastic fiber microfibrils), form a uniform population of fibrous structures. Furthermore, sulphydryl and/or disulfide bridges seem to be more abundant than sulphate groups in the endothelial cell membranes, especially in certain sites on the abluminal side where the anchoring filaments attach to the vessel wall. Overall the results tend to confirm further the close molecular similarity between the different types of microfibrils which we have previously described as an integral part of the "Fibrillar Elastic Apparatus" of the lymphatic capillaries.

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