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ULTRASTRUCTURAL LOCALIZATION OF ELASTIN-LIKE IMMUNOREACTIVITY IN THE EXTRACELLULAR MATRIX AROUND HUMAN SMALL LYMPHATIC VESSELS

P. Poggi, A. Casasco, C. Marchetti, M. Casasco, A. Cornaglia, A. Calligaro, C. Lethias, D.J. Hartmann

Institute of Human Anatomy (PP), Institute of Histology & Embryology (AC,CM,MC,AIC,AC), Pavia, Italy, and Centre de Radioanalyse, Institut Pasteur (CL,DJH), Lyon Cedex, France

ABSTRACT

The distribution of elastin-like immunoreactivity around small lymphatic vessels was investigated in three different human tissues (skin, heart and dental pulp) using high resolution immunocytochemistry. Quantitative assessment of the immunogold reaction was performed with an image analysis system. Intense and moderate elastin-immunoreactivity was detected in the extracellular matrix around small lymphatic vessels of the skin and heart, respectively. By contrast, absence of immunostaining was observed around lymphatic vessels in the dental pulp. Although the staining was mostly detectable on the non-fibrillar amorphous component of the extracellular matrix, some microfibrils were also immunostained in close proximity to the lymphatic vessel wall. These findings support the concept that small lymphatic vessels may be heterogeneous with respect to the composition of the extracellular matrix around their wall. The observation that it is possible to observe small lymphatic vessels displaying low or no elastin-immunoreactivity in the adjoining matrix militates against the hypothesis that elastic fibers play a pivotal role in the mechanisms that regulate the function of small lymphatic vessels.

The extracellular matrix (ECM) plays an important role in regulating the function of vascular endothelium in tissues (1). In order to clarify the possible involvement of ECM in lymph formation and drainage, many studies have addressed and characterized anchoring filaments and other components of the ECM that surround small lymphatic vessels (SLV)(2-5). The occurrence of elastic fibers around the lymphatic endothelial wall has been investigated in various organs using orcein staining (6-10). Recently, the possible existence of a *fibrillar elastic apparatus* around SLV has been proposed by Gerli et al (4). According to this study, such an apparatus consists of oxytalan fibers close to the endothelial wall, then elaunin fibers, and externally elastic fibers. Thus, it has been proposed that the wall of lymphatic capillaries in close continuity with the elastic network of the connective tissue and this *fibrillar elastic apparatus* shows similar characteristics in all organs (5). However our investigations on lymphatic vessels of various organs in humans have demonstrated some notable differences in the structure and composition of the fibrillar network surrounding the lymphatic endothelial wall (11).

Immunogold cytochemistry allows high resolution detection of antigens in the ECM and a semi-quantitative evaluation of the immunocytochemical detection which is not possible using light microscopy immunostaining or immunoperoxidase electron microscopy (12-14). To clarify the arrange-

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ment of the fibrillar network around SLV, we investigated the distribution of elastin-like immunoreactivity in the ECM around SLV in different human tissues using high resolution immunogold cytochemistry.

MATERIALS AND METHODS

Electron microscopic immunocytochemistry

Samples of human tissues (skin, heart and dental pulp) were obtained from adult subjects of both sexes according to the ethical standards of the S. Matteo Hospital, Pavia, in which they were collected. Skin samples (n=10) were obtained from skin biopsies of lower legs from healthy subjects. Heart samples (n=5) were obtained from normal donor hearts which could not be used for transplantation because of recipient problems. Dental pulp was obtained from healthy teeth (n=10) extracted under local anesthesia for orthodontic purposes.

Tissue samples were immediately immersed in a solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 6 h. They were then post-fixed for 1 h in osmium tetroxide (1.33%) in 0.1 M collidine buffer. The tissues were dehydrated through graded alcohols and embedded in Epon 812.

The morphological identification of SLV was accomplished in semithin sections stained with toluidine blue, as described previously (8,14).

Ultrathin sections containing SLV were collected on uncoated nickel grids. The high resolution protein A-gold immunocytochemical technique was performed according to Bendayan and Zollinger (15). Briefly, sections were incubated for 30 min at room temperature in saturated aqueous solution of sodium metaperiodate, a strong oxidizing agent that unmasks antigenic sites in osmium-fixed tissues. After washing in 0.05 M *Tris* buffer, pH 7.6, containing 0.1 mol/l NaCl (0.15 M *Tris* buffered saline, TBS), the grids were incubated in 10% normal goat serum solution in TBS for 1 h and then transferred on a drop of rabbit antiserum anti-elastin, diluted 1:1000 in TBS containing 1% bovine serum albumin (TBS-BSA), overnight at 4°C. Preparation, purification and immunochemical characterization of the polyclonal antibodies to elastin have been described previously (16). The polyclonal antiserum cross-reacts with multiple isoforms of elastin. After thorough rinsing in TBS-BSA, the sites of reaction of the primary antiserum were revealed by incubation with protein A coupled with 15 nm-gold particles (Biocell Research Laboratories, Cardiff, U.K.), diluted 1:30 in TBS-BSA, for 1 h at room temperature. The sections were then washed in TBS, rinsed in distilled water, dried and finally counterstained with uranyl acetate and lead citrate. Immunostained sections were examined in a Zeiss EM10 transmission electron microscope.

Controls of the immunocytochemical reaction included: 1) incubation of sections in the primary elastin-antiserum which had been absorbed (liquid phase) with corresponding and inappropriate antigens; 2) incubation of sections with elastase (5 mg/ml in 0.5 M Tris buffer, pH 8.8) for 1 h at room temperature; 3) omission of the primary antisera; 4) substitution of the primary antisera with non-immune goat, rabbit and mouse sera; 5) substitution of corresponding gold-tagged probes with inappropriate gold conjugates (13).

With the exception of primary elastin antiserum and protein A-gold conjugates, all reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Morphometric evaluation of the immunogold reaction

Quantitative evaluations of the immunolabeling was performed as described previously (12,14,18) and expressed as the number of gold particles per square micrometer. The protocol of tissue fixation and embedding resulted in good ultrastructural

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morphology, thus permitting a precise localization of gold particles over intra- and extracellular structures. At least 3 samples of each tissue (skin, heart and dental pulp) were examined. Within each sample, sections obtained at 3 different levels were immunostained. At least 3 different SLV, each one cut at different levels, were studied within each sample. Electron micrographs were taken from randomly-selected fields of immunostained sections (magnification 12,500 and 20,000x) by two different observers (PP, AC). Density of immunolabeling was evaluated within at least 9 micrographs of each samples. The following intracellular and extracellular compartments were examined: nuclei of endothelial cells and fibroblasts, lymphatic vessel lumen, collagen and perivascular area. Perivascular area was arbitrarily assumed as the area of the extracellular matrix within 1 μm from the abluminal side of the lymphatic vessel endothelium.

Quantitative assessment of the number of gold particles was performed on electron micrograph negatives using an automated-interactive computerized image analysis system (Kontron-Zeiss IBAS II). The image of the negatives, through a high geometric linearity TV camera, was displayed on a TV color monitor with a resolution of 512 x 512 pixels^2. Images were first stored in the image memory and then processed by the image analyzer. A window of 1 square micrometer was applied over different intracellular and extracellular compartments, thus allowing the measurement of gold particles per μm^2.

The data were stored in a floppy disk and submitted to basic statistical evaluation (mean value and standard error) with an IBM computer.

RESULTS

SLV were characterized by a thin and irregular wall *(Figs. 1,2)*. Lymphatic endothelial cells showed scanty cytoplasm containing few organelles. Close to the nucleus, some ribosomes, mitochondria, Weibel-Palade bodies and a Golgi apparatus could be observed. Plasmalemmal and pinocytotic vesicles were a conspicuous feature of lymphatic endothelial cells *(Figs. 1,2)*. The ECM intimately connected to the abluminal side of the endothelial wall was mainly composed of numerous thin filaments showing no cross striations (the so-called *"anchoring filaments"*). Striated collagen fibers were not detectable in close proximity to the endothelial wall. Discontinuities in the basal lamina were consistently observed in the basement membrane around SLV *(Figs. 1,2)*.

The sites of elastin-like immunoreactivity were revealed at the electron microscopical level by the labeling with 15 nm-gold particles. Although immunoreactivity was mainly observed on the amorphous component of the elastic fibers, gold particles could also be observed on elastin-associated microfibrils *(Fig. 2)*.

In the skin, elastin-like immunoreactivity was continuous and intense all around the wall of lymphatic vessels *(Figs. 1A, 2A,B)*.

In the heart, the ECM around SLV showed weak and discontinuous elastin-immunoreactivity; rare and small clusters of gold particles were detectable around the vessel wall *(Figs. 1B,2C,D)*.

In the dental pulp, no immunoreactivity could be detected around SLV nor elsewhere in the extracellular matrix *(Fig. 1C)*.

Significant differences in the density of immunolabeling were measured in the perivascular areas of SLV from different tissues examined *(Table 1)*.

Although the possible occurrence of specific elastin-immunoreactivity within *any* cellular or extracellular compartment cannot not be excluded *a priori*, it is reasonable to presume that the immunostaining observed over vessel lumen, nuclei and collagen fibers represents non-specific *background* staining. The measurement of the density of gold-particles over these structures served as a control of the immunocytochemical reaction.

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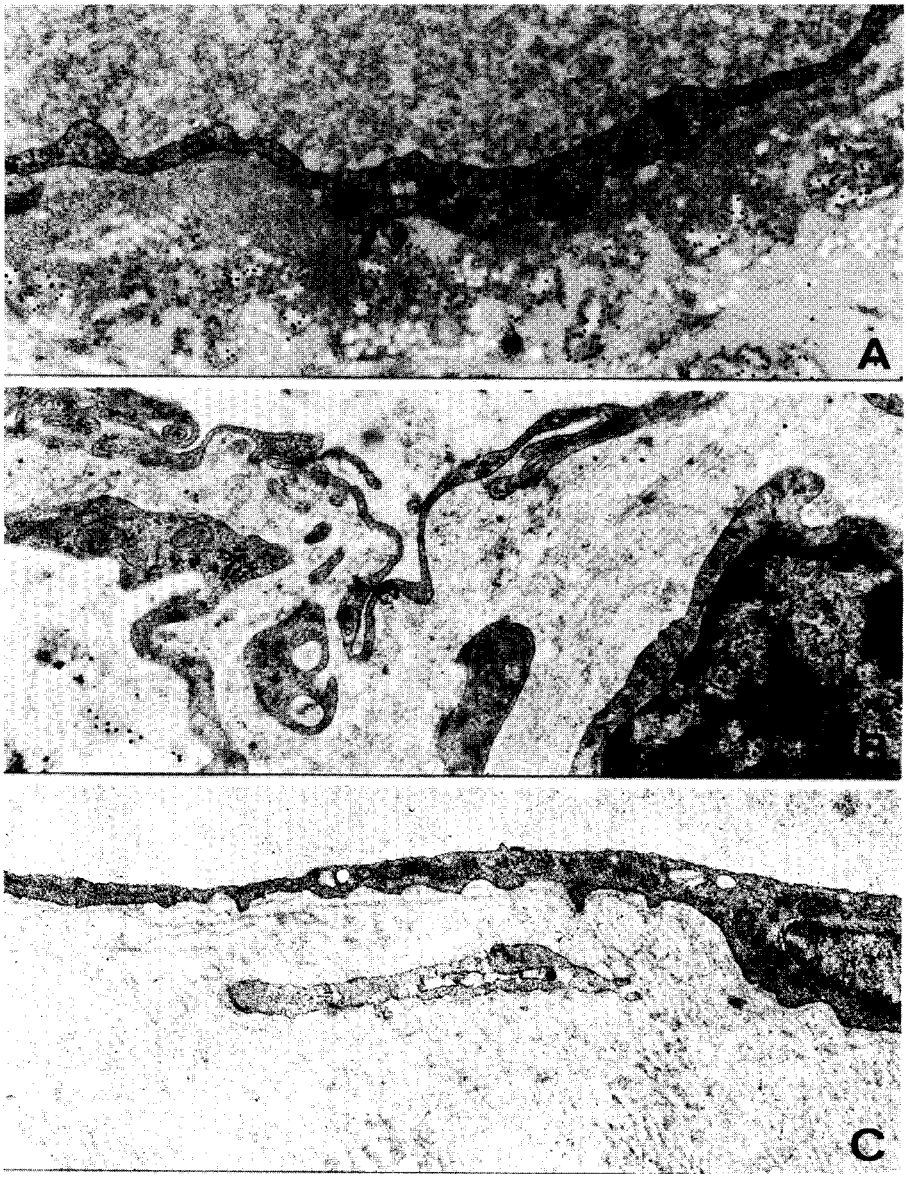

Fig. 1. Immunogold identification of elastin-like immunoreactivity around small lymphatic vessels in human tissues. Skin (A): intense immunoreactivity is continuous all around the vessel wall. Heart (B): weak and discontinuous immunoreactivity is visible in the perivascular area. Dental pulp (C): no immunoreactivity can be detected in the extracellular matrix around the lymphatic vessel. x15,000.

DISCUSSION

Many studies have focused on the so-called *anchoring filaments* that mediate the relationships between SLV and the connective tissue matrix (2,3,19-21). In this regard, the distribution of elastic fibers around lymph vessels have been previously investigated using histochemical methods (4-6,10). In this study we have employed high resolution immunocytochemistry to detect elastin-immunoreactivity around human SLV. Previously, the same technique has been used to identify at the electron microscopical level elastin-immunoreactivity around blood vessels (17,22). This technique, besides being highly specific, allows the evaluation of the immunostaining, provided that standard procedures have been applied in tissue processing and staining.

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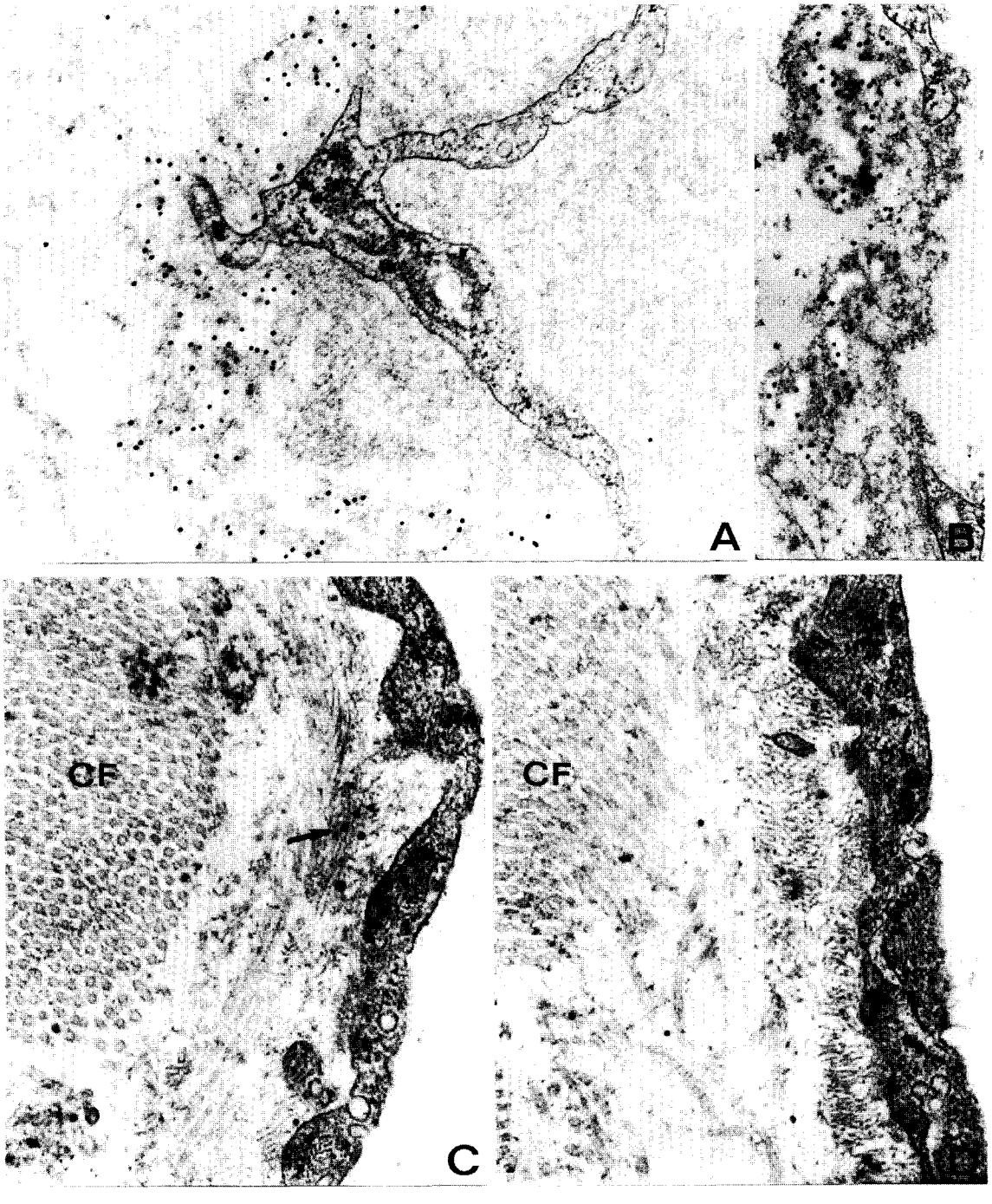

Fig. 2. Immunogold identification of elastin-like immunoreactivity around small lymphatic vessels in human skin (A,B) and heart (C,D). The immunoreactivity is detectable in the extracellular matrix close to the lymphatic vessel wall (A,C,D) as well as in the matrix adjoining the so-called open junctions between the endothelial cells (B). Although immunoreactivity is mainly detectable on the amorphous component of the elastic fibers (B), some small microfibrils close to the endothelial wall are also immunolabeled (C, arrow). Note absence of specific immunostaining on collagen fibers (CF). A,B: x33,200; C,D: x44,200.

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TABLE 1
Density of Labeling* With Anti-Elastin/Protein A-Gold Over Intracellular and Extracellular Compartments in Human Skin (Dermis), Heart and Dental Pulp.

	vessel lumen	nucleus[a]	collagen fibers[b]	perivascular area[c]
Skin	1.1±0.42	0.93±0.33	1.25±0.7	30.64±4.69
Heart	0.4±0.15	0.80±0.28	0.71±0.3	3.6±0.58
Dental Pulp	0.8±0.6	0.25±0.2	0.8±0.4	0.6±0.4

*Labeling is expressed as the number of gold particles per μm^2; values are means ± standard error
[a]Non-specific nuclear labeling was measured over the nuclei of randomly-selected fibroblasts and endothelial cells.
[b]Collagen fibers were identified as striated fibers with a periodicity of about 64nm
[c]Perivascular area was arbitrarily assumed as the area of the extracellular matrix within 1µm from the abluminal side of the lymphatic vessel endothelium. This area contains a discontinuous basal lamina and associated fibrillar structures.

The occurrence of elastin-like immunoreactive material all around the wall of skin SLV is in agreement with the data reported by Gerli et al (4). Nevertheless, the distribution as well as the intensity of elastin-like immunoreactivity around SLV was found to be different in three different human tissues, viz. skin, heart and dental pulp. Specifically, no immunostaining could be detected in the extracellular matrix around dental lymphatic vessels. Our results thus suggest that SLV may be heterogeneous with respect to the composition of the extracellular matrix around their wall. On the other hand, elastic fibers consistently surround lymphatic vessels, but not blood vessels, in the skin (4,23). The variable amount of elastin-immunoreactivity around these vessels in different tissues, however, makes elastin an unreliable *marker* of SLV. Moreover, the observation that it is possible to observe SLV displaying low or no elastin-immunoreactivity in the adjoining matrix argues against the hypothesis that elastic fibers play a pivotal role in the mechanisms that regulate the function of lymphatic vessels.

Elastic fibers are composed of either amorphous material or fibrillar components. Although in our samples the immunostaining is most detectable on the non-fibrillar amorphous component of the ECM, some microfibrils are also immunostained. This observation is in agreement with previous immunocytochemical studies regarding elastogenesis in developing tissues (22,24-26). During early stages of elastogenesis, elastin-like immunoreactivity is first detectable in microfibrils, whereas the amorphous component is absent. Once amorphous material has accumulated in the later stages of elastogenesis, elastin-like immunoreactivity is mainly observed in such material, whereas the adjoining microfibrils are poorly labeled. We are unclear whether immunostained fibrils observed in our specimens represent immature elastic fibers, referred to as elaunin or oxytalan fibers, that have been previously described around SLV (5).

In conclusion, this study demonstrates that elastic fibers are not uniformly present in the ECM that surrounds human SLV and supports the view that SLV may be heterogeneous with respect to the ECM around their wall. We believe that further studies should be addressed to clarify the biochemical nature of fine anchoring filaments that are tightly associated with the lymphatic wall.

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Prof. Paola Poggi
Istituto di Anatomia Umana
via Forlanini, 8
1-27100 Pavia (Italy)

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