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THE PATTERN OF FIBRILLIN DEPOSITION CORRELATES WITH MICROFIBRIL-ASSOCIATED GLYCOPROTEIN 1 (MAGP-1) EXPRESSION IN CULTURED BLOOD AND LYMPHATIC ENDOTHELIAL CELLS

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ABSTRACT

Fibrillins constitute the major structural components of 10-12nm microfibrils of the extracellular matrix of several elastic and non elastic tissues and of initial lymphatic vessel anchoring filaments. Microfibril-Associated Glycoprotein-1 (MAGP-1) binds fibrillin to tropoelastin during elastogenesis. We recently reported that cultured blood endothelial cells deposit fibrillin in a honeycomb pattern, whereas lymphatic endothelial cells form an irregular web. The aim of this immunohistochemical study was to verify whether the deposition pattern of fibrillin is related to the expression of MAGP-1 in confluent and post-confluent cultures of bovine aortic (AEC), pulmonary artery (PAEC) and lymphatic endothelial cells (LEC). In AEC and PAEC, MAGP-1 and fibrillin co-localized and their deposition increased with time in culture. In AEC, both proteins formed a honeycomb pattern. In LEC, MAGP-1 deposition was still negligible when fibrillin formed an irregular web covering the entire surface. PAEC, which in vivo are exposed to physiological conditions intermediate between AEC and LEC, had an intermediate pattern of deposition of fibrillin and MAGP-1. Assuming that early elastogenesis is an intrinsic functional need for the aorta, but not for the thoracic duct, we propose that delayed appearance of MAGP-1 in LEC may correlate with their irregular fibrillin deposition. Different fibrillin scaffolds could in turn account for the specificity of elastic fibers in compliance with the specific functional requirements of the tissue.

Fibrillins are large glycoproteins of about 350 kDa with a high cysteine content, which constitute the major structural components of 10-12 nm microfibrils of the extracellular matrix. There are two isoforms of fibrillin: fibrillin-1 (1) and fibrillin-2 (2,3), encoded by different genes. The two isoforms have significant similarities in primary sequence and predicted protein domains, but differ in a region near the aminoterminal domain which is rich in proline in fibrillin-1 and glycine in fibrillin 2. Fibrillins have a multidomain structure comprising 47 epidermal growth factor-like domains, 43 of which are of the calcium binding type, interspersed with 8 cysteine motifs with homology to the transforming growth factor ß binding protein. Fibrillin 1 and 2 both contain an RGD sequence that may bind $\alpha_v\beta_3$ integrins (4). Fibrillin-2 has an additional RGD site (3) which is, however, unavailable for cell-matrix interactions (5).

Isolated fibrillin-containing microfibrils have a "beads on a string appearance" with a periodicity of 50-56 nm. The globular beads are connected by bundles of linear arms (6, 7). Fibrillin molecules are secreted as monomers by several types of cells: fibroblasts (1), smooth muscle cells (8), osteoblasts (9), chondroblasts (10), keratinocytes (11) and

endothelial cells from blood and lymphatic vessels (12). In the pericellular space they polymerize by head-to-tail alignment and undergo conformational maturation that presumably renders the molecule extensible (7). The next step is assemblage with other molecules onto thread-like filaments which in turn give rise to macroaggregates with or without elastin (5).

Fibrillin-containing microfibrils are distributed in a wide variety of tissues and organs, either associated with elastin in elastic fibers or as elastin-free bundles (the latter are also known as oxytalan fibers). Elastin-associated microfibrils are found in elastic tissues, subjected to stretching and expansible forces, where they form the external coating of elastic fibers. They are thought to act as a scaffold for tropoelastin deposition and alignment. Both fibrillins guide elastogenesis. Indeed microfibrils appear before tropoelastin deposition, however they intervene sequentially: fibrillin-2 is presumably involved in morphogenesis of elastic fibers during development since it appears before elastogenesis, whereas fibrillin-1 is probably involved in the homeostasis of elastic fibers throughout life (13).

Microfibrils devoid of elastin are found in elastic and non elastic tissues, especially in those subjected to mechanical stress. In elastic tissues, they connect elastic fibers among themselves and to other structural and cellular components (5); in non-elastic tissues, like ciliary zonules (14), they have an anchoring function.

We previously used immunohistochemical techniques (15) to show that the microfibrils of anchoring filaments of human skin initial lymphatic vessels, contain fibrillin. We also recently reported that fibrillin deposition differs in cultured blood and lymphatic endothelial cells (12). Blood endothelial cells deposit fibrillin in a honeycomb pattern with empty spaces through which endothelial cells are visible; in lymphatic endothelial cells fibrillin covers the entire monolayer forming a thick, irregular web. Looking for similar findings in literature, we noticed a deposition pattern of fibrillin resembling that of lymphatic endothelial cells in fetal bovine chondroblasts (16) and a honeycomb pattern resembling that of blood endothelial cells in immortalized ciliary body pigmented epithelial cells. Interestingly, the authors, who do not mention this morphology, report that the expression of microfibril-associated glycoprotein-1 (MAGP-1) was much lower in fetal bovine chondroblasts than in ciliary body pigmented epithelial cells (16).

MAGPs, originally identified in bovine nuchal ligament, are a family of small structurally related glycoproteins that include two molecules specifically associated with fibrillin-containing microfibrils: MAGP-1, 31-kDa, and MAGP-2, 25-kDa (17-19). These molecules undergo posttranslational modifications that may influence their interaction with microfibrillar proteins. It has been shown that MAGP-1 is covalently bound by disulfide bonds to fibrillin beads (19) and that its N-terminus may also bind the C-terminus of tropoelastin (20,21). Binding of MAGP-1 to tropoelastin may serve during development to guide elastogenesis in relation to the functional requirements of the tissue and its exposure to stress and, at more mature stages, to stabilize the interactions between microfibrils and the elastic core of elastin (22). MAGP-2 has an $\alpha_v\beta_3$-integrin-binding RGD motif near the N terminus. This raises the possibility that MAGP-2 may play a unique function, within microfibrils, as a molecular bridge between microfibrils and the cell surface (22).

The aim of this study was to verify whether differences in MAGP-1 production might relate to the different fibrillin deposition patterns of cultured bovine aortic (AEC) and lymphatic (LEC) endothelial cells. Pulmonary artery endothelial cells (PAEC) were also investigated under the assumption that their deposition pattern of fibrillin and MAGP-1 production might have intermediate characteristics between those of AEC and LEC. The pulmonary artery is a low pressure vessel with the structure of an artery. Its

endothelium is exposed to intraluminal systolic pressure, much lower than that of arteries but higher than that of lymphatic vessels in the absence of obstruction to lymph flow. We provide evidence that differences in MAGP-1 do correlate with differences in the deposition pattern of fibrillin.

MATERIALS AND METHODS

Cell Cultures

All cells were bovine: LEC were obtained from the thoracic duct (23), AEC from the thoracic aorta and PAEC from the pulmonary trunk and left and right pulmonary arteries. Cells were detached by enzyme digestion with 0.05% collagenase type 2 (Worthington, Lakewood, NJ, USA, 239 U/mg) and seeded into four-well Nunc plates (1.5 cm diameter), containing round glass cover slips coated with 0.1% gelatin (Difco, Detroit, Michigan, USA, in phosphate buffered saline, PBS, without calcium and magnesium). Culture medium was Dulbecco's modified Eagle's MEM (DMEM, GIBCO, Life Technologies Ltd, Paisley, Scotland, UK) containing 20% fetal bovine serum (FBS, GIBCO), 100 µg/ml endothelial cell growth supplement (ECGS, Sigma, St. Louis, MO, USA), 100 µg/ml kanamycin (Sigma) and 2 mM glutamine (Sigma). FBS was reduced to 10% the day before confluence, and to 2% the next day to minimize outgrowth of contaminating smooth muscle cells. For the same reason, ECGS was no longer added to culture medium as soon as cells reached confluence (24). Fibrillin and MAGP-1 distribution was evaluated in ex vivo cells (freshly isolated) and in primary culture cells at confluence and at days 2 and 4 after confluence, since it is known that fibrillin deposition progressively increases after confluence (25).

Immunohistochemistry

Cultures were washed in PBS containing 0.5% bovine serum albumin (BSA), fixed for 5 minutes in acetone at -20°C and washed again extensively. Unspecific binding sites were blocked with 3% BSA in PBS. Cells were then incubated overnight at 4°C either with a monoclonal antibody to fibrillin (Chemicon, MAb 1919) or a polyclonal antibody to MAGP-1 (EPC Elastin Products Company, Owensville, Missouri U.S.A.) diluted 1:50 and 1:100 respectively in PBS containing 0.5% BSA. After incubation with the primary antibody, cells were washed and incubated for 2 h with fluorescein isothiocyanate–labeled goat anti- mouse or anti-rabbit IgG secondary antibodies (Sigma) diluted 1:100 in PBS with 0.5% BSA, washed again and mounted upside-down on glass slides with Vectashield mounting media (Vector). Controls were obtained omitting the primary antibodies.

Double immunolabeling was also performed on cells at day 4 after confluence. After fixation and blockade of unspecific binding sites, cells were incubated overnight at 4°C with the polyclonal antibody to MAGP-1, then washed and incubated with a goat anti-rabbit FITC-conjugated secondary antibody for 2 hours at room temperature. After thorough washing, unspecific binding sites were blocked with 3% BSA and 5% goat serum in PBS for 40 min., then the cells were incubated for 2 hours at room temperature with the monoclonal antibody to fibrillin, after which they were washed and incubated for 2h at room temperature with a rabbit anti-mouse TRITC-conjugated secondary antibody.

Quantitative Analysis

MAGP-1 deposition was evaluated with a morphometric program, "Lucia G," by Nikon, on at least 500 cells/condition. The amount of MAGP-1 deposited was expressed as Integral Density (I.D.) which is the sum of individual optical densities of each pixel in the area being measured. In this program, Optical Density (O.D.), the negative logarithm of the transmittance is evaluated

according to the formula: O.D. = -log((Pixel Gray Value+ 0.5)/ 62.5).

The program automatically selected positive areas and calculated their integral density. The data were corrected for the percentage of the field covered by the positive area.

RESULTS

Immunohistochemistry

Fibrillin deposition

We failed to detect fibrillin in ex vivo AEC, LEC and PAEC (data not shown). In culture, fibrillin deposition increased as a function of time in all cell types. At day 4 after confluence of AEC *(Fig. 1a)* fibrillin formed a honeycomb pattern of filaments arranged in strands surrounding fibrillin-free spaces. Fibrillin deposition in LEC was less regular: fibrillin microfibrils arranged in filaments of different size that eventually formed a sort of web covering the cell monolayer *(Fig. 1b)*. Cultured PAEC produced fibrillin with a deposition pattern which was intermediate between that of AEC and LEC *(Fig. 1c)*: in some areas filament bundles of various dimensions formed a web covering the culture like in LEC, in other areas of the same culture, fibrillin filaments formed a honeycomb pattern with thick fibrillin microfibrils surrounding fibrillin-free spaces as in AEC.

MAGP-1 deposition

In AEC, MAGP-1 deposition mimicked that of fibrillin. At confluence MAGP-1 positive thin filaments could be detected in some areas of the culture *(Fig. 2a)*. MAGP-1 deposition increased gradually with time and eventually, 4 days after confluence, a honeycomb pattern was formed *(Fig. 2b)*. Through MAGP-1 free spaces in the wide mesh of MAGP-1 positive strands, the cell monolayer could still be recognized. Some of these spaces

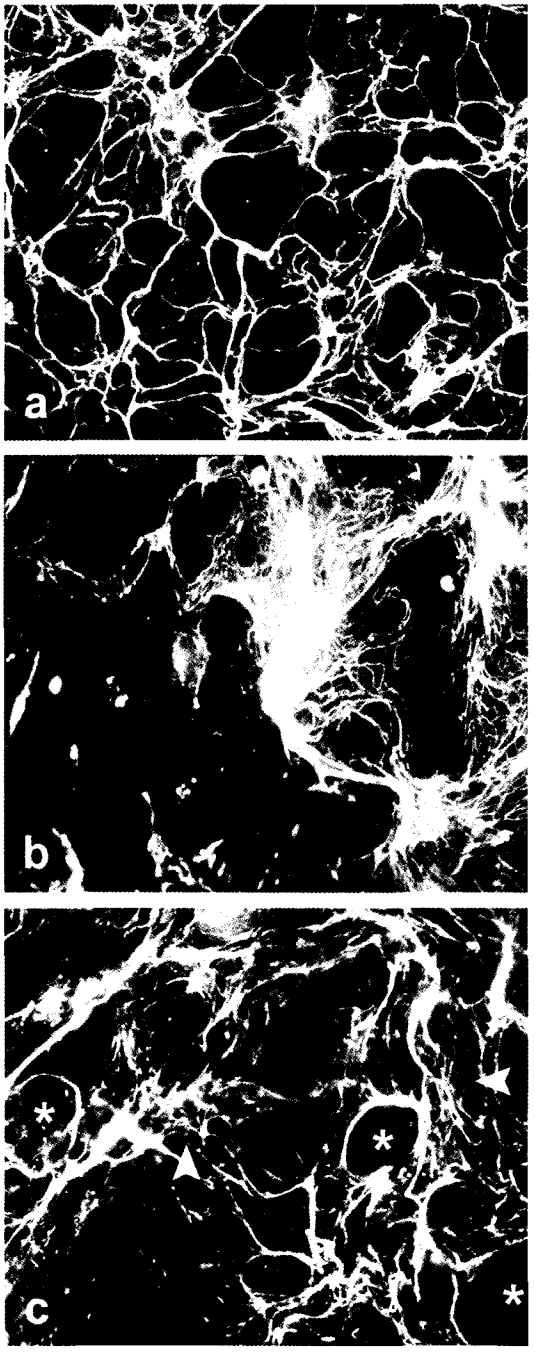

Fig.1. Fibrillin deposition at day 4 after confluence. a) AEC deposit fibrillin in a honeycomb pattern; b) LEC in an irregular web; c) PAEC with an intermediate pattern between AEC and LEC: honeycomb pattern (*) and irregular web (arrowheads). Orig. mag. x40.

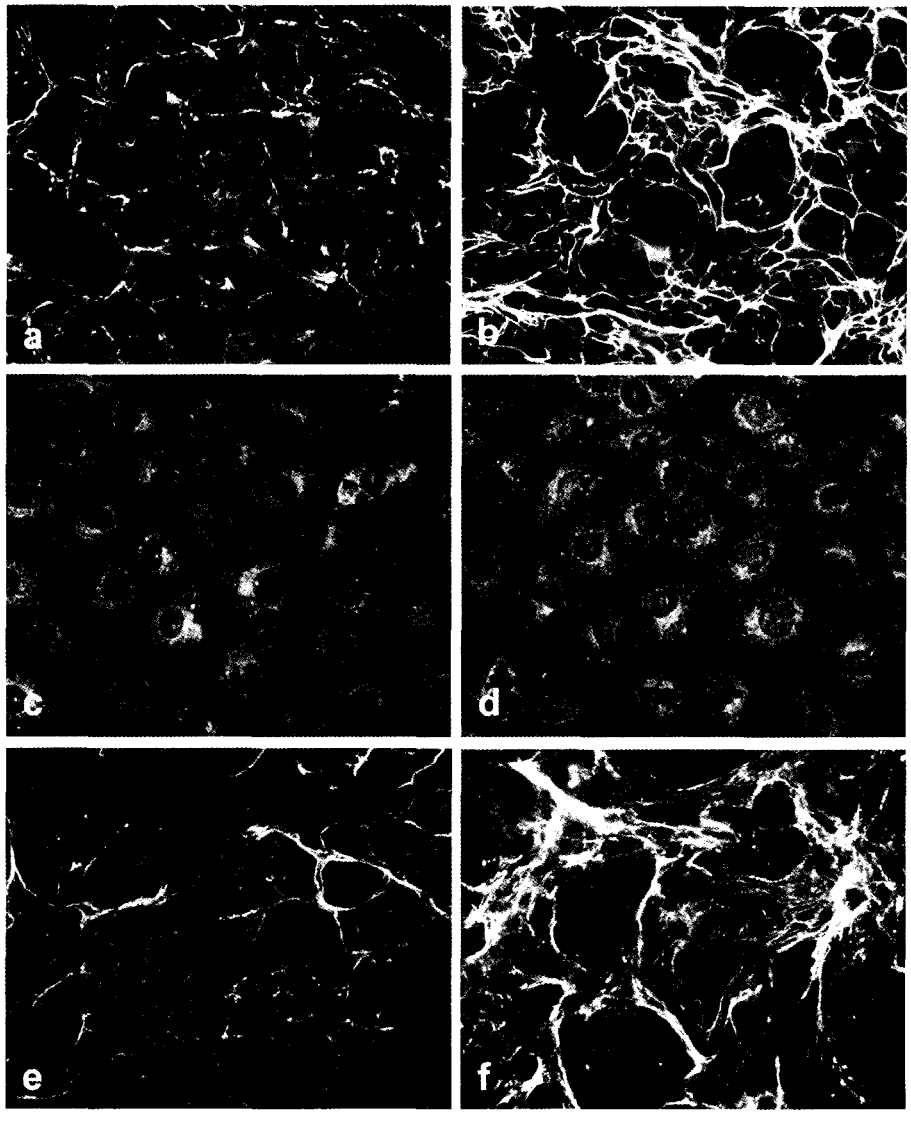

Fig. 2. MAGP-1 deposition in AEC (a and b), LEC (c and d) and PAEC (e and f) at confluence (a, c and e) and at day 4 after confluence (b, d and f). MAGP-1 deposition mimics that of fibrillin in AEC (a and b) and PAEC (e and f), whereas LEC do not deposit MAGP-1 either at confluence (c) or 4 days later (d). Honeycomb pattern (*) and irregular web (arrowheads). Orig. mag. x40.

were further divided into a closer mesh by finer filaments sprouting from the wider mesh, creating a complex mesh structure.

PAEC also deposited MAGP-1 with a pattern resembling that of fibrillin. At confluence MAGP-1 was only detected in some areas of the culture *(Fig. 2e)*. In these areas, MAGP-1 was deposited as thin filaments and thicker strands. Some meshes were formed, but thin filaments also formed a delicate web. At day 4 after confluence *(Fig. 2f)* some wide meshes including MAGP-1 free spaces, like in AEC, were formed. These meshes alternated with the web-like deposition characteristic of LEC.

No MAGP-1 was ever detected in

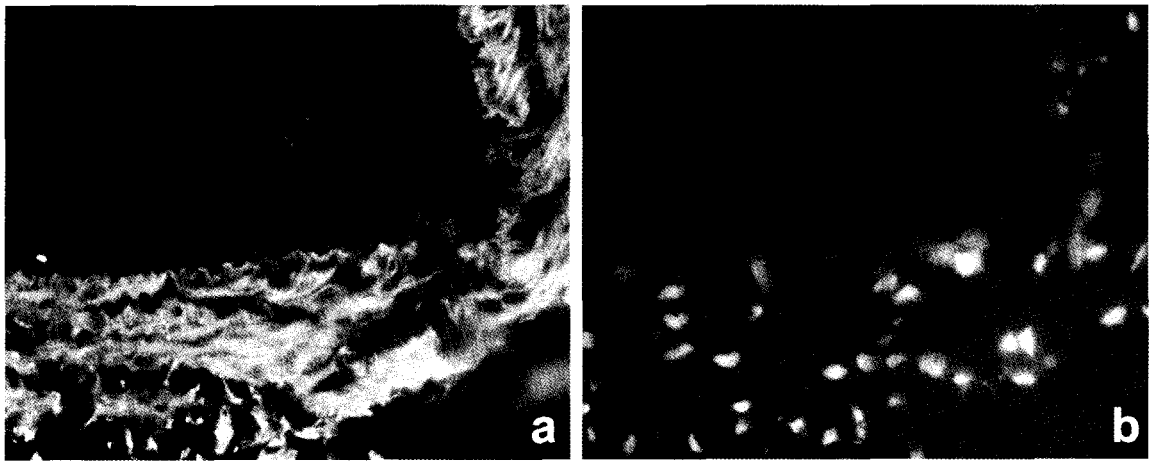

Fig. 3. A cryostat section of bovine thoracic duct: a) the endothelium is labeled by the polyclonal antibody to MAGP-1, b) nuclei are stained with Hoechst solution. Orig. mag. x40.

cultured LEC at confluence *(Fig. 2c)*. In most cases there was still no detectable MAGP-1 at day 4 after confluence *(Fig. 2d)*. In three cases out of eight, a few small areas containing some MAGP-1 positive microfibrils were found 4 days after confluence in correspondence with the thickest strands of fibrillin, although most of the coverslip was still MAGP-1 negative. Fibrillin, by this time, was abundant and present all over the culture. To verify whether MAGP-1 is expressed in vivo by lymphatic endothelium, we stained cryostat sections of bovine thoracic duct with the antibody to MAGP-1. The endothelium of bovine thoracic duct was labeled by the antibody *(Fig. 3)*.

Double labeling

There was perfect co-localization of fibrillin and MAGP-1 in AEC *(Figs. 4a and b)* and PAEC *(Figs. 4e and f)*, whereas most of LEC cultures *(Figs. 4c and d)* stained only for fibrillin. When MAGP-1 was detected in LEC cultures, it co-localized with the thicker strands of fibrillin.

Quantitative Analysis

Quantitative analysis of integral density *(Fig. 5)* showed that MAGP-1 production significantly increased from confluence to day 4 in AEC and PAEC ($p<0.001$). There was no significant quantitative difference between MAGP-1 production in AEC and PAEC either at confluence or on day 4.

DISCUSSION

The role of fibrillin and MAGPs in tropoelastin assemblage, as evinced from the literature (22, 26-28) is shown in *Fig. 6*. Fibrillin microfibrils may attach to $\alpha_v\beta_3$ integrins on the plasma membrane of endothelial cells via an RGD sequence. MAGP-1, together with other less investigated proteins, binds to fibrillin with its C-terminus and to tropoelastin with its N-terminus. It may thus promote elastogenesis on the scaffold of fibrillin. MAGP-2, which also contains an RGD motif that recognizes $\alpha_v\beta_3$ integrins (22), may unite fibrillin with the cells and thus determine the specificity of elastic fibers in relation to tissue requirements.

In our experimental conditions, MAGP-1 always co-localized with fibrillin. In AEC, both fibrillin and MAGP-1 were deposited in

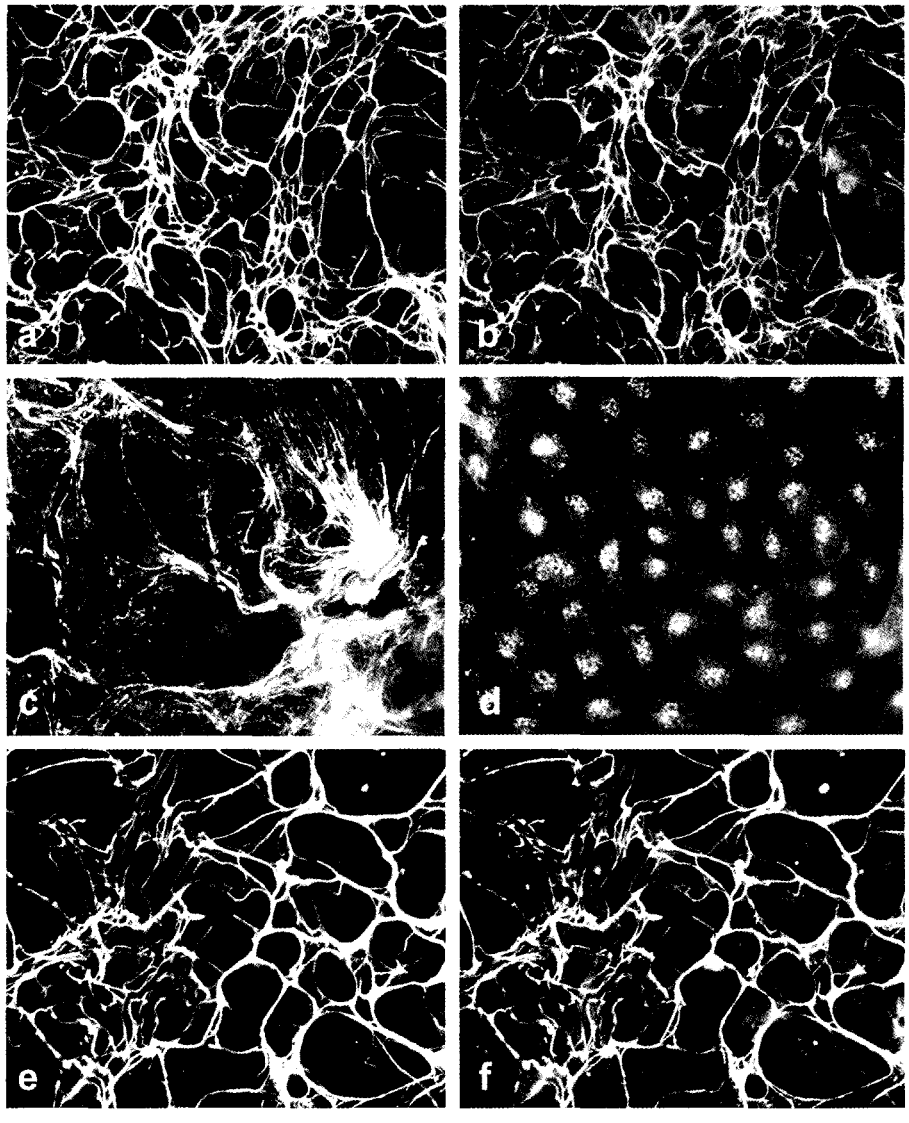

Fig. 4. Double labeling with monoclonal antibody to fibrillin (a, c and e) and antiserum to MAGP-1 (b, d and f) at day 4 after confluence. Fibrillin and MAGP-1 co-localize in AEC (a and b) and PAEC (e and f). LEC stain for fibrillin (c) but not for MAGP-1 (d). Orig. mag. x40.

a honeycomb pattern. In PAEC, areas with a honeycomb pattern coexisted with areas where fibrillin and MAGP-1 formed an irregular web. This is in line with the observation that MAGP-1 associates with fibrillin molecules, specifically the beads (19). On the other hand, LEC, that also deposit fibrillin in vitro (12), were mostly negative to immunolabeling with the polyclonal antibody to MAGP-1 used even at day 4 when fibrillin is abundant all over the culture.

It is not clear why LEC behave so differently from the other endothelial cells tested. Since small amounts of MAGP-1 are present 4 days after confluence, when fibrillin deposition has already taken place, we hypothesize that LEC require a longer time to express MAGP-1. Alternatively, culture

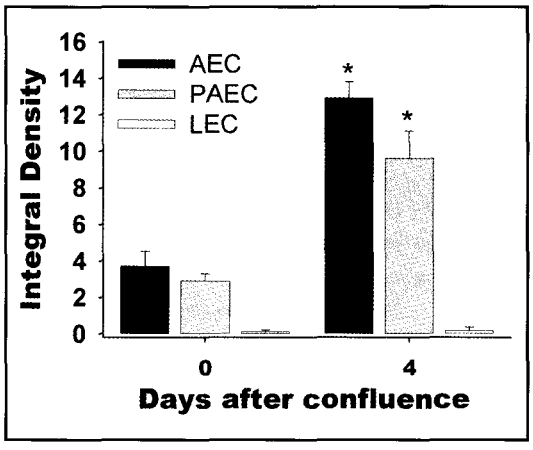

Fig.5. Integral density, expressing MAGP-1 deposition. MAGP-1 deposition increased from confluence to day 4 in AEC and PAEC ($p<0.001$). The difference between the amount of MAGP-1 produced by AEC and PAEC was not significant at confluence or at day 4. LEC did not deposit MAGP at confluence and only negligible amounts by day 4.

conditions and particularly the addition of serum to the medium, may better mimic the normal situation of blood than of lymphatic endothelial cells which are in vivo exposed to lymph.

We think it unlikely that LEC do not express MAGP-1 at all. MAGP-1 is a key molecule for elastogenesis and the endothelium of all lymphatic vessels, particularly that of initial lymphatic vessels, has close connections with elastic fibers (15,29-32). Transmission electron micrographs often catch elastic fibers contained in small "pocket-like" invaginations of the abluminal side of lymphatic endothelial cells. This finding strongly suggests that the cells themselves produce and secrete elastin (27,30). Initial lymphatic vessels are always surrounded by elastic fibers. Some of us described a fibrillo-elastic apparatus that

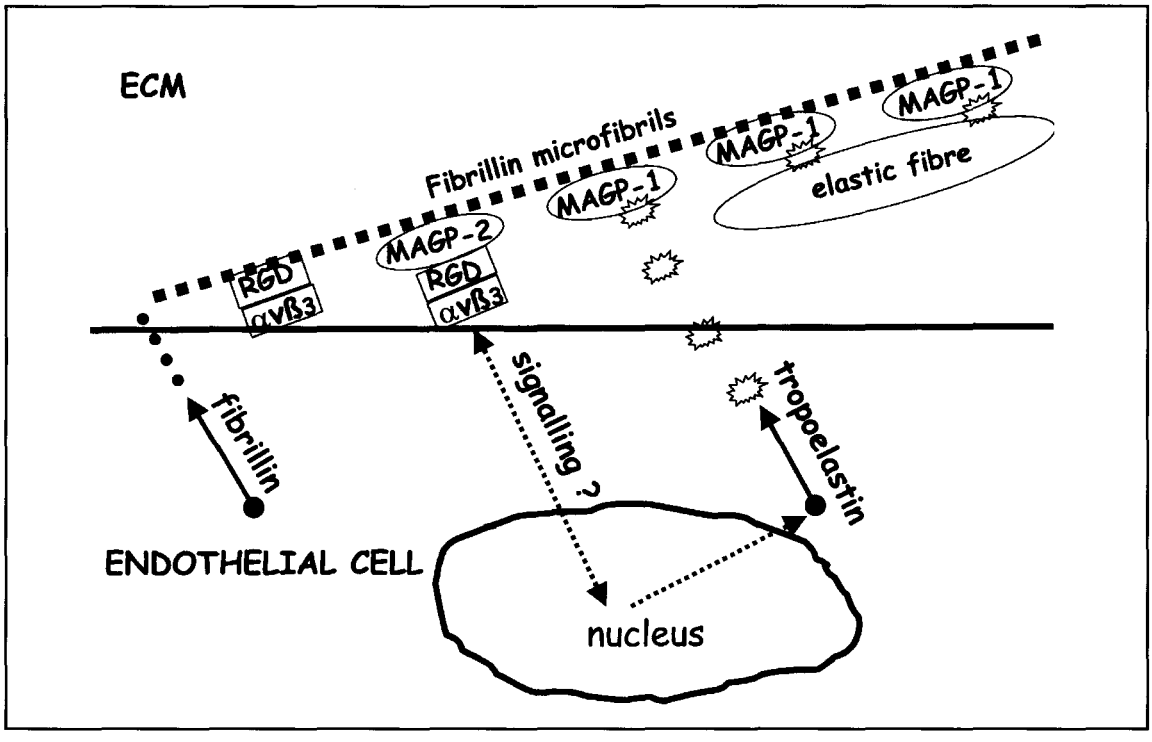

Fig. 6. Schematic representation of a possible molecular mechanism of elastogenesis in endothelial cells. Fibrillin microfibrils may attach to $\alpha_v\beta_3$ integrins on the plasma membrane of endothelial cells either via an RGD sequence of their own or via an RGD sequence of MAGP-2. The latter may correlate elastogenesis with cell specificity. MAGP-1 regulates the polymerization of tropoelastin molecules on the scaffold of fibrillin microfibrils.

unites endothelial cells with elaunin fibers (33) and the latter with the elastic fibers of the extracellular matrix by means of anchoring filaments (15,30). Blood capillaries are never surrounded by elastic fibers (29) and their endothelium therefore presumably does not produce elastic fibers. A profound difference exists between the endothelium of blood capillaries and lymphatic initial vessels in this respect. It may have repercussions on the deposition pattern of fibrillin, the time required for its secretion, the morphology of its network and also on MAGP-1 production by the two types of cells.

The cells that we used in this study are not microvascular, they were taken from large vessels, where other factors may be relevant during elastogenesis. The walls of the thoracic duct and large arteries contain smooth muscle cells and fibroblasts, which are also known to produce fibrillin and elastic fibers. Culture conditions deprive cells of the possibility to interact with other cell types and to respond to signals and stimulations of the extracellular matrix and a dynamic environment. Lymphatic endothelial cells may depend more than blood endothelial cells on these external stimuli and interactions. It is even feasible that the elastic fibers surrounding lymphatic vessels are mostly produced by cells other than endothelial.

It might be interesting to evaluate whether cultured LEC deposit tropoelastin and whether other microfibril-associated glycoproteins, like MAGP-2, which also specifically associates with fibrillin-containing microfibrils, but has a more restricted pattern of tissue localization than MAGP-1 (34), is expressed by cultured LEC earlier and more abundantly. Lymphatic (12,35) and blood endothelial cells (36) possess α_v integrins. Gibson et al (22) suggest that MAGP-2 may be relevant in modulating the interactions of microfibrils with cell surfaces in particular tissue environments. Whether lymphatic vessels belong to these environments is unknown. Unfortunately, the antibody to MAGP-2 is not yet commercially available.

We suggest that elastogenesis is an earlier event in the aorta than in the thoracic duct. The aorta has an intrinsic functional need for elastic fibers and resilience in an early phase of development. In line with this, MAGP-1 deposition in AEC is perfectly correlated with that of fibrillin from the first days of culture. Early elastogenesis is not a requirement for the thoracic duct, the wall of which can form and develop more slowly. Hence, MAGP-1 is not expressed in LEC at early times of culture and is not correlated with fibrillin deposition. PAEC, which are derived from a vessel that has intermediate structural and functional characteristics between the aorta and the thoracic duct and an intermediate exposure to oxygen and intraluminal pressure, also have intermediate deposition patterns of fibrillin and MAGPs. In our opinion, the timing of MAGP-1 deposition may influence the pattern of fibrillin deposition in the three endothelial cell types tested. Different fibrillin scaffolds could in turn account for the specificity of elastic fibers in compliance with the specific functional requirements of the aorta, the thoracic duct and the pulmonary artery.

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