Histol Histopathol (2011) 26: 711-724

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Laminin isoforms in atherosclerotic arteries from mice and man

Uwe Rauch[1], Amit Saxena[1], Stefan Lorkowski[2], Jürgen Rauterberg[3], Harry Björkbacka[4], Madeleine Durbeej[1] and Anna Hultgårdh-Nilsson[1]

[1]Institute of Experimental Medical Sciences, Lunds University, Lund, Sweden, [2]Institute of Nutrition, Friedrich Schiller University, Jena, Germany, [3]Leibniz Institute of Arteriosclerosis Research, Münster, Germany, and [4]Institute of Clinical Science, Lunds University, Malmö, Sweden

Summary. The properties of the arterial vasculature depend to a large extent on the activities of smooth muscle cells, which, in turn, are determined by their extracellular environment. During pathological conditions, such as atherosclerosis, this interaction is altered. In close proximity to medial smooth muscle cells are basement membrane components, such as different isoforms of laminin. These proteins can have great impact on cellular function via interaction with cell surface integrins. However, knowledge of laminins in smooth muscle cell basement membranes during normal and pathological conditions is scarce.

Therefore, we have analyzed the presence of laminin isoforms in atherosclerotic lesions of apolipoprotein E (ApoE)-deficient mice. Our study revealed that the laminin chain isotype composition within atherosclerotic plaque tissue was different from the chain composition in the media. In addition, obvious differences in laminin chain composition could be observed in areas of the media, which were or were not associated with plaque tissue. Our major findings demonstrate that laminin gamma3 was exclusively present in media associated with plaque tissue. Laminin alpha2 was also enriched in these medial areas. Plaque tissue was predominantly enriched in laminin alpha5 chains. This general distribution applied to lesions both with and without a fibrous cap-like structure. The differential distribution of laminin chains were partially accompanied by changes in the presence of the integrin alpha subunits 7 and V.

The distribution of laminin chains in human atherosclerotic arteries, with different size and morphology, grossly resembled their distribution in mouse arteries.

Key words: Laminin, Atherosclerosis, Smooth muscle cell

Offprint requests to: Dr. Uwe Rauch, Institute of Experimental Medical Sciences, Lunds University, MBC B12, 22184 Lund, Sweden. e-mail: uwe.rauch@med.lu.se

Introduction

The arterial vasculature has to deal with considerable pressure changes, while maintaining an appropriately sized lumen. Medial smooth muscle cells (SMCs) contribute to this ability by their elastinogenic activity and contractile strength. The SMCs are also implicated in the initiation and development of one of the most common vascular diseases, atherosclerosis. Their atherogenic activities include migration from the media into the subendothelial space, proliferation and production of extracellular matrix molecules and lipoprotein-trapping proteins (Newby and Zaltsman, 1999). Mainly proteoglycans, but also laminins have been implicated in this latter function (D'Angelo et al., 2005; Doran et al., 2008).

In established atherosclerotic plaques SMC proliferation can have a beneficial effect by the generation of a stable fibrous cap, preventing plaque rupture and concomitant downstream embolic vascular occlusions (Newby and Zaltsman, 1999). Consequently, apoptosis in vascular SMCs is associated with features of plaque vulnerability, such as a thin fibrous cap (Clarke et al., 2008). Taken together, it seems desirable to have plaques populated with SMCs with restricted proliferative, but good survival ability.

Many cellular functions are determined by the extracellular environment, which includes both the

extracellular matrix and soluble factors such as cytokines. The basement membrane components (laminins, collagen type IV, heparan sulfate proteoglycans) underlying endothelial cells and encasing medial SMCs are important extracellular matrix structures for vascular integrity (Dingemans et al., 2000). Vascular SMCs deprived of their in vivo environment require cultivation on a laminin substrate to maintain to some extent their contractile characteristics (Hedin et al., 1988).

Laminins represent a family of large heterotrimeric glycoproteins, each composed of one alpha, one beta, and one gamma chain derived from eleven individual genes coding for five different alpha, three different beta, and three different gamma chains (Miner, 2008). The linkage of the cytoskeleton to laminins can be mediated by members of the integrin family and/or by dystroglycan, which have both been observed in vascular SMCs (Yao et al., 1997; Barresi et al., 2000). Integrins have been shown not only to transmit force across the cell membrane, but also to be able to influence growth factor signal transmission (Clemmons and Maile, 2003; Cabodi et al., 2004; Dekkers et al., 2007). Distinct laminin isotypes interacting with different types of integrins can therefore likely induce different responses in medial SMCs (Genersch et al., 2003; Hallmann et al., 2005; von der Mark et al., 2007).

An early investigation of developing and mature human vessels demonstrated changes in beta chain expression in coronary arteries with atherosclerotic plaques, while alpha5 (in the study named A-) chains were the only alpha chains detected (Glukhova et al., 1993). The same chains have consecutively also been observed in rodent aortic media (Sorokin et al., 1997), and a more recent expression analysis on mouse aorta using microarrays indicated the presence of alpha2, alpha4, alpha5, beta1, beta2 and gamma1 chains (Kelleher et al., 2004). However, the overall information on laminin isoform expression in arteries, in particular atherosclerotic arteries, is sparse.

In this study we have investigated whether the distribution of laminin isoforms differs in atherosclerotic lesions compared to normal media. This may have a great impact on SMC behavior. Thus, we made an immunohistochemical survey of arteries from ApoE-deficient mice fed with a cholesterol-rich diet.

This analysis revealed that the laminin chain composition within atherosclerotic plaque from mouse indeed was different from the chain composition in the media. In addition, obvious differences in laminin chain composition could be observed in areas of the media, which were associated with plaque tissue (plm, for definitions see also Fig. 1) or were not associated with plaque tissue (nm). In particular, significant expression of laminin gamma3 chain was found in mouse plm. The changes in laminin chains were partially accompanied by changes in the expression of integrins. Further analysis of human atherosclerotic plaques, although limited in number, revealed a consistent distribution of laminin chains within this material. It grossly resembled the laminin chain distribution in mouse arteries.

Materials and methods

ApoE-/- mice on B6.129P2 background were obtained from Taconic, Lille Skensved, Denmark. Female animals 16-20 weeks of age were fed a cholesterol-rich diet (containing 15% (w/v) cacoa butter and 0.25% (w/v) cholesterol) for twelve weeks. Mice were perfusion fixed using PBS followed by Histochoice (Amresco Inc., Solon, OH, USA) injection via the right atrium under constant pressure and drained via the femoral artery. All experiments were performed in compliance with institutional (Lunds University, Sweden) and national guidelines.

Aortas were dissected out and stored in Histochoice at 4°C. Aortas were removed from Histochoice and dehydrated in 30% sucrose phosphate buffer and embedded in OCT (Tissue-Tek OCT, Sakura Japan) compound for sectioning. Aortic arches were frozen and sectioned (6 μm).

Human coronary arteries were obtained at Papworth Hospital (Cambridge, UK) from hearts explanted during heart transplantation for advanced coronary heart disease as part of a tissue bank of human coronary arteries established by the MAFAPS consortium (Bellosta et al., 2002). The explantation procedure was approved by the local Ethics Committee of Papworth Hospital. The arteries were cut into 1 cm sections and snap-frozen in liquid nitrogen-cooled isopentane within minutes after explantation. Thereafter, coronary arteries were embedded in GSV tissue embedding medium (Slee Technik; Germany) and stored at -80°C until use.

Frozen tissue specimens were cut with a Microm HM 560 microtom into 6 μm sections and air dried on Superfrost plus slides (Menzel, Germany) for 30 minutes and stored at -80°C for further use. For immuno-fluorescence detection of antigenes, frozen slides were left to dry at room temperature and submerged for 10 minutes in -20°C methanol, transferred to PBS and blocked with 5% goat serum in PBS for 1 h at room temperature except stainings for alpha smooth muscle actin and stainings of human tissue for laminin beta1, gamma1 and CD68, which were blocked with MOM-block, further processed following the basic protocol of the MOM-kit (Vector labs), and visualized with Cy3-streptavidin (Sigma). For all other stainings the goat serum blocking solution was exchanged against antibodies (plus biotinylated Wisteria Floribunda Agglutinin (WFA, Vector labs) for mouse tissue) diluted in blocking solution overnight. Slides were washed with PBS and incubated with Cy3-linked secondary antibody (goat anti rat Ig or goat anti rabbit Ig, Jackson) in PBS for 1 h at room temperature, washed again and mounted with vectashield (Vector labs). The secondary antibody solution for slides with mouse sections contained in addition FITC-linked streptavidin (Amersham). Images were taken on a Zeiss Axiophot 2 with a Hamamatsu

C4742-95 camera and Openlab 5 software (Improvision).

Antibodies against laminins alpha2 (Schuler and Sorokin, 1995; Ringelmann et al., 1999), alpha4 (Ringelmann et al., 1999), alpha5 (Sorokin et al., 1997), beta1 (Sixt et al., 2001), beta2 (Sasaki et al., 2002; Zenker et al., 2004), gamma1 (Wewer et al., 1983; Sixt et al., 2001) from Lydia Sorokin, Muenster, against laminin gamma3 (Gawlik et al., 2006a; Schlötzer-Schrehardt et al., 2007) from Takako Sasaki, Erlangen, and against integrin alpha7B (Cohn et al., 1999; Gawlik et al., 2006b) from Ulrike Mayer, Manchester, were kind gifts. Antibodies against alpha smooth muscle actin (1A4, Sigma; Ström et al., 2004), CD68 (KP1, DAKO; Goncalves et al., 2008), integrin alphaV (AB 1923, Chemicon; Beauvais et al., 2009) and integrin beta1 (9EG7, Pharmingen; Lenter et al., 1993) were commercially obtained.

Total RNA from aortas stripped of adventitial and endothelial layer was isolated independently from eight wild type and eight ApoE-deficient mice (16-20 weeks old) using TRIzol reagent (Invitrogen) and RNeasy columns (Qiagen) according to the manufacturer's specifications. RNA integrity and purity was analyzed with an Experion Automated Electrophoresis System (Bio-Rad) and quantified using a ND-1000 spectrophotometer (NanoDrop Technologies). Purified total RNA was reverse transcribed with MMLV Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was carried out using SYBR Green PCR Master Mix and a 7900HT Fast Real-time PCR System (Applied Biosystems). Relative mRNA expression was calculated from standard curves, constructed by serial dilution of gel-purified PCR products, and normalized to GAPDH mRNA expression as housekeeping gene.
Primer sequences (5'-3'):
alpha5 Fw:GTACAGCTGCCAACAGCAAC;
alpha5 Rev:TTGGCAAACTTGATGAGCAC;
beta2 Fw: GTGTGGCTTGCATAGCCCT;
beta2 Rev:TCCGATGACTATTTGGGTTGTCT;
GAPDH Fw:AGGTCGGTGTGAACGGATTTG;
GAPDH Rev:TGTAGACCATGTAGTTGAGGTCA.

Results

Immunohistochemical detection of laminin chains in atherosclerotic aortas from mice

In this study we have used ApoE-deficient mice which, when fed a cholesterol-rich diet, develop arterial lesions with structural features similar to human atherosclerotic lesions. In nm positive signals with antibodies against the alpha2, 4, and 5 chain, beta2 chain and gamma1 chain could be detected (Figs. 2A-C, E, F, 3A-C, E, F). Positive staining for the laminin chain beta1 was restricted to plaque, whereas prominent gamma3 immunoreactivity was only present in plm (Figs. 2D,G, 3D,G). It was noticed that most atherosclerotic plaques were well-recognized by the lectin WFA, while medial tissue in general exhibited a lower binding to the lectin (Figs. 2, 3). Therefore, the biotinylated lectin was employed as counterstain.

Analyzed atherosclerotic plaques from mouse arteries could be grossly divided into two groups depending on their morphology; plaques not exhibiting (Fig. 2) and exhibiting (Fig. 3) a distinct fibrous cap-like structure at the luminal border. This morphological distinction was in agreement and supported by the distribution of CD68-positive macrophages (Figs. 2H, 3H) in the superficial tissue of the plaque not exhibiting such a distinct structure (Fig. 2H). In contrast, alpha smooth muscle actin was, apart from being expressed in the media (Figs. 2I, 3I), localized to the superficial tissue of plaques with a distinct fibrous cap-like structure along the lumen (Fig. 3I). This alpha smooth muscle actin immunoreactivity (Fig. 3I, arrow) was not present in a CD68-enriched shoulder region of the plaque (Fig. 3H, arrow). The individual laminin chains showed a grossly similar distribution in these different types of plaque (Figs. 2A-G, 3A-G).

Laminin alpha2 chains were detectable throughout the media (Figs. 2A, 3A). The signal intensity was markedly increased in plm, whereas the signal was minute in the plaque itself, where only occasionally a faint staining delineating the border to the lumen could be seen (open arrow head, Fig. 3A).

Laminin alpha4 chains were ubiquitously present in the media and plaque tissue (Figs. 2B, 3B). Although the signal was apparent throughout the media, the intensity was reduced in plm compared with nm (Fig. 2B). Within plaque tissue staining was heterogeneous, in most parts at the same level as in plm, but occasionally small patches with high intensity could be seen (Fig. 2B).

Laminin alpha5 chains were detected ubiquitously, most prominently in plaque tissue, where in several areas laminin alpha5 immunoreactivity was more intense as compared to plm. In turn, compared to nm, plm exhibited to some extent a stronger but less homogenous laminin alpha5 staining. The most intense plaque staining was present in the superficial area (Figs. 2C, 3C), and particularly in association with fibrous cap-like structures along the lumen (Fig. 3).

Laminin beta1 chains were almost exclusively observed within the plaque, in particular in superficial plaque tissue (Figs. 2D, 3D).

Laminin beta2 chains were present throughout media and plaque tissue (Figs. 2E, 3E). However, staining intensity within the plaque was reduced compared to the media (Figs. 2E, 3E).

Laminin gamma1 chains were evenly distributed throughout the media as well as in plaque tissue (Figs. 2F, 3F), where stronger immunoreactivity co-localized with the fibrous cap-like structure.

Laminin gamma3 chain staining was exclusively detected in plm, in particular in the inner plm (Figs. 2G, 3G).

Increased expression of laminin alpha5 and laminin beta2 mRNAs in aortic medias from ApoE-deficient mice

To confirm differences in laminin beta2 and alpha5 chain expression detected by immunofluorescence, quantitative RT-PCR experiments were performed with aortas isolated from C57BL/6 mice and from ApoE-deficient mice fed a cholesterol-rich diet. Aorta segments were stripped of adventitial and intimal tissue before the medial tissue was processed, allowing analysis of laminin beta2 and alpha5 chain expression exclusively in SMCs. This experiment revealed that expression of laminin alpha5 and laminin beta2 mRNAs were significantly increased in the media from ApoE-deficient mice. On average both mRNAs were increased about twofold in aortas from ApoE-deficient mice as compared to wild-type mice (Fig. 4). The analyses also revealed that in relation to GAPDH the absolute level of laminin beta2 mRNA is about 50-fold higher than the level of laminin alpha5 mRNA (Fig. 4).

Immunohistochemical analysis of integrin beta1, alphaV, and alpha7 chains in atherosclerotic aortas from mice

Previous studies have shown that integrin alpha7, alphaV and beta1 subunits are present in aortic tissue (Cheuk and Cheng, 2004; Heerkens et al., 2006), where they can interact with alpha2 and alpha5 chains containing laminin isoforms (Genersch et al., 2003; Hallmann et al., 2005; von der Mark et al., 2007). Immunofluorescence staining confirmed localization of these integrin subunits in the media and within plaque tissue (Figs. 2, 3J-L). Beta1 integrin staining was ubiquitously distributed with minor alterations in intensity (Figs. 2J, 3J). The two alpha chains showed more intense staining in the inner plm and deeper plaque areas (Figs. 2, 3K,L).

Although distribution of the two alpha chains appeared similar, the pattern of staining was clearly different. A more homogenous pattern was observed for alpha7 integrin (Figs. 2L, 3L), while distribution of alphaV integrin appeared in a "polka dot" pattern, which is in line with previous reports (Sakai et al., 2001; Beauvais et al., 2009) (Figs. 2K, 3K).

Distribution of laminin chains in human atherosclerotic plaques

We investigated five human atherosclerotic lesions with different size and morphology: Specimen V exhibited a large advanced plaque with no clear lumen, but obvious areas of necrosis or lipid deposition; specimen IV exhibited extended plaque tissue containing obvious areas of necrosis or lipid deposition surrounding a small central remaining lumen; specimen III exhibited extended plaque tissue, which on one side contained obvious areas of necrosis or lipid deposition, surrounding a large central lumen; specimen II exhibited extended plaque tissue on one side of a more laterally located lumen, with minor plaque tissue on the opposite side; specimen I exhibited the least advanced lesion of just minor plaque tissue. Structures of neo-vascularisation were usually observed within extended (specimens II-V), but not within minor (specimens I and II) plaque tissues.

The immunohistochemical analysis demonstrated laminin alpha2 deposition in all specimens (I-V), both in the SMC layers in the media as well as in the plaque (Fig. 5A,C,E,G,H). In specimens IV and V the laminin alpha2 staining observed in the plaques was weaker compared to the staining in the medial tissue (Fig. 5G,H). In specimens II, III and IV, laminin alpha2 deposition colocalized with the plaque border, surrounding the remaining lumen (Fig. 5C,E,G). Diffuse

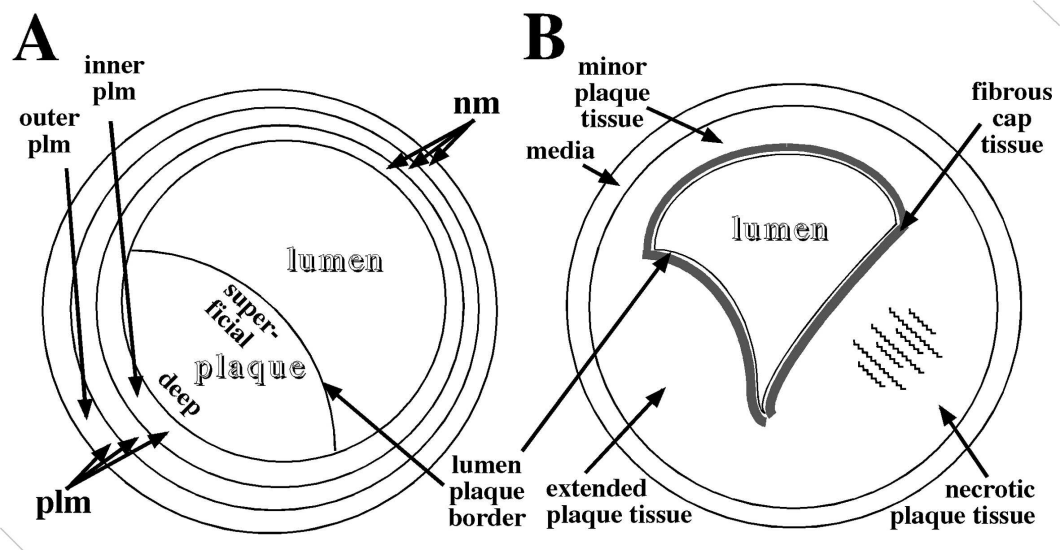

Fig. 1. Schematic figure of atherosclerotic plaques in mouse **(A)** and human **(B)** arteries. nm: media not associated with plaque; plm: media associated with plaque.

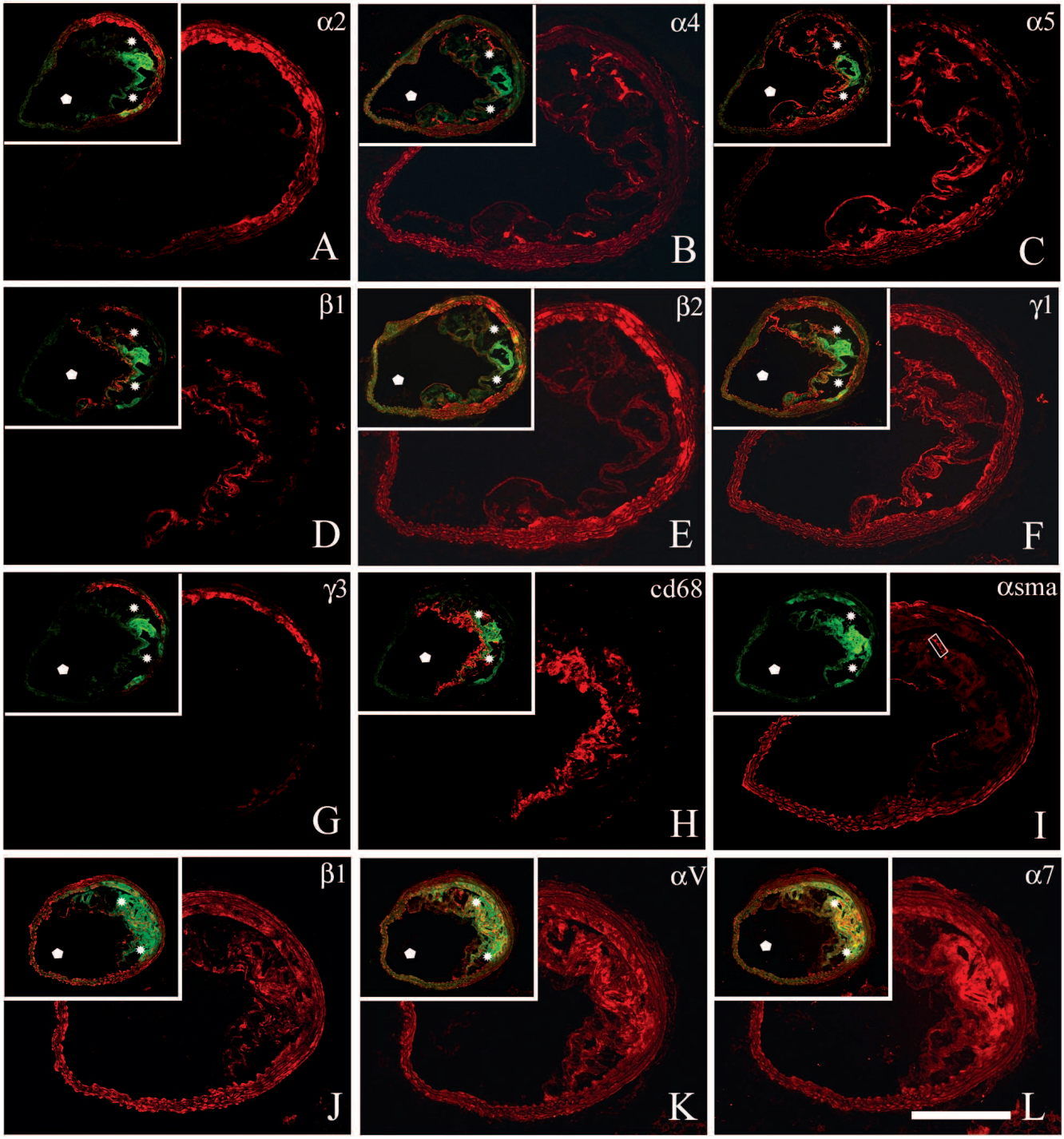

Fig. 2. Immunofluorescence staining of laminin chains and integrin subunits in atherosclerotic plaques from ApoE-deficient mice, lacking a fibrous cap-like structure. Frozen sections were incubated with antibodies against laminin alpha2 **(A)**, alpha4 **(B)**, alpha5 **(C)**, beta1 **(D)**, beta2 **(E)**, gamma1 **(F)**, and gamma3 chains **(G)**, CD68 **(H)**, alpha smooth muscle actin **(I)** (not in insert), and integrin beta1 **(J)**, alpha **(K)** and alpha7 subunits **(L)** (all in red). Plaque tissue is indicated by gear wheels and the vascular lumen by a pentagon within the inserted images, which are counterstained with biotinylated WFA (in green). The boxed area in I) indicates a staining signal, which was also evident with secondary reagents (MOM-kit) alone. Bar: 0.25 mm.

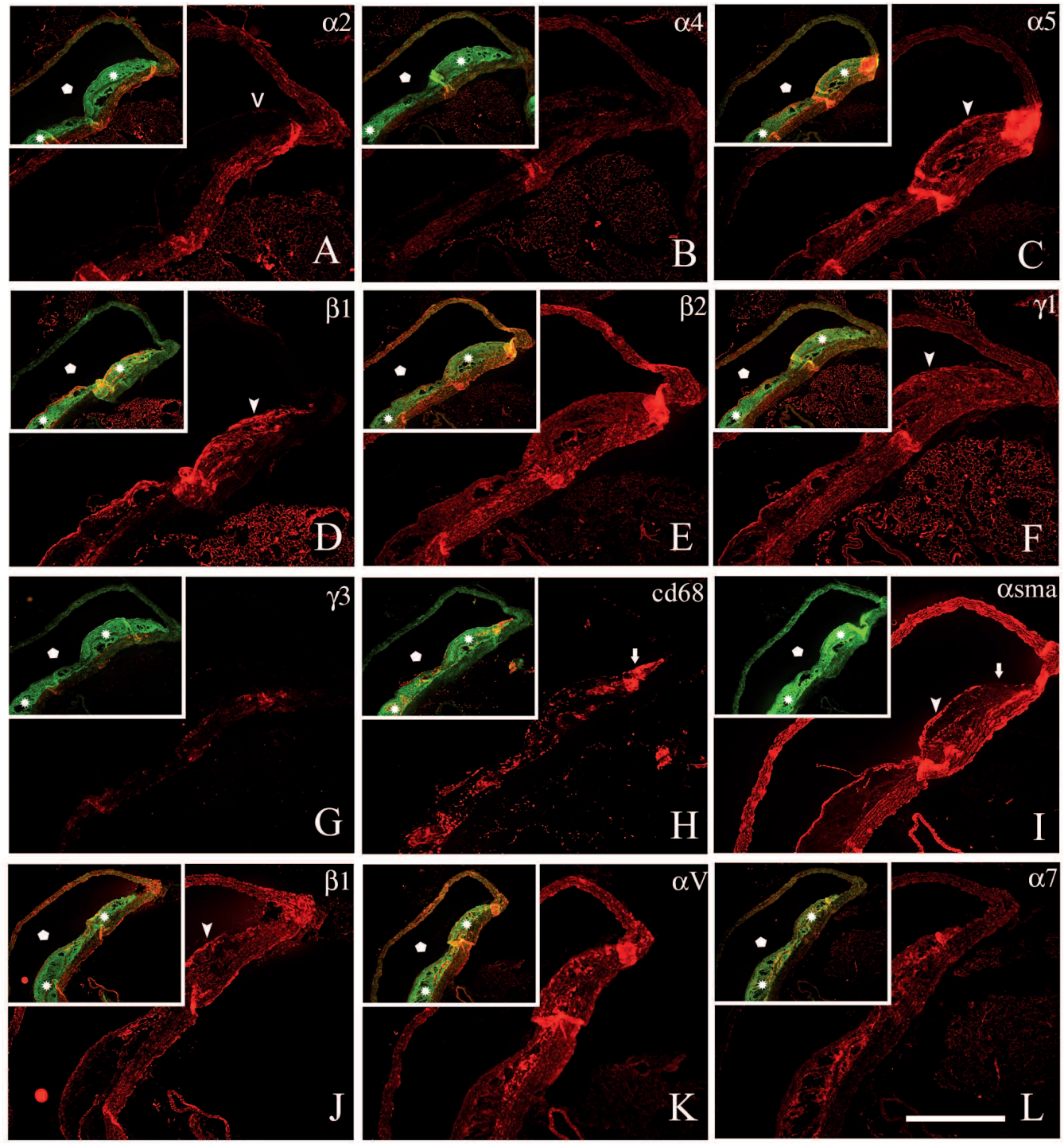

Fig. 3. Immunofluorescence staining of laminin chains and integrin subunits in atherosclerotic plaques from ApoE-deficient mice exhibiting a fibrous cap-like structure. Frozen sections were incubated with antibodies against laminin alpha2 **(A)**, alpha4 **(B)**, alpha5 **(C)**, beta1 **(D)**, beta2 **(E)**, gamma1 **(F)**, and gamma3 chains **(G)**, CD68 **(H)**, alpha smooth muscle actin **(I)** (not in insert), and integrin beta1 **(J)**, alphaV **(K)** and alpha7 subunits **(L)** (all in red). Plaque tissue is indicated by gear wheels and the vascular lumen by a pentagon within the inserted images, which are counterstained with biotinylated WFA (in green). The fibrous cap-like structure is marked with a filled arrow head. Bar: 0.5 mm.

laminin alpha2 staining could also be observed within the minor plaque tissue in specimen II (Fig. 5C) and in specimen I this isoform to some extent colocalized with the luminal border. The media in specimen I bound laminin alpha2 antibodies less efficiently than medias of more advanced lesions (Fig. 5A,C,E,G,H). Structures of neovascularisation, which were detected in extended areas of advanced human plaques by antibodies against alpha4 and alpha5 laminin chains (Fig. 6A,B), did not exhibit a similar clear laminin alpha2 chain staining (Fig. 5C).

Laminin alpha4 chains were shown to be present in very low amounts in the media of arteries with less advanced lesions, as shown for specimen II (Fig. 6A), and were essentially absent in the media of human arteries with more advanced lesions as shown for specimen IV (Fig. 7D,D'). Within plaques laminin alpha4 staining was mainly present around the remaining lumen, particularly evident in specimens II (Fig. 6A) and III (not shown). Apart from neo-vascular structures the staining intensity in the plaque tissue was otherwise weak compared with the staining of peripheral nerves and micro-vasculature located on the adventitial outside of the coronary artery (Figs. 6A, 7D,D').

Laminin alpha5 chains were clearly present in the media of all five human specimens and exhibited an even more intense immunoreactivity in plaques from the more advanced lesions (II-V) (Figs. 6B, 7A,G). In specimens IV (Fig. 7A), III (not shown) and II (Fig. 6B) intense staining was present in the luminal plaque border and in the fibrous cap tissue region. This distribution resembled the localization of laminin alpha5 in mice (Figs. 2C, 3C).

Laminin beta1 chains were essentially absent from

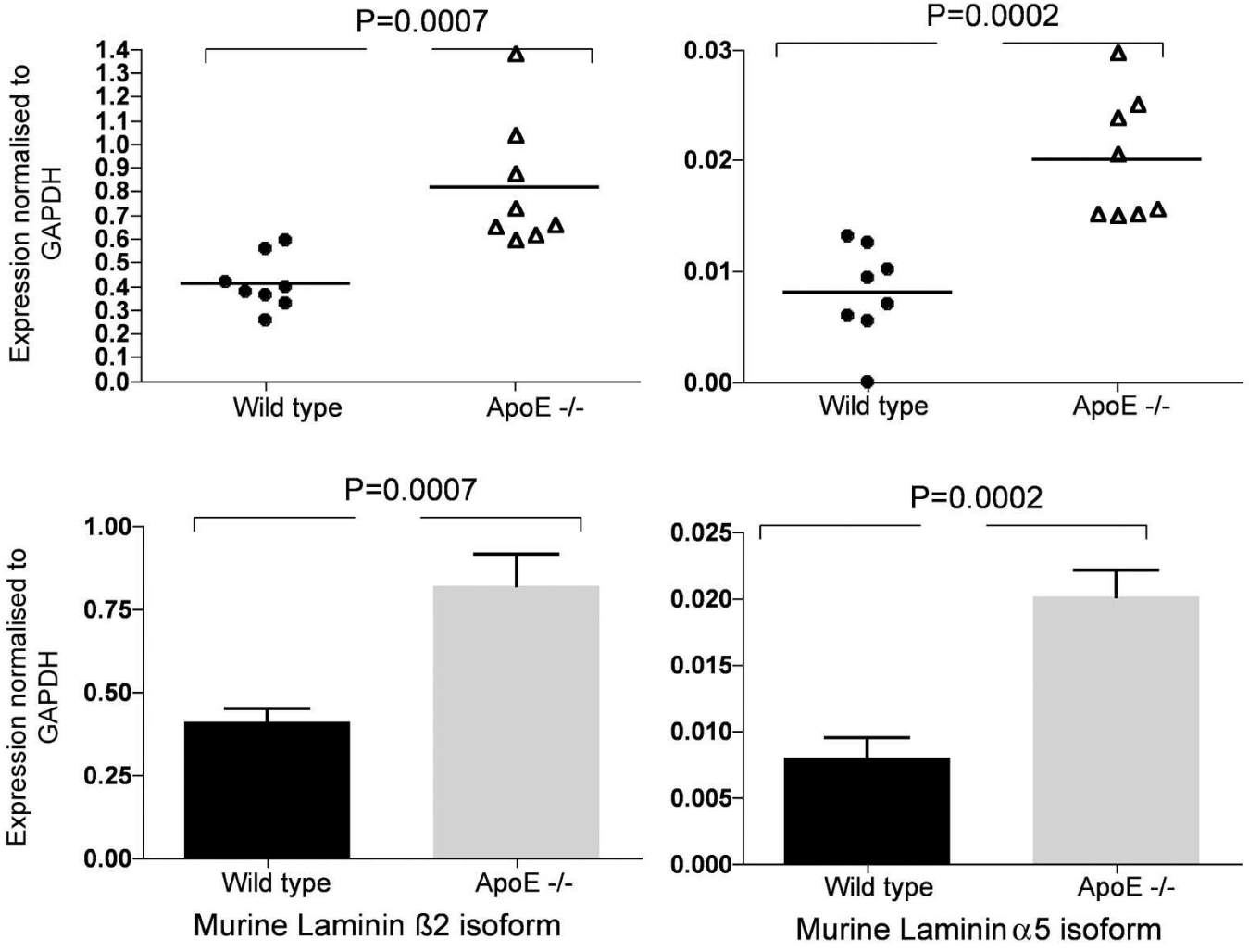

Fig. 4. RT-PCR analyses of aortic tissue from ApoE-deficient and wild-type B6 mice. Aortas from individual age-matched wild-type B6 mice (dots) and from ApoE-knock out mice (peaks) on Western diet for twelve weeks were cleaned from adventitial and intimal tissue and homogenized. The extracted RNA was subjected to RT-PCR with primers for laminin beta2-, alpha5 chains, and GAPDH.

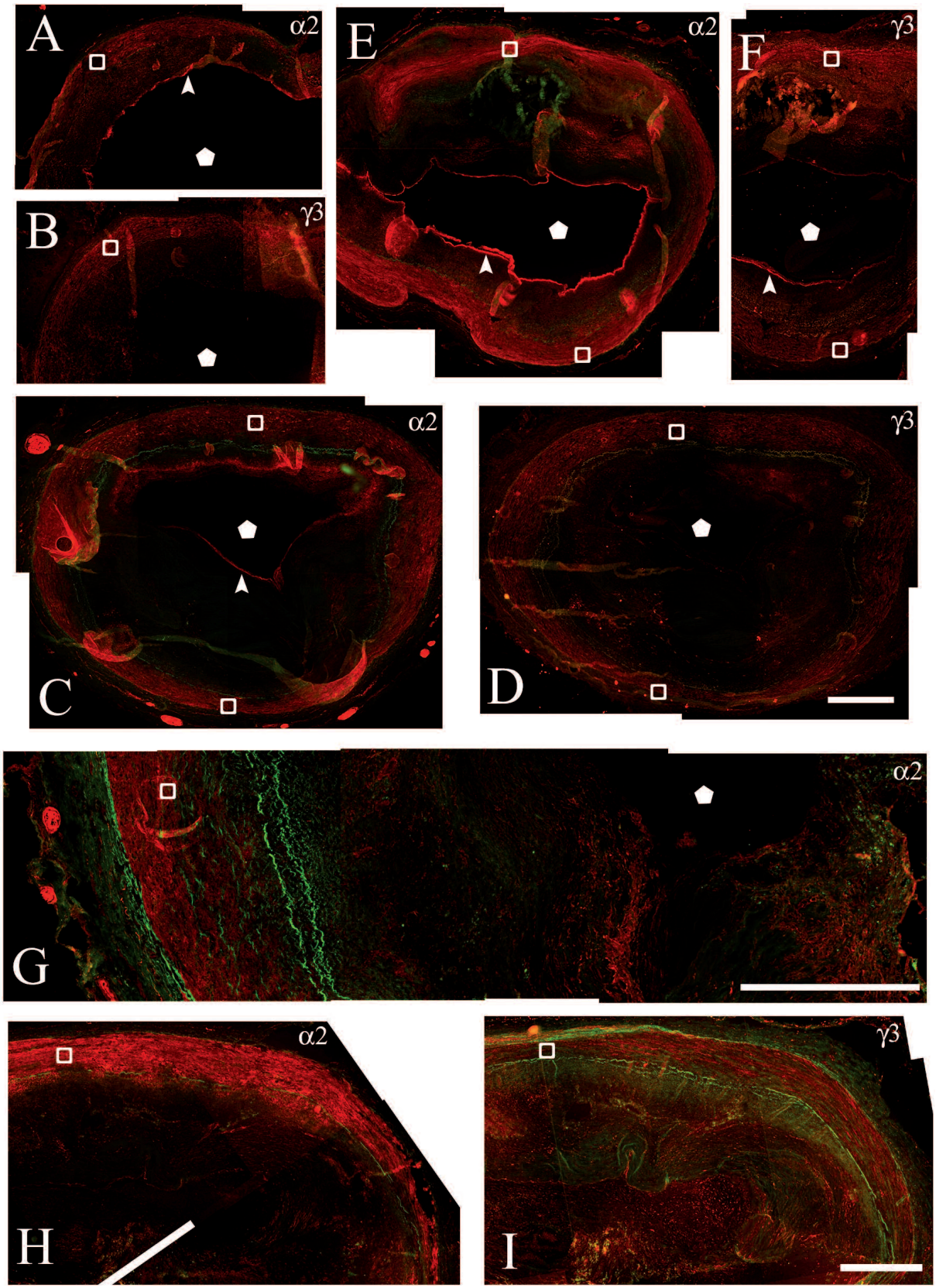

Fig. 5. Immunofluorescence stainings of alpha2 and gamma3 laminin on human atherosclerotic plaques. Frozen sections of human specimen I (**A, B**), II (**C, D**), III (**E, F**), IV (**G**), and V **(H, I)** were incubated with antibodies against alpha2 (**A, C, E, G and H)** and gamma3 (**B, D, F and I)** laminin chains (both in red). Autofluorescence of the tissue in the FITC channel (490 nm) is presented in green. Arrow heads indicate extended plaque tissue-associated luminal borders. Pentagons indicate the remaining vascular lumen, and open squares the media. Bars: D (for A-F), G, and I (for H and I), 1 mm.

the media, but were present in areas of the fibrous cap tissue in advanced lesions, as shown for specimens II (Fig. 6C) and IV (Fig. 7E). This distribution was shown to be similar to that in mice (Figs. 2D, 3D). The presence of laminin beta1 chains was pronounced around narrow extensions of the lumen into the plaque tissue. Beta1

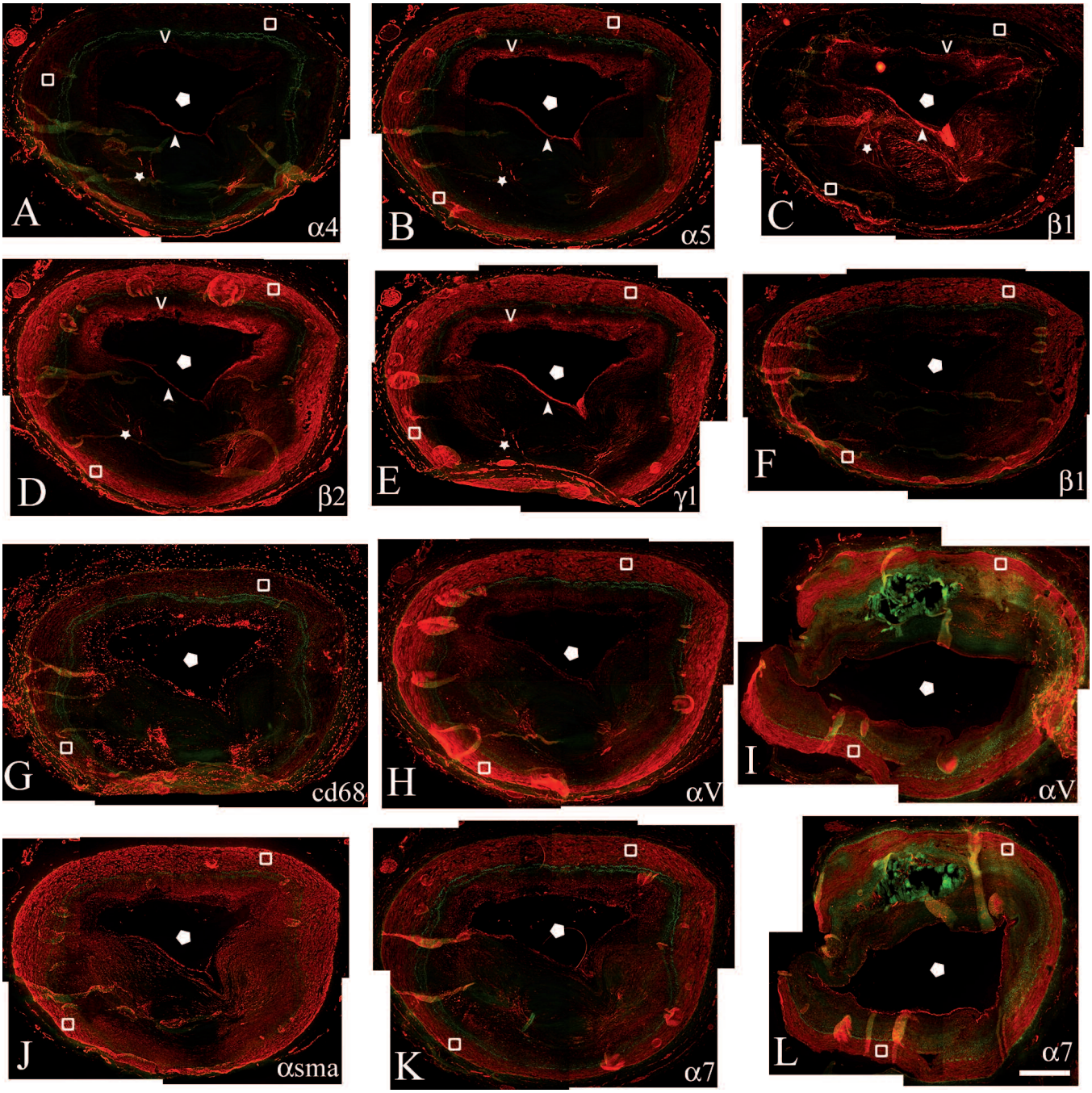

Fig. 6. Immunofluorescence stainings of human atherosclerotic aortic tissue specimens II and III. Consecutive frozen sections from specimens II (**A-H, J and K**) and III (**I, L**) were incubated with antibodies against laminin alpha4 **(A)**, laminin alpha5 **(B)**, laminin beta1 **(C)**, laminin beta2 **(D)**, laminin gamma1 **(E)**, beta1 integrin **(F)**, CD68 **(G)**, alpha7 integrin **(H and I)**, alpha smooth muscle actin **(J)**, alphaV integrin **(K and L)** (all in red). Autofluorescence of the tissue in the FITC channel is presented in green. Laminin staining of extended plaque tissue-associated luminal borders is indicated by closed arrow heads and minor plaque tissue by open arrow heads. Stars indicate neovascular structures. Pentagons indicate the remaining vascular lumen, and open squares the media. Bar: 1 mm.

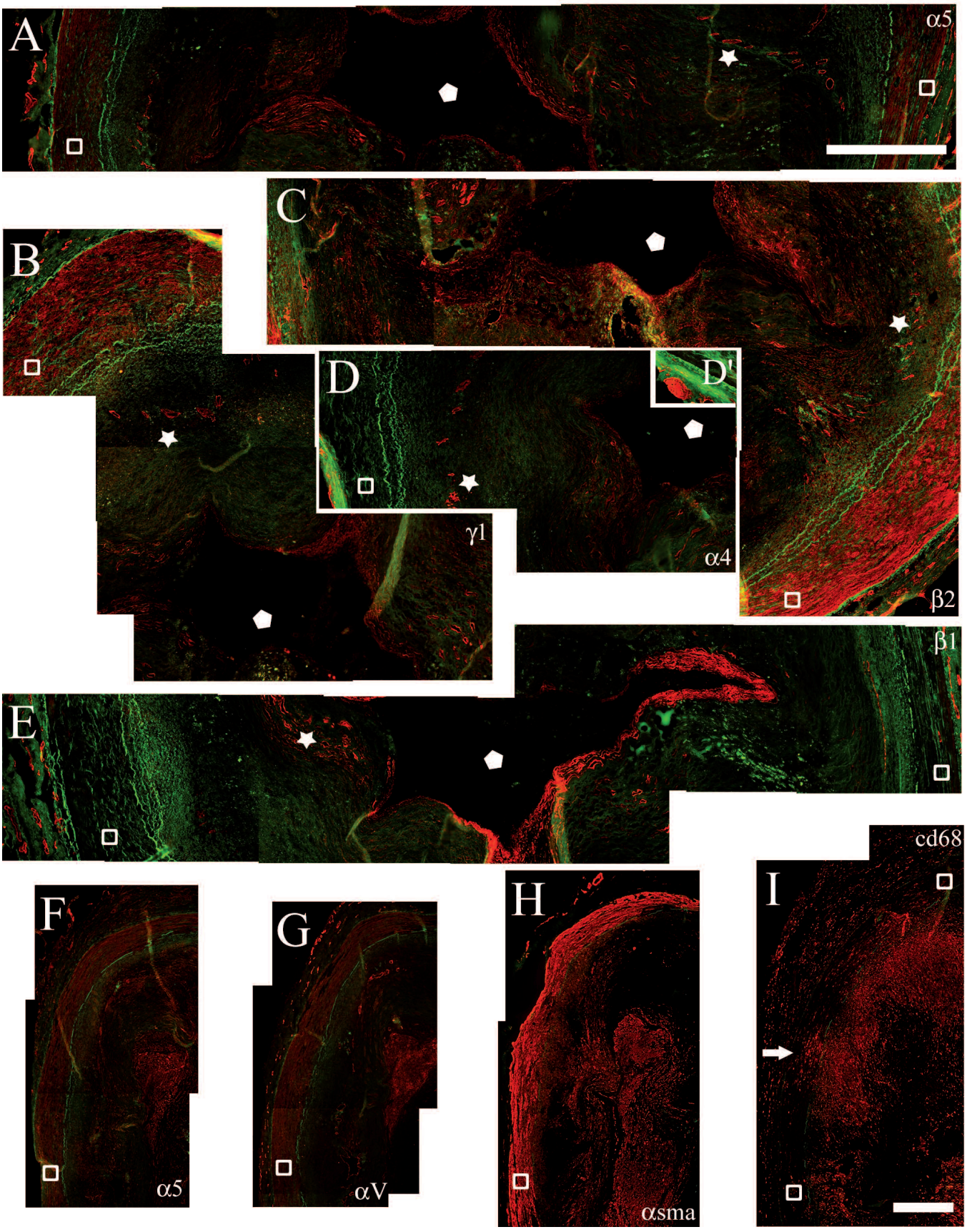

Fig. 7. Immunofluorescence stainings of human atherosclerotic aortic tissue specimens IV and V. Frozen sections of specimen VI (A-E) and specimen V (F-I) were incubated with antibodies against laminin alpha5 **(A and G)**, laminin gamma1 **(B)**, laminin beta2 **(C)**, laminin alpha4 **(D and D')**, and laminin beta1 **(E)**, alphaV integrin **(F)**, alpha smooth muscle actin **(H)**, and CD68 **(I)** (all in red). Autofluorescence of the tissue in the FITC channel is presented in green. Arrow indicates infiltration of by CD68 positive macrophages in the media. Pentagons indicate the remaining vascular lumen, open squares the media, and stars neovascular structures. Bars: A (for A-E) and F (for F-I), 1 mm.

chains were also observed in structures of neovascularization (Figs. 6C, 7E).

Laminin beta2 chains were clearly present in the media of all human specimens and in structures of neovascularization of plaque tissue (Figs. 6D, 7C). In specimens II (Fig. 6D) and III (not shown) laminin beta2 chains were detectable in the luminal plaque border and present in the fibrous cap tissue in specimen IV (Fig. 7C). However, compared to the media, laminin beta2 chains were, as in mouse tissue, less apparent in the plaque tissue.

Laminin gamma1 chains were, similar to mouse tissue, generally present, including structures of neovascularization and the luminal plaque border (Figs. 6E, 7B).

Laminin gamma3 chain staining with the same rabbit antiserum as used for mouse tissue, which has previously been proven to function on human tissue (Schlötzer-Schrehardt et al., 2007), exhibited an overall weak signal. In the media, as well as in the plaque and at the luminal plaque border, the signal could only be detected by image enhancement (Fig. 5B,D,F,I). As a consequence, autofluorescent signals of the necrotic tissue became visible in red (compare Fig. 5F with Fig. 5E).

Analysis of cell composition and integrin chains in human atherosclerotic plaques

The presence of macrophages was confirmed by CD68 antigen reactivity in the plaques and was localized to areas surrounding the remaining vascular lumen, but also elsewhere (Figs. 6G, 7I, and not shown). In specimen V macrophages could also be detected in the media (Fig. 7I). Alpha smooth muscle actin was clearly detectable in the media of all specimens (Figs. 6J, 7H), and was also present at the luminal plaque border (Fig. 6J).

Beta1, alpha7 and alphaV integrin staining could be observed in the media of all specimens (Figs. 6F,H,I,K,L, 7F). Beta1 and alpha7 integrin staining intensity was usually lower within the atherosclerotic plaque tissue, including the luminal plaque border (Fig. 6F,H,I). AlphaV integrin staining in less affected arteries (specimen II and III) showed similar intensity in the media and at the luminal plaque border (Fig. 6K,L). This was also the case in distinct areas of advanced plaques in specimen IV (not shown) and V (Fig. 7F). An intriguing co-distribution of the staining for alphaV integrin and for alpha5 laminin chains was observed occasionally (Fig. 7F,G).

Discussion

In this study we have found distinct changes in the presence and in particular the distribution of laminin chains in atherosclerotic arteries from ApoE-deficient mice fed a cholesterol-rich diet. These changes include differences in the presence of laminin isotypes between nm, plm, and plaque tissue. Our findings indicate that different laminin isoforms may be important for SMC function in the development of an atherosclerotic lesion and thus also for the outcome of the disease process. In particular, we revealed the presence of laminin alpha2 chains predominantly associated with plm, and laminin alpha5 chains associated with plaque tissue. Moreover, we detected the laminin gamma3 chain exclusively in plm. It was recently discovered that this laminin chain may be a dominant negative inhibitor of integrin binding (Ido et al., 2008). This observation might indicate a so far undetected biological function of the laminin gamma3 chains in a mechanism to modulate integrin-mediated signaling in medial SMCs.

No clear signal above background staining could be observed for the laminin alpha3 and beta3 chains. It is of note that the same antisera gave a clear positive staining of epithelial basement membranes in co-processed esophagus tissue. Traces of these two and laminin gamma2 chains, which together constitute the major epithelial laminin, have been detected in rat aortas, but only by using special fluorescence amplification and photobleaching methods (Kingsley et al., 2002). In addition, an antiserum, which detects laminin alpha1 chains in brain, revealed no clear signals in the atherosclerotic specimens (U. R., unpublished observations).

Even though the human atherosclerotic specimens were limited in number, our observation of the presence of laminin alpha2 chains within the media, and the presence of laminin alpha5 chains predominantly in plaque tissue, is in line with the findings in mouse tissue. Thus, these and other similarities indicate that the ApoE-deficient mouse appears to constitute an appropriate animal model to investigate the role of basement membrane components in the atherosclerotic process.

A difference observed between human and mouse was the clear occurrence of laminin alpha2 chains in human, but not mouse plaque tissue. In particular, laminin alpha2 chains were detected at the luminal plaque border in human atherosclerotic tissue of specimen II, III and IV. Interestingly, all investigated laminin chains and integrin alphaV subunits were distributed in a similar way at this location, where they were associated with lined up alpha smooth muscle actin positive cells. The specimens used in the present study came most likely from patients with stable coronary artery disease. A more comprehensive human material including stable as well as vulnerable lesions is needed to further investigate the role of laminin isoforms in the development of human atherosclerosis.

The present study shows distinct different compositions of laminin isoforms in atherosclerotic tissue compared to nm from mouse and human. We speculate that these extracellular environments differ significantly in their ability to interact and communicate with cells. Particularly, the obvious paucity of laminin alpha2 and laminin beta2 chains in the plaque tissue might have a strong effect on the ability of cells to avidly

interact with basement membrane structures. In neuroskeletal muscular junctions of mice lacking laminin alpha2 or laminin beta2 chains, the result was a reduction in the interdigitation between the muscle cell surface and the basement membrane via folds in the muscle cell membrane (Patton, 2000). SMCs are known to form caveolae, which can similarly interdigitate with basement membranes and extend the contact area of the SMC surface (Halayko et al., 2008). SMCs, lacking the ability to form caveolae, are more proliferative and migratory, activities, which enhance neointima formation during vascular injury (Hassan et al., 2004). Based on these observations it is tempting to speculate that a lower level of laminin alpha2 and beta2 chains might augment the ability of SMCs to migrate.

In contrast to alpha2 and beta2 chains, increased amounts of alpha5 chain containing laminins were observed in the plaque tissue. While alpha7beta1 may be the only integrin able to bind to laminin alpha 2 chains, alpha5 chain containing laminins appear to be much more promiscuous ligands. In lymphocytes, glioma and epithelial cells, integrin alpha6beta1 (Gorfu et al., 2008), integrin alpha3beta1 (Kawataki et al., 2007), and integrin alphaVbeta3 (Genersch et al., 2003), respectively, can bind alpha5 chain containing laminins. Thus, the increased amount of laminin alpha5 chains in the plaque tissue could, at least partially, be associated with the observation of integrin alphaV chains. Integrin alphaV has been shown to be rapidly upregulated after wire-induced lesion formation in ApoE-knockout mouse carotid arteries (Sadeghi et al., 2004). Ligand binding of alphaVbeta3 integrin has been shown to induce increased SMC migration and proliferation as a response to IGF-I stimulation (Maile et al., 2007). AlphaVbeta3 intergrin-laminin alpha5 interaction has also been shown to affect growth factor response in epithelial cells (Genersch et al., 2003). Another example for integrin isoform-dependent alterations in growth factor responses is that oligodendrocyte precursor cells grown on the alphaVbeta3 integrin ligand vitronectin show a proliferative response upon PDGF stimulation while alpha2 chain containing laminin substrate primarily promoted survival of these cells (Baron et al., 2003). In muscle tissue of mice and of man deficient in laminin alpha2 chains apoptosis is indeed a hallmark and this pathology can be ameliorated by anti-apoptotic agents (Erb et al., 2009). Interestingly, while cell binding-induced survival signals are generally considered as integrin-mediated, has anti-apoptotic activity of laminin alpha2 chains recently been demonstrated to be mediated by dystroglycan (Munoz et al., 2010).

It has been observed that the proportion of SMCs undergoing apoptosis is significantly increased in unstable versus stable atherosclerotic lesions (Bauriedel et al., 1999). Therefore, the presence of laminin isotypes favoring survival of differentiated cells in the plaque tissue may be of advantage and laminin alpha2 chains may have such an effect. These considerations appear to mark the laminin alpha5 chains and their substitution by laminin alpha2 in the plaque tissue as a possible therapeutic target. However, while the presence of laminin alpha2 chains in the media affects only smooth muscle cells, the expression of these chains in the intima is likely to affect also endothelial cells by being integrated into the endothelial basement membrane, a condition, which has to be carefully evaluated for a potential impairment of endothelial function.

In conclusion, our most important findings of mouse and human atherosclerotic aortic tissue demonstrate the presence of laminin alpha2 chains predominantly in the media, and the presence of laminin alpha5 chains predominantly in plaque tissue, in both species. Our findings indicate that the ApoE-deficient mouse constitutes an appropriate animal model to investigate basement membrane related mechanism in the atherosclerotic process. These observations open up possibilities for further experimental approaches to modulate the atherosclerotic process in mice via alterations of basement membrane components.

Acknowledgements. We thank Lydia Sorokin for antibodies and continuous practical and intellectual support, Takako Sasaki and Ulrike Mayer for antibodies, Gunnel Roos for experimental assistance, and the Swedish Science Research Council, the Swedish Heart Lung foundation, the Greta and Johan Kocks foundation, the Alfred Österlund foundation, the Thelma Zoega foundation, the European Union and the Deutsche Infarktforschungshilfe for financial support.

References

Baron W., Decker L., Colognato H. and ffrench-Constant C. (2003). Regulation of integrin growth factor interactions in oligodendrocytes by lipid raft microdomains. Curr. Biol. 13, 151-155.

Barresi R., Moore S.A., Stolle C.A., Mendell J.R. and Campbell K.P. (2000). Expression of gamma -sarcoglycan in smooth muscle and its interaction with the smooth muscle sarcoglycan-sarcospan complex. J. Biol. Chem. 275, 38554-38560.

Bauriedel G., Hutter R., Welsch U., Bach R., Sievert H. and Luderitz B. (1999). Role of smooth muscle cell death in advanced coronary primary lesions: implications for plaque instability. Cardiovasc. Res. 41, 480-488.

Beauvais D.M., Ell B.J., McWhorter A.R. and Rapraeger A.C. (2009). Syndecan-1 regulates alphavbeta3 and alphavbeta5 integrin activation during angiogenesis and is blocked by synstatin, a novel peptide inhibitor. J. Exp. Med. 206, 691-705.

Bellosta S., Bernini F., Chinetti G., Cignarella A., Cullen P., von Eckardstein A., Exley A., Freeth J., Goddard M., Hofker M., Kanters E., Kovanen P., Lorkowski S., Pentikainen M., Printen J., Rauterberg J., Ritchie A., Staels B., Weitkamp B. and de Winther M. (2002). Macrophage function and stability of the atherosclerotic plaque: progress report of a European project. Nutr. Metab. Cardiovasc. Dis. 12, 3-11.

Cabodi S., Moro L., Bergatto E., Boeri Erba E., Di Stefano P., Turco E., Tarone G. and Defilippi P. (2004). Integrin regulation of epidermal growth factor (EGF) receptor and of EGF-dependent responses. Biochem. Soc. Trans. 32, 438-442.

Cheuk B.L. and Cheng S.W. (2004). Differential expression of integrin

alpha5beta1 in human abdominal aortic aneurysm and healthy aortic tissues and its significance in pathogenesis. J. Surg. Res. 118, 176-182.

Clarke M.C., Littlewood T.D., Figg N., Maguire J.J., Davenport A.P., Goddard M. and Bennett M.R. (2008). Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. Circ. Res. 102, 1529-1538.

Clemmons D.R. and Maile L.A. (2003). Minireview: Integral membrane proteins that function coordinately with the insulin-like growth factor I receptor to regulate intracellular signaling. Endocrinology 144, 1664-1670.

Cohn R.D., Mayer U., Saher G., Herrmann R., van der Flier A., Sonnenberg A., Sorokin L. and Voit T. (1999). Secondary reduction of alpha7B integrin in laminin alpha2 deficient congenital muscular dystrophy supports an additional transmembrane link in skeletal muscle. J. Neurol. Sci. 163, 140-152.

D'Angelo A., Geroldi D., Hancock M.A., Valtulina V., Cornaglia A.I., Spencer C.A., Emanuele E., Calligaro A., Koschinsky M.L., Speziale P. and Visai L. (2005). The apolipoprotein(a) component of lipoprotein(a) mediates binding to laminin: contribution to selective retention of lipoprotein(a) in atherosclerotic lesions. Biochim. Biophys. Acta 1687, 1-10

Dekkers B.G., Schaafsma D., Nelemans S.A., Zaagsma J. and Meurs H. (2007). Extracellular matrix proteins differentially regulate airway smooth muscle phenotype and function. Am. J. Physiol. Lung Cell Mol. Physiol. 292, L1405-1413.

Dingemans K.P., Teeling P., Lagendijk J.H. and Becker A.E. (2000). Extracellular matrix of the human aortic media: an ultrastructural histochemical and immunohistochemical study of the adult aortic media. Anat. Rec. 258, 1-14.

Doran A.C., Meller N. and McNamara C.A. (2008). Role of smooth muscle cells in the initiation and early progression of atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 28, 812-819.

Erb M., Meinen S., Barzaghi P., Sumanovski L.T., Courdier-Fruh I., Ruegg M.A. and Meier T. (2009). Omigapil ameliorates the pathology of muscle dystrophy caused by laminin-alpha2 deficiency. J. Pharmacol. Exp. Ther. 331, 787-795.

Gawlik K.I., Li J.Y., Petersen A. and Durbeej M. (2006a). Laminin alpha1 chain improves laminin alpha2 chain deficient peripheral neuropathy. Hum. Mol. Genet. 15, 2690-2700.

Gawlik K.I., Mayer U., Blomberg K., Sonnenberg A., Ekblom P. and Durbeej M. (2006b). Laminin alpha1 chain mediated reduction of laminin alpha2 chain deficient muscular dystrophy involves integrin alpha7beta1 and dystroglycan. FEBS Lett. 580, 1759-1765.

Genersch E., Ferletta M., Virtanen I., Haller H. and Ekblom P. (2003). Integrin alphavbeta3 binding to human alpha5-laminins facilitates FGF-2- and VEGF-induced proliferation of human ECV304 carcinoma cells. Eur. J. Cell Biol. 82, 105-117.

Glukhova M., Koteliansky V., Fondacci C., Marotte F. and Rappaport L. (1993). Laminin variants and integrin laminin receptors in developing and adult human smooth muscle. Dev. Biol. 157, 437-447.

Goncalves I., Ares M.P.S., Moberg A., Moses J., To F., Montan J., Pedro L.M., Dias N., Fernandes e Fernandes J., Nordin Fredrikson G., Nilsson J., Jovinge S. and Bengtsson E. (2008). Elastin- and collagen-rich human carotid plaques have increased levels of the cysteine protease inhibitor cystatin C. J. Vasc. Res. 45, 395-401

Gorfu G., Virtanen I., Hukkanen M., Lehto V.P., Rousselle P., Kenne E., Lindbom L., Kramer R., Tryggvason K. and Patarroyo M. (2008). Laminin isoforms of lymph nodes and predominant role of alpha5-laminin(s) in adhesion and migration of blood lymphocytes. J. Leukoc. Biol. 84, 701-712.

Halayko A.J., Tran T. and Gosens R. (2008). Phenotype and functional plasticity of airway smooth muscle: role of caveolae and caveolins. Proc. Am. Thorac. Soc. 5, 80-88.

Hallmann R., Horn N., Selg M., Wendler O., Pausch F. and Sorokin L.M. (2005). Expression and function of laminins in the embryonic and mature vasculature. Physiol. Rev. 85, 979-1000.

Hassan G.S., Jasmin J.F., Schubert W., Frank P.G. and Lisanti M.P. (2004). Caveolin-1 deficiency stimulates neointima formation during vascular injury. Biochemistry 43, 8312-8321.

Hedin U., Bottger B.A., Forsberg E., Johansson S. and Thyberg J. (1988). Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. J. Cell Biol. 107, 307-319.

Heerkens E.H., Shaw L., Ryding A., Brooker G., Mullins J.J., Austin C., Ohanian V. and Heagerty A.M. (2006). alphaV integrins are necessary for eutrophic inward remodeling of small arteries in hypertension. Hypertension 47, 281-287.

Ido H., Ito S., Taniguchi Y., Hayashi M., Sato-Nishiuchi R., Sanzen N., Hayashi Y., Futaki S. and Sekiguchi K. (2008). Laminin isoforms containing the gamma3 chain are unable to bind to integrins due to the absence of the glutamic acid residue conserved in the C-terminal regions of the gamma1 and gamma2 chains. J. Biol. Chem. 283, 28149-28157.

Kawataki T., Yamane T., Naganuma H., Rousselle P., Anduren I., Tryggvason K. and Patarroyo M. (2007). Laminin isoforms and their integrin receptors in glioma cell migration and invasiveness: Evidence for a role of alpha5-laminin(s) and alpha3beta1 integrin. Exp. Cell Res. 313, 3819-3831.

Kelleher C.M., McLean S.E. and Mecham R.P. (2004). Vascular extracellular matrix and aortic development. Curr. Top. Dev. Biol. 62, 153-188.

Kingsley K., Rust W.L., Huff J.L., Smith R.C. and Plopper G.E. (2002). PDGF-BB enhances expression of, and reduces adhesion to, laminin-5 in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 294, 1017-1022.

Lenter M., Uhlig H., Hamann A., Jeno P., Imhof B. and Vestweber D. (1993). A monoclonal antibody against an activation epitope on mouse integrin chain beta 1 blocks adhesion of lymphocytes to the endothelial integrin alpha 6 beta 1. Proc. Natl. Acad. Sci. USA 90, 9051-9055.

Maile L.A., Capps B.E., Ling Y., Xi G. and Clemmons D.R. (2007). Hyperglycemia alters the responsiveness of smooth muscle cells to insulin-like growth factor-I. Endocrinology 148, 2435-2443.

Miner J.H. (2008). Laminins and their roles in mammals. Microsc. Res. Tech. 71, 349-356.

Munoz J., Zhou Y. and Jarrett H.W. (2010). LG4-5 domains of laminin-211 binds alpha-dystroglycan to allow myotube attachment and prevent anoikis. J. Cell Physiol. 222, 111-119.

Newby A.C. and Zaltsman A.B. (1999). Fibrous cap formation or destruction--the critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. Cardiovasc. Res. 41, 345-360.

Patton B.L. (2000). Laminins of the neuromuscular system. Microsc. Res. Tech. 51, 247-261.

Ringelmann B., Roder C., Hallmann R., Maley M., Davies M., Grounds M. and Sorokin L. (1999). Expression of laminin alpha1, alpha2, alpha4, and alpha5 chains, fibronectin, and tenascin-C in skeletal

muscle of dystrophic 129ReJ dy/dy mice. Exp. Cell Res. 246, 165-182.

Sadeghi M.M., Krassilnikova S., Zhang J., Gharaei A.A., Fassaei H.R., Esmailzadeh L., Kooshkabadi A., Edwards S., Yalamanchili P., Harris T.D., Sinusas A.J., Zaret B.L. and Bender J.R. (2004). Detection of injury-induced vascular remodeling by targeting activated alphavbeta3 integrin in vivo. Circulation 110, 84-90.

Sakai T., Johnson K.J., Murozono M., Sakai K., Magnuson M.A., Wieloch T., Cronberg T., Isshiki A., Erickson H.P. and Fassler R. (2001). Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. Nat. Med. 7, 324-330.

Sasaki T., Mann K., Miner J.H., Miosge N. and Timpl R. (2002). Domain IV of mouse laminin beta1 and beta2 chains. Eur. J. Biochem. 269, 431-442.

Schlotzer-Schrehardt U., Dietrich T., Saito K., Sorokin L., Sasaki T., Paulsson M. and Kruse F.E. (2007). Characterization of extracellular matrix components in the limbal epithelial stem cell compartment. Exp. Eye Res. 85, 845-860.

Schuler F. and Sorokin L.M. (1995). Expression of laminin isoforms in mouse myogenic cells in vitro and in vivo. J. Cell Sci. 108 (Pt 12), 3795-3805.

Sixt M., Engelhardt B., Pausch F., Hallmann R., Wendler O. and Sorokin L.M. (2001). Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. J. Cell Biol. 153, 933-946.

Sorokin L.M., Pausch F., Frieser M., Kroger S., Ohage E. and Deutzmann R. (1997). Developmental regulation of the laminin alpha5 chain suggests a role in epithelial and endothelial cell maturation. Dev. Biol. 189, 285-300.

Ström A., Ahlqvist E., Franzen A., Heinegard D. and Hultgardh-Nilsson A. (2004). Extracellular matrix components in atherosclerotic arteries of Apo E/LDL receptor deficient mice: an immunohistochemical study. Histol. Histopathol. 19, 337-347.

Wewer U., Albrechtsen R., Manthorpe M., Varon S., Engvall E. and Ruoslahti E. (1983). Human laminin isolated in a nearly intact, biologically active form from placenta by limited proteolysis. J. Biol. Chem. 258, 12654-12660.

von der Mark H., Poschl E., Lanig H., Sasaki T., Deutzman R. and von der Mark K. (2007). Distinct acidic clusters and hydrophobic residues in the alternative splice domains X1 and X2 of alpha7 integrins define specificity for laminin isoforms. J. Mol. Biol. 371, 1188-1203.

Yao C.C., Breuss J., Pytela R. and Kramer R.H. (1997). Functional expression of the alpha 7 integrin receptor in differentiated smooth muscle cells. J. Cell Sci. 110 (Pt 13), 1477-1487.

Zenker M., Aigner T., Wendler O., Tralau T., Muntefering H., Fenski R., Pitz S., Schumacher V., Royer-Pokora B., Wuhl E., Cochat P., Bouvier R., Kraus C., Mark K., Madlon H., Dotsch J., Rascher W., Maruniak-Chudek I., Lennert T., Neumann L.M. and Reis A. (2004). Human laminin beta2 deficiency causes congenital nephrosis with mesangial sclerosis and distinct eye abnormalities. Hum. Mol. Genet. 13, 2625-2632.

Accepted December 22, 2010