Cytokeratins 8 and 18 in Smooth Muscle Cells
Detection in Human Coronary Artery, Peripheral Vascular, and Vein Graft Disease and in Transplantation-Associated Arteriosclerosis

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During development of atherosclerotic lesions, vascular smooth muscle cells (SMCs) undergo changes both phenotypically and in their cytoskeleton composition. An expression of cytokeratins 8 and 18 in SMCs in plaques of the human superficial femoral artery and of cytokeratin 8 in lesions of the aorta was recently described. Since cytokeratins are epithelial markers generally not found in normal adult vascular SMCs, we performed a detailed immunofluorescence microscopy study using a large panel of antibodies against the various cytokeratin polypeptides and other elements of the cytoskeleton. We included lesions of carotid, common and superficial femoral, iliac, and popliteal arteries; the abdominal aorta; and saphenous vein bypass grafts, as well as primary, restenotic, and transplantation-associated lesions of coronary arteries (n=33). Cytokeratins 8 and 18 were present in myointimal cells of all pathological specimens. Colocalization with smooth muscle α-actin identified most cytokeratin-positive cells as SMCs. Only very few cells cosynthesized cytokeratin and desmin, whereas the majority of cytokeratin-positive cells were vimentin-positive. This pattern of cytoskeletal protein synthesis is similar to that found in some fetal and/or neonatal SMCs. These findings suggest that the synthesis of cytokeratins in a subset of SMCs of atherosclerotic lesions is a common phenomenon in coronary artery and peripheral vascular disease as well as graft disease and transplantation-associated arteriosclerosis and that the state of these SMCs is of a "dedifferentiated" fetal type. (Arterioscler Thromb. 1993;13:1631-1639.)

Key Words • atherosclerosis • smooth muscle cells • cytokeratins • cytoskeletal proteins

Proliferation of vascular smooth muscle cells (SMCs) is a major cause of clinical complications of peripheral and coronary artery disease, neointimal healing after vessel injury, bypass graft failure, and transplantation-associated arteriosclerosis.1-4 In vitro experiments showed that the onset of SMC proliferation is preceded by a phenotypic modulation of contractile SMCs into a synthetic state.5,6 Ultrastructurally, synthetic SMCs show abundant rough endoplasmic reticulum, a high density of Golgi complexes, and few myofilaments, indicating substantial changes in their state of differentiation. Recent studies using antibodies against polypeptides known to be markers of tissue differentiation, such as intermediate filament proteins and the proteins of the contractile apparatus, have characterized SMCs in normal vessels and atherosclerotic lesions. During the development of atherosclerotic lesions, actin and myosin expression shifts to isoforms found predominantly in fetal vessels,7-11 although a complete "on" or "off" switch of either isoform was reported only for an embryonic smooth muscle myosin isoform.12

The intermediate filament proteins seen in SMCs of normal and atherosclerotic vessels are desmin and/or vimentin, with a lower number of desmin-expressing cells in diseased compared with normal tissue.10,13 Strikingly, cytokeratins 8 and 18 together were found in SMCs in atherosclerotic plaques of the superficial femoral artery and in one peripheral saphenous vein graft14,15 and cytokeratin 8 was found in lesions of the aorta.16,17 Since cytokeratins are known to be hallmarks of epithelial differentiation and usually are not found in muscle tissue, these initial observations were unexpected and described a neoeexpression of two genes during atherogenesis, in contrast with the partial upregulation or downregulation of most isoforms of the contractile proteins.

However, these studies were limited by their focusing on only the early stages of the atherosclerotic lesion or a single localization.15-17 and two of these studies did not include monospecific antibodies against cytokeratins other than cytokeratin 8.16,17 Using a large panel of antibodies against the various cytokeratin polypeptides and against other elements of the cytoskeleton, we now analyzed a variety of atherosclerotic lesions by immunofluorescence microscopy, including specimens of ca-
rotid, iliac, common femoral, superficial femoral, and popliteal arteries; abdominal aorta; peripheral and coronary vein grafts; and primary, restenotic, and heart transplantation-associated lesions of the coronary arteries.

Methods

Tissues

Tissue samples from human coronary arteries (n=7) and stenosed aortocoronary vein bypass grafts (n=2) were obtained by percutaneous atherectomy. Segments from atherosclerotic human carotid (n=4), iliac (n=3), common femoral (n=3), superficial femoral (n=5), and popliteal arteries (n=2); abdominal aorta (n=2); and peripheral saphenous vein bypass grafts (n=2) were obtained during bypass surgery or endatherectomy (Department of Vascular Surgery, Surgical Clinics, University of Heidelberg). Lesions, classified as described by Ross,18 ranged from intimal thickenings to complicated lesions. Femoral artery and aorta segments with mild intimal thickenings and samples from patients with transplantation-associated arteriosclerosis (n=3) were obtained from autopsies. Tissue samples were immediately snap-frozen in isopentane precooled to approximately −130°C in liquid nitrogen.

Antibodies

The following murine monoclonal antibodies against cytokeratins were used: Ks 8.13, KL 1, and lu-5 reacting with a broad range of cytokeratin polypeptides; Ks 8.17.2 and CAM 5.2 against cytokeratin 8; Ks 18.174, Ks 18.18, Ks 18.27, and RGE-53 against cytokeratin 18; 6B10 against cytokeratin 4; 1C7 against cytokeratin 13; and Ks 19.1 and Ks 19.2 against cytokeratin 19. We also used affinity-purified guinea pig antibodies raised against cytokeratins 8 and 18. References and sources of these antibodies are given in Reference 19. Antibodies against vimentin (clone 3B4 from Progen, Heidelberg, Germany; clone V9 from Boehringer Mannheim, Indianapolis, Ind) and desmin (DE-B-5, from Boehringer Mannheim) were commercially available monoclonal antibodies, as were the antibodies against desmplakins (DP1 and 2-2.15) and the smooth muscle–specific isoform of α-actin (clone asm-1), both from Progen. Monoclonal antibodies against proliferating cell nuclear antigen (PCNA, clone 19F4) were from Boehringer Mannheim. To identify endothelial cells, we used rabbit antibodies against factor VIII–related antigen (from Behringwerke, Marburg, Germany). Secondary antibodies were Texas red– or fluorescein isothiocyanate–coupled, affinity-purified donkey anti-mouse, anti-rab-
Immunofluorescence Microscopy

Cryosections 5 μm thick were thaw-mounted on glass slides and air-dried. Sections were fixed in cold acetone (5 minutes at −20°C) followed by a short dip in cold methanol (−20°C). After air-drying, the tissue was rehydrated directly by applying primary antibodies that were incubated for 30 minutes in a humid chamber. After three washes in phosphate-buffered saline (PBS, pH 7.4) for 5 minutes each, secondary antibodies were applied for 30 minutes. For double-label experiments, primary and secondary antibodies were applied simultaneously at 2× concentration. Slides were again washed three times in PBS, briefly rinsed with deionized water, dehydrated in 100% ethanol for 3 minutes, air-dried, and mounted in Mowiol (Calbiochem, San Diego, Calif).

Results

Immunofluorescence Microscopic Analysis of Lesions in Peripheral Vascular Disease

In this study, we examined peripheral lesions from different sites of the cardiovascular system ranging from mild intimal thickenings to complicated lesions (for numbers and classification of lesions, see "Methods"). When we looked at intimal thickenings with fewer than eight cell layers, we found no or only sparsely scattered cells in the intima that reacted with antibodies against cytokeratins 8 and 18, whereas the vast majority of cells of the intima and all cells of the media were cytokeratin-negative (Fig 1a and 1b). However, we consistently found few cytokeratin-containing cells in intimal thickenings of more than 8 to 10 cell layers and many in atherosclerotic aneurysms of the abdominal aorta (Fig 1c). These cytokeratin-producing cells were negative with antibodies specific to cytokeratins other than cytokeratins 8 and 18 as well as with antibodies against desmoplakins. An example of such a lesion with a relatively high number of cytokeratin-producing cells is shown in Fig 2a and 2b. Most cytokeratin-positive cells were found in the innermost cell layers of the intima, often arranged in patches of positive cells. In more advanced lesions, we found cytokeratin-positive cells at a much higher frequency. The highest number of cytokeratin-producing cells was found in the cap region of advanced lesions with a high proportion of SMCs. A complicated fibromuscular lesion of a superficial femoral artery with a fibrous cap, very little lipid deposit, and a very high number of cytokeratin-producing cells is shown in Fig 2c and 2d. Double-label immunofluorescence microscopy using guinea pig antibodies against cytokeratins 8 and 18 together with a monoclonal antibody against the smooth muscle–specific isoform of α-actin (asm-1), performed on all specimens included in...
FIG 3. Double-label immunofluorescence photomicrographs of a common femoral artery with diffuse intimal thickening using monoclonal antibody K8.17.2 against cytokeratin 8 (a) in combination with a rabbit serum against von Willebrand factor (b; c, same field, phase optics). Cytokeratin immunoreactivity is restricted to intimal cells, whereas endothelial cells, labeled by antibodies to von Willebrand factor (arrowheads in a and b), and cells of the media are absolutely negative. The internal elastic lamina (arrows) shows moderate autofluorescence. Reaction of the same antibody against cytokeratin 8 on a peripheral saphenous vein bypass graft, with proliferation of medial cells that critically narrowed the lumen of the vessel, is shown in d. Corresponding field under phase-contrast microscopy is shown in e. Bars=20 μm.

this study, confirmed the muscle differentiation of these cells. Desmin was coexpressed only in a minor portion of these cytokeratin-containing SMCs. SMCs of the media and adventitia were only in very rare and exceptional cases cytokeratin-positive, even in vessels in which virtually all SMCs of the intimal lesion were cytokeratin-positive. Desmoplakin antibodies and cytokeratin antibodies not reacting with cytokeratins 8 or 18 were negative on both cells of the media as well as on intimal cells, as opposed to antibodies to vimentin that were positive on almost all cells of the lesions. All antibodies reacting with either cytokeratin 8 or 18 showed a similar staining pattern.

Antibodies to PCNA showed a nuclear reaction in <1% of cytokeratin-producing cells (not shown). Since cytokeratin 8– and 18–synthesizing endothelial cells were reported in synovial tissue, we routinely performed double-label immunohistochemistry with antibodies against both cytokeratins and von Willebrand factor, but we never found such cytokeratin-synthesizing endothelial cells in either type of lesion (see Fig 3a through 3c).

Lesions in Coronary Artery Disease

When we examined specimens that were obtained by percutaneous coronary atherectomy, including two specimens of the left anterior descending coronary artery with restenotic lesions, we found a high frequency of cytokeratin-producing intimal SMCs. In some specimens, almost all intimal SMCs were cytokeratin-positive, as demonstrated by an overview photomontage in Fig 4. Fig 5 shows the typical pattern of cytokeratin (Fig 5a, 5d, and 5f), smooth muscle α-actin (Fig 5b and 5g), and desmin immunostaining (Fig 5e) of lesions of the coronary arteries in double-label immunohistochemistry. Most of these cytokeratin-expressing cells were found in intimal SMCs. Occasionally, however, scattered SMCs of the media showed weak cytokeratin immunoreactivity and usually coexpressed desmin and smooth muscle α-actin. We also found cytokeratins 8
Lesions in Vein Graft Disease

We included in this study four diseased saphenous vein grafts, two peripheral (see Fig 3d and 3e), and two coronary artery bypass grafts. Histologically, lesions in all grafts were of the fibromuscular type. In all specimens, we frequently found cytokeratin-synthesizing cells that also contained vimentin and smooth muscle α-actin. These cells usually did not show any desmin reactivity. Differences between peripheral and coronary artery grafts with regard to the composition of cytokeratins, vimentin, desmin, and smooth muscle α-actin in SMCs of the lesions were not observed.

Lesions in Transplantation-Associated Arteriosclerosis

Transplantation-associated arteriosclerotic lesions were concentric, with a preserved internal elastic lamina. Most cells were SMCs, as assessed by immunostaining using monoclonal antibody asm-1 against smooth muscle α-actin (not shown). Cytokeratin 8- and 18-positive cells were observed in the intima, usually in clusters close to the luminal surface, whereas adjacent areas without any obvious histological differences did not contain any cytokeratin-positive cells. An area of such a lesion with cytokeratin 18-positive cells is shown in Fig 7. As in all other lesions examined, we could not detect desmoplakins or other cytokeratin polypeptides.

Discussion

We have investigated the occurrence of cytokeratins, generally known as markers of epithelial differentiation, in SMCs of a variety of atherosclerotic lesions from different sites of the cardiovascular system. Strikingly, we found cytokeratin 8- and 18-synthesizing cells in all specimens examined, including two forms of vascular lesions with potentially different pathogeneses, the restenotic coronary lesion after invasive intervention and heart transplantation-associated arteriosclerosis. Double-label immunofluorescence microscopy clearly identified the majority of these cells as SMCs by their positive reaction with antibodies against smooth muscle α-actin. The frequency of these cytokeratin-containing cells was somewhat variable but generally increased with the severity of the lesion from intimal thickening to complicated lesions. Mild intimal thickenings showed

and 18 in vascular SMCs in a few vasa vasorum of the adventitia and of the lesions (Fig 5a). When we looked at restenotic lesions of the coronary arteries, we found the same pattern of intermediate filament protein composition in SMCs as in primary stenoses. Most intimal cells displayed vimentin and less cytokeratin immunoreactivity, whereas only a small number were desmin-positive. The majority of cytokeratin-positive cells also contained smooth muscle α-actin (see Fig 6). As in the peripheral lesions, we did not find any evidence for an expression of desmoplakins or cytokeratin polypeptides other than numbers 8 and 18.

Fig 4. Photomontage of cytokeratin immunoreactivity of predominantly intimal tissue obtained by percutaneous atherectomy from a primary stenosis of the left coronary artery. Note that almost all cells of this fibromuscular lesion show immunoreactivity with the monoclonal antibody K518.174, which is specific for cytokeratin 18. Bar=40 μm.
Fig 5. Double-label immunostaining of atherectomy specimens of the left anterior descending coronary artery obtained from primary stenoses. a, d, and f, Epifluorescence using a guinea pig serum against cytokeratins 8 and 18; b and g, anti-smooth muscle α-actin (monoclonal antibody asm-1); and e, desmin immunoreactivity of the corresponding fields. c Shows the same field as a and b under phase-contrast microscopy. Note that most cells positive for cytokeratin are also smooth muscle α-actin-positive (a and b, f and g) but desmin negative (d and e). Examples of cells reacting with both smooth muscle α-actin and cytokeratin antibodies are marked by arrows (f and g). One cell containing only cytokeratins is marked by an arrowhead (f). Groups of cells corresponding to the vasa vasorum in the lesions are marked with curved arrows (a, b, f, and g). Most smooth muscle cells of these vessels are cytokeratin-negative. The former luminary side of the vessels is marked by an L. Bars=50 μm.

Only scattered, if any, cytokeratin-positive cells, whereas in advanced lesions of arteries of the muscular type, almost all SMCs were positive by immunostaining with antibodies against cytokeratins 8 and 18. In most fibromuscular lesions, particularly in vein grafts, cytokeratin-positive cells occurred in clusters, raising the possibility...
FIG 6. Double-label immunofluorescence microscopy of a restenotic lesion from a left coronary artery that occurred 3 months after the primary intervention. The specimen was obtained by coronary atherectomy. The left panel shows a cytokeratin reaction with a guinea pig serum against cytokeratins 8 and 18; b, epifluorescence of the smooth muscle α-actin reaction; d, vimentin reaction. Most of the cells containing cytokeratins coexpress smooth muscle α-actin (arrowheads in a and b) and vimentin (c and d). Arrows mark two cytokeratin-positive cells that are negative for the smooth muscle α-actin antibody. Bars=50 μm.

that a diffusible factor synthesized by cells in the vessel wall might be involved in the induction of cytokeratin expression. Conversely, endothelial cells were never found to be cytokeratin-positive, even when they were in the direct vicinity of cytokeratin-positive SMCs (for cytokeratin-producing endothelial cells in other tissues, see References 19 and 20).

In addition to SMCs of atherosclerotic lesions, expression of cytokeratins 8, 18, and some additional cytokeratin 19 was described not only in a subset of fetal and embryonic vascular SMCs, but in certain vessels of synovial tissue from patients with rheumatoid arthritis, and in trabecular arteries of the spleen but also in variable frequencies in certain nonvascular SMCs of

FIG 7. Cytokeratin immunostaining of a coronary artery with heart transplantation–associated arteriosclerosis. a, Monoclonal antibody Ks18.174 against cytokeratin 18; b, corresponding phase-contrast photomicrograph. The intima is concentrically thickened, with most cytokeratin-positive cells in the portion near the lumen. The internal elastic lamina shows bright autofluorescence (arrows). Bar=20 μm.
leiomyosarcomas, myomas, myometrium, \textsuperscript{23-28} trabeculae of the spleen,\textsuperscript{20} and in embryonic stages of the muscular ring layer of the rectum.\textsuperscript{20} These cytokeratins are the most “fundamental” (simple epithelial) ones, the earliest in embryogenesis and practically the only ones that can appear—mostly in pathological situations—in certain nonepithelial cells such as smooth muscles and in myocyte-derived tumors.

The advent of cytokeratins in mesenchymally derived SMCs does not necessarily imply that these cells initiate a broader epithelial program of differentiation. We have not detected any SMCs with morphological alterations indicative of epithelial cells, nor have we noticed reactions with antibodies against desmosomal proteins, supporting the hypothesis of Knapp and Franke\textsuperscript{30} that the genes encoding cytokeratins 8 and 18 are under a “loose” control and are readily inducible under certain conditions without concomitant expression of other epithelial markers. Quantitative Western blot analysis using antibodies also used in the present study had revealed a very low level of expression of cytokeratins 8, 18, and 19 in SMCs of the human umbilical cord vessels.\textsuperscript{22} That 5-azacytidine can induce the expression of cytokeratins 8 and 18 in some nonepithelial transformed cells, including SMC-derived cell lines, suggests that hypomethylation of cytosine residues may be involved in the induction of those genes.\textsuperscript{30-32}

Since cytokeratins 8 and 18 are also transiently expressed in another mesenchymal cell type with a high rate of proliferation, the blastema cells of the regenerating newt limb,\textsuperscript{23} and can appear in some cells of rapidly proliferating cultures of transformed fibroblasts,\textsuperscript{30,31} we have also examined the hypothesis that cytokeratin-positive SMCs may have a higher proliferation rate than “normal,” cy, cytokeratin-negative, SMCs. We used an antibody against PCNA as a proliferation marker; however, we found $<1\%$ PCNA-positive cells, even in lesions with $>95\%$ SMCs containing cytokeratins, indicating that there is no direct correlation between proliferation and cytokeratin expression in SMCs. This finding is also supported by our observation that in restenotic coronary lesions that occurred within 6 months after invasive intervention, the number of cytokeratin-expressing cells is not higher than in primary lesions.

Cytokeratin-positive SMCs often did not synthesize desmin, the intermediate filament protein typically found in muscle cells. This observation is in agreement with previous reports about lesions in peripheral blood vessels\textsuperscript{10,11,19} and does not contradict the muscle character of these cells, since the filaments of SMCs of normal vessels, particularly of the elastic type, such as human and rat thoracic aorta, often do not contain desmin but instead vimentin.\textsuperscript{10,34,37} The muscle differentiation of desmin-negative SMCs in atherosclerotic lesions was confirmed in a number of studies, including the present one, showing that these cells contain smooth muscle-type α-actin.\textsuperscript{2,3,9}

Functional differences between desmin-positive and desmin-negative SMCs have been reported using primary culture SMCs of rat aorta. When cultured in the presence of low-density lipoprotein (LDL), desmin-negative/vimentin-positive SMCs accumulated more LDL than those SMCs that contained both desmin and vimentin.\textsuperscript{40} This observation also may provide a link between the differential expression of intermediate filament proteins and a change of function, which might be important in the generation of SMC-derived foam cells.

Interestingly, the pattern of synthesis of intermediate filament proteins in SMCs of atherosclerotic lesions resembles the one found in the innermost layers of the human umbilical cord arteries, since they contain no desmin but rather vimentin and cytokeratins 8 and 18.\textsuperscript{10,38} Moreover, intercellular adhesion molecule 1, a polypeptide found in fetal mesenchymally derived cells but not in normal adult vascular tissue, is induced during atherogenesis in different cell types, including SMCs,\textsuperscript{41} as is the embryonic isoform of smooth muscle myosin in experimental atherosclerosis in rabbits.\textsuperscript{12}

Whether these similarities of expression in certain fetal SMCs and in SMCs of atherosclerotic lesions reflect common functions different from those of other SMCs remains to be seen in future experiments also using primary cultures from atherosclerotic lesions.

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