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Proliferation and Extracellular Matrix Synthesis of Smooth Muscle Cells Cultured From Human Coronary Atherosclerotic and Restenotic Lesions

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Objectives. The purpose of this study was to examine the proliferative capacity and extracellular matrix synthesis of human coronary plaque cells in vitro.

Background. Common to both primary atherosclerosis and restenosis are vascular smooth muscle cell proliferation and production of extracellular matrix proteins. The applicability to humans of experimental animal models of these processes has been questioned.

Methods. Primary atherosclerotic and restenotic lesions were excised by percutaneous directional coronary atherectomy in 93 patients. Smooth muscle cells were cultivated by an explant technique and identified by their morphology in culture, ultrastructural features under electron microscopy and immunostaining using monoclonal antibodies to smooth muscle cell alpha-actin. Proliferation in secondary culture was assessed with growth curves and the synthesis of collagen and sulfated glycosaminoglycans by the incorporation of ^{3}H-proline and ^{35}S-sulfate, respectively. These studies were also performed in cells derived from human umbilical artery media.

Results. Success rates for primary (45%) and secondary (12%) culture of coronary cells were not influenced by clinical variables or lesion category. Primary culture success was improved by the presence of organized thrombus in the plaque and in relation to increased maximal cell density of the atherectomy specimen. Restenotic cells displayed more rapid growth than did cells of primary atherosclerotic origin, which grew in a manner similar to that of umbilical artery cells. Mean calculated population-doubling times for the three cell groups were 52 h (95% confidence interval [CI] 48 to 58 h), 71 h (95% CI 62 to 83 h) and 74 h (95% CI 65 to 84 h), respectively. Restenotic and primary atherosclerotic cells did not differ in the synthesis of collagen ([mean ± SEM] 0.034 ± 0.004 vs. 0.033 ± 0.004 nmol isotope·μg protein^{-1}, p = NS) or sulfated glycosaminoglycans (11.47 ± 1.07 vs. 15.37 ± 3.10 nmol isotope·μg protein^{-1}, p = NS), but the coronary cells synthesized significantly more collagen and sulfated glycosaminoglycans than did umbilical artery cells (0.019 ± 0.004 and 5.43 ± 1.00 nmol isotope·μg protein^{-1}, respectively, both $p < 0.05$).

Conclusions. These data indicate that increased smooth muscle cell proliferation contributes to coronary restenosis in humans and support the concept that the extracellular matrix synthesis of adult smooth muscle cells is important to lesion formation.

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Migration, proliferation and extracellular matrix synthesis are properties of the vascular smooth muscle cell central to both primary atherosclerosis and restenosis, the vascular response to injury that may be provoked by balloon dilation (1–8). In cell culture, vascular smooth muscle cells retain many of their in vivo characteristics, thus allowing the study of specific cellular properties under controlled conditions in vitro. Early experimental work with smooth muscle cells derived from human atherosclerotic lesions depended on tissue removed from peripheral arteries at surgery or autopsy (3,9–11), but fresh human atherosclerotic tissue can now be retrieved from both peripheral and coronary arteries by directional atherectomy (12,13). The feasibility of culture, characterization and investigation of smooth muscle cells from tissue obtained by directional atherectomy has been demonstrated (14–17).

Recent reviews of mechanisms in atherosclerosis (6) and in the arterial response to mechanical injury (7,8) have focused more on the migration and proliferation of vascular smooth muscle cells than on extracellular matrix synthesis. However, the contribution of the extracellular matrix to primary atherosclerotic and restenotic lesions has been well defined histologically (3,18,19), and there is experimental evidence that after balloon injury, extracellular matrix dep-

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Table 1. Clinical Characteristics of 93 Patients With Directional Coronary Atherectomy

	No. (%)
Age (yr) (mean ± SEM)	58 ± 1
Men	77 (83)
Women	16 (17)
Stable angina	48 (52)
Unstable angina	45 (48)
Primary atherosclerotic lesion	69 (74)
Restenotic lesion	24 (26)
Vessel	
LAD	59 (63)
LCx	11 (12)
RCA	19 (21)
SV Graft	4 (4)

LAD = left anterior descending coronary artery; LCx = left circumflex coronary artery; RCA = right coronary artery; SV Graft = saphenous vein coronary bypass graft; Unstable angina = typical pain occurring at rest, with or without accompanying electrocardiographic changes, despite maximal medical therapy.

osition outlasts cellular proliferation and accounts for the bulk of the resulting intimal lesion (20). It has been proposed, too, that the extracellular matrix may exert important effects on the migration and proliferation of the vascular smooth muscle cell (21,22).

We cultivated smooth muscle cells from primary atherosclerotic and restenotic coronary lesions in humans, obtained by directional atherectomy, to study both the proliferative behavior and the synthesis of collagen and sulfated glycosaminoglycans, principal components of the extracellular matrix, of the cultured cells. In establishing this in vitro model of processes central to the formation of vascular intimal lesions, we also examined whether adult coronary plaque smooth muscle cells displayed properties in culture different to those of smooth muscle cells from an alternative immature arterial source—the normal media of human umbilical arteries.

Methods

Patients and procedure. A series of 93 patients underwent percutaneous directional coronary atherectomy. Clinical characteristics are summarized in Table 1. Lesions were designated restenotic when associated with a ≥50% diameter stenosis at the site of a previous intervention including conventional (balloon) percutaneous transluminal coronary angioplasty (n = 18 [75%]); stents (n = 4 [17%]); and directional atherectomy (n = 2 [8%]). The target vessels included four long-standing saphenous vein grafts, all with restenotic lesions. Informed consent was obtained from all patients before the procedure, which was performed using the Simpson Coronary AtheroCath (Devices for Vascular Intervention) according to a protocol approved by our hospital ethics committee and described elsewhere (23). Under sterile conditions, specimens were flushed from the device with 0.9% saline solution and placed immediately in serum-free M199 culture medium for transfer to the culture laboratory.

Cell culture. Excised coronary tissue was placed in cell culture according to the method described by Bauriedel et al. (14). Tissue explants were placed on round glass coverslips coated with fibronectin (10 $\mu g \cdot cm^{-2}$) in 2-cm^2 wells, and culture medium (M199 supplemented with glutamine, 10% human serum, 10% fetal calf serum, penicillin [100 $IU \cdot ml^{-1}$] and streptomycin [0.1 $mg \cdot ml^{-1}$]) was added. For primary cultures, conditioned medium from actively growing umbilical artery medial smooth muscle cell strains was mixed 1:1 with the standard culture medium. In secondary culture, the culture medium was not supplemented with conditioned medium. For human umbilical artery smooth muscle cell culture, 3- to 4-mm^3 pieces of arterial wall were dissected free from fresh human umbilical cord sections and cultured in fibronectin-coated 24-well plates using standard unconditioned culture medium. All cultures were incubated at 37°C in a moist atmosphere under 5% carbon dioxide. At confluency, cells were subcultured by trypsinizing.

Histology. A small portion of the atherectomy specimen was set aside for histology. This tissue was divided into approximately 1-mm^3 pieces with a sterile scalpel, fixed in 10% buffered formalin and processed for light microscopy with hematoxylin-azofloxin and Verhoeff-van Gieson stains.

Assessment of maximal cell density. Early studies used tissue from the initial 12 patients to assess maximal cell density. Cell nuclei were counted in several microscope fields in hematoxylin-azofloxin–stained sections by means of a computerized morphometric system (IBAS, Kontron). The maximal value recorded was used for the estimation of maximal cell density, expressed as $cells \cdot mm^{-2}$.

Identification of smooth muscle cells by immunostaining and electron microscopy. Cells from passages 2 to 4 were seeded on round glass coverslips at a density of 5,000 $cells \cdot cm^{-2}$. After 48 h, the coverslips were rinsed in phosphate-buffered saline solution (pH 7.4) before fixing in acetone at −20°C. Immunostaining was performed with monoclonal antibodies directed against smooth muscle cell alpha-actin (DAKO) using an indirect immunoperoxidase procedure (24). For electron microscopy, cells cultured in 10-cm^2–diameter petri dishes were fixed in a phosphate-buffered mixture of 4% paraformaldehyde and 1% glutaraldehyde, postfixed in 1% osmium tetraoxide and dehydrated in alcohol. Thereafter, embedding capsules filled with Epon were placed upside down on the cell monolayer, and after polymerization of the Epon, the Epon blocks were separated from the plastic by immersion in liquid nitrogen. Ultrathin sections were cut on a Reichert OmLI3 Ultramicrotome, stained with uranyl acetate and lead citrate and examined with a Philips E.M. 400 electron microscope.

Proliferation studies. Cells from passages 2 to 5 were seeded in 10-cm^2 culture plates at a density of 2,000 $cells \cdot cm^{-2}$. One day after seeding and on every 3rd day thereafter, the medium was exchanged. At appropriate times, cells of two to four wells were washed with phosphate-buffered

saline solution, trypsinized for 3 to 5 min at 37°C and counted in a hematocytometer. Cell numbers were use to plot growth curves.

Extracellular matrix synthesis. To determine collagen synthesis, subconfluent cells of passage 2 to 4 were incubated for 48 h at 37°C in culture medium containing 2 μmol/liter ascorbic acid and 10 $\mu Ci \cdot ml^{-1}$ ^{3}H-proline (specific activity 231 $mCi \cdot mg^{-1}$). Incubations were performed in triplicate in 2-cm^2 culture wells. Subsequently, the medium was removed and combined with two cell layer washes (phosphate-buffered saline solution) before precipitating with 20% trichloroacetic acid/1 mmol/liter of proline (24 h, 4°C). After centrifugation, the pellet was washed with graded concentrations of trichloroacetic acid/1 mmol/liter of proline and dissolved in 0.2N sodium hydroxide overnight. Thereafter, collagen and noncollagen protein synthesis were quantified by a bacterial collagenase digestion method (*Clostridium histolyticum* type III, Calbiochem) modified from Peterkofsky and Diegelmann (25). Collagen synthesis was expressed as nmol of ^{3}H-proline·μg total cell $protein^{-1}$.

Sulfated glycosaminoglycan synthesis was determined as follows. In triplicate 2-cm^2 culture wells, subconfluent cells of passages 3 to 5 were incubated for 48 h at 37°C in culture medium containing 0.5 ml of ^{35}S-sulfate (specific activity 20 $\mu Ci \cdot ml^{-1}$). Thereafter, the medium was washed with phosphate-buffered saline solution to a final volume of 2.5 ml, and 250 μl of this solution was applied to a disposable Sephadex G-25M gel filtration column (PD10, Pharmacia) equilibrated and run in 8 mol/liter of urea/0.5% TritonX100/0.15 mol/liter of sodium chloride/50 mmol/liter of sodium acetate (pH 6). Column fractions of 300 μl were collected, and fractions 8 to 45 were counted in Instagel II. The synthesis of sulfated glycosaminoglycans, expressed as nmol of ^{35}S-sulfate·μg total cell $protein^{-1}$, was derived from the sum of the counts in the initial peak.

For total cell protein, cells were detached from culture wells by gentle scraping, washed in phosphate-buffered saline solution and precipitated in 20% trichloroacetic acid (24 h, 4°C). After centrifugation, the pellet was dissolved in 0.2N sodium hydroxide overnight, and the total protein was estimated according to the Pierce BCA assay (Pierce Rochford) using bovine serum albumin as the standard (26).

Statistical analysis. Results are presented as mean value ± SEM. Group differences in initial smooth muscle cell outgrowth (primary culture); success of secondary culture, patient age, gender, anginal class, medications; and histologic features were assessed by means of chi-square tests for categoric variables with a Yates correction where appropriate. Cell population doubling times, expressed as mean value and 95% confidence intervals (CI), were derived after linear regression of log-transformed cell population growth curves. Otherwise, comparisons between primary atherosclerotic and restenotic coronary artery smooth muscle cells and human umbilical artery smooth muscle cells were performed with a two-sample *t* test. Significance was accepted at the 5% level.

Results

Culture success: relation to clinical variables and histologic features. Initial cell outgrowth (primary culture) was seen in 37 (40%) of 93 attempted cultures, and successful secondary culture (up to seven serial passages) was achieved in 11 (12%). Clinical data for the patients whose tissue provided secondary cultures were the following: eight men (73%), three women (27%), aged 56 ± 1 years; stable angina (six patients [55%]), unstable angina (five patients [45%]), primary atherosclerotic lesions (seven patients [64%]) and restenotic lesions (four patients [36%]).

Cells started to grow from explants after 4 to 8 days, and confluent multilayer primary cultures from the 11 patients were established after 4 to 6 weeks. The success of the initial outgrowth of coronary smooth muscle cells and of serial passage in secondary culture was not related to the nature of the lesion, primary atherosclerotic or restenotic, or to any patient characteristic, including current medication. Cell outgrowth was, however, related to the presence of organized thrombus in the plaque. The rate of outgrowth in the presence of thrombus was 80% (8 of 10) but was 35% (29 of 83) in the absence of thrombus ($p < 0.025$). In the initial 12 patients, cell outgrowth improved with greater tissue maximal cell densities (outgrowth 922 ± 43 vs. no outgrowth 213 ± 34 $cells \cdot mm^{-2}$, $p < 0.01$). The four successful secondary cultures from restenotic lesions were not associated with unusually aggressive restenosis (interval from previous intervention 130 ± 36 days).

Morphology and identification of cells cultured from human coronary lesions. Subconfluent cultures took the form of a network of multilayered elongated cells in interlacing bands separated by lacunae. In confluent multilayer cultures, the cells appeared as whorls producing the "hill-and-valley" pattern typical of vascular smooth muscle cells in culture. This pattern was observed for all serially passaged cells. Electron microscopy of cultured coronary cells revealed features of synthetic phenotype (27), with abundant perinuclear organelles and peripheral myofibrils (Fig. 1). Immunostaining for alpha-actin was positive for both coronary (Fig. 2) and umbilical cell cultures, identifying the cells as smooth muscle cells. However, a lesser proportion, approximately 20%, of the coronary cells showed typical staining with stress fibers, consistent with the electron micrographic evidence of synthetic phenotype.

Proliferation studies. When subcultured at the same initial densities, the three groups of cells grew similarly until day 5 (Fig. 3). Thereafter, the cells of restenotic origin demonstrated accelerated proliferation with significantly greater cell numbers compared with the primary atherosclerotic cells whose growth pattern closely resembled that of the umbilical artery cells. Mean derived population-doubling times were 52 h (95% CI 48 to 58 h) for restenotic cells, 71 h (95% CI 62 to 83 h) for primary atherosclerotic cells and 74 h (95% CI 65 to 84 h) for umbilical artery cells. The correlation coefficients for the regressions were 0.95 (restenotic cells),

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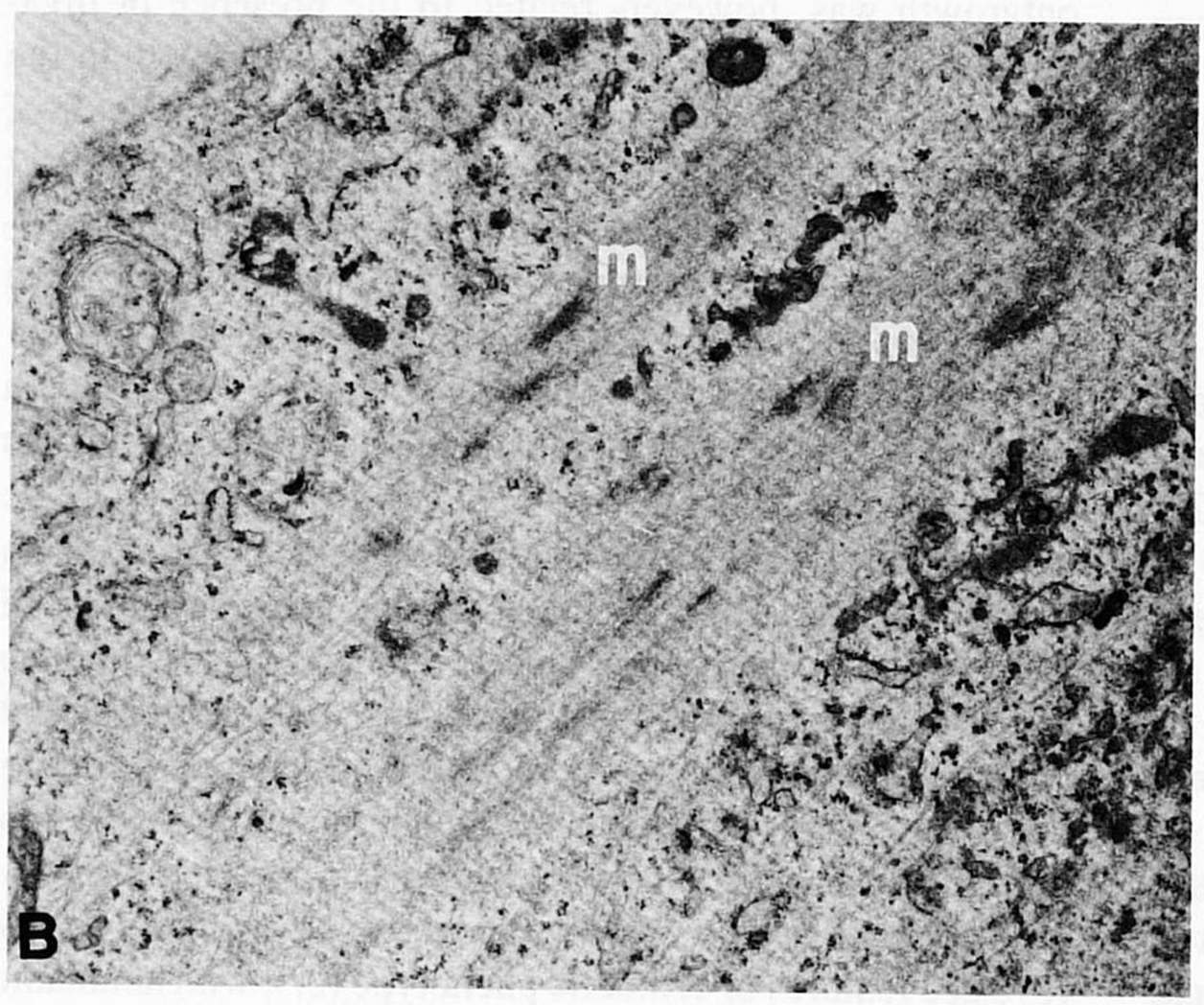

Figure 1. Transmission electron micrographs illustrating the synthetic phenotype of cultured coronary smooth muscle cells. **A,** Dense perinuclear (N) organelles with endoplasmic reticulum (**white arrow**), Golgi apparatus (**black arrow**) and abundant ribosomes. ×12,000, reduced by 35%. **B,** Peripheral myofibrils (m). ×8,000, reduced by 35%.

0.87 (primary atherosclerotic cells) and 0.88 (umbilical artery cells).

Extracellular matrix synthesis. Collagen synthesis, reflected by the incorporation of ^{3}H-proline, did not differ between restenotic and primary atherosclerotic cells in culture but was, however, significantly greater for cells of coronary than umbilical artery origin (Table 2). Production of sulfated glycosaminoglycans, as assessed by the incorporation of ^{35}S- sulfate, was less for restenotic than for primary atherosclerotic smooth muscle cells, although not significantly so, and was more than two times greater for coronary than for umbilical artery cells.

Discussion

It is accepted that the proliferation and extracellular matrix synthesis of vascular smooth muscle cells are phenomena important in atherosclerosis and highly relevant to restenosis. We describe the first attempt to investigate both of these processes in smooth muscle cells cultured from human coronary plaque tissue. In particular, we compared primary atherosclerotic lesions with restenotic lesions and adult coronary cells with cells derived from the media of umbilical arteries.

Proliferation of smooth muscle cells cultivated from coronary lesions. The difference in proliferative behavior that we show for human coronary restenotic and primary atherosclerotic smooth muscle cells in secondary culture is in close agreement with that reported for cells from human peripheral arterial lesions in primary and early subculture (15,17). Our data suggest that the proliferative behavior of restenotic cells in vitro reflects a previous phenomenon of phenotypic modulation and selection in vivo, rather than some effect of the cell culture process, because the success rates of primary

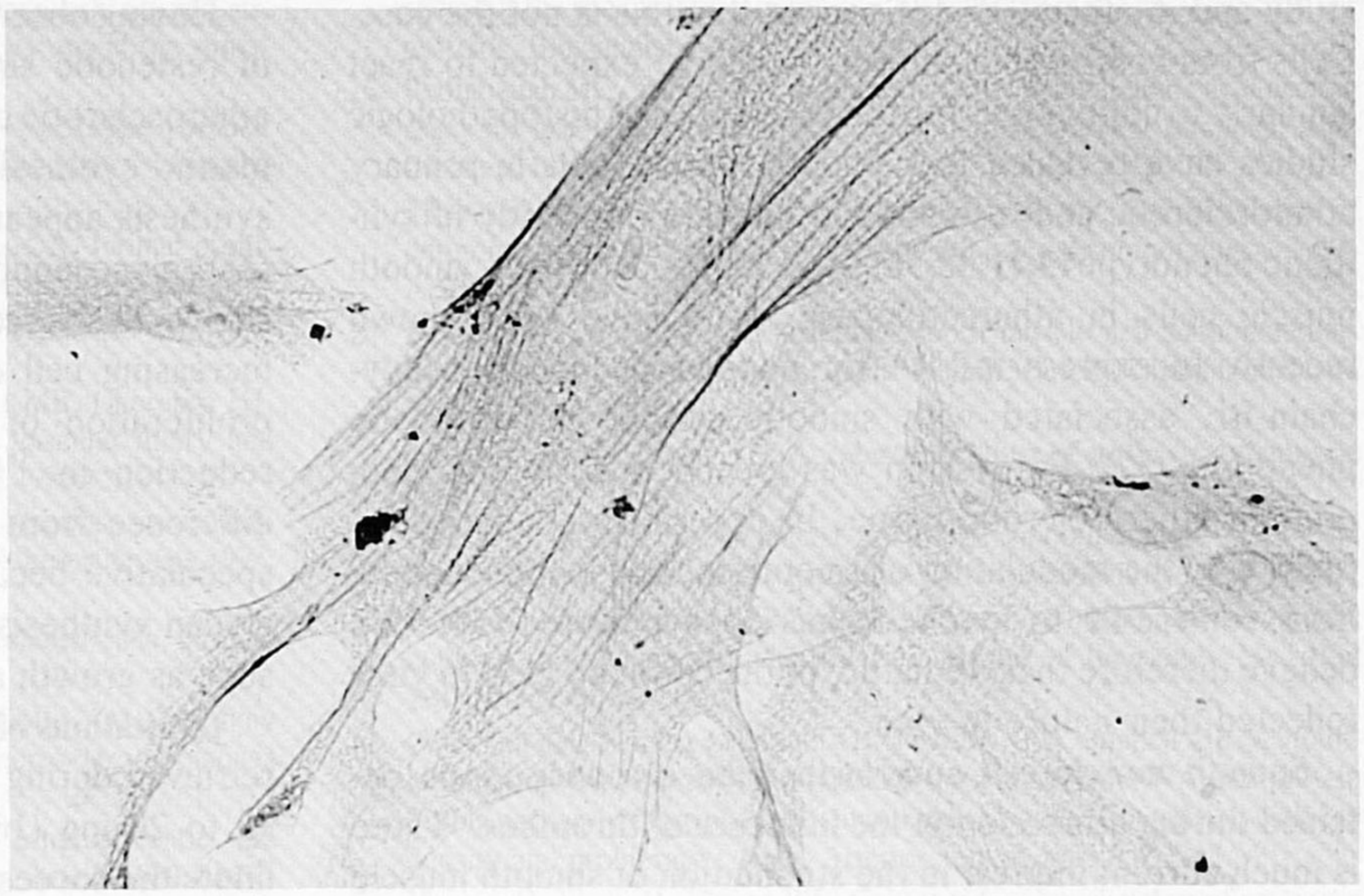

Figure 2. Monoclonal antibody (1/400 dilution) directed against smooth muscle cell-specific alpha-actin. Stress fibers are clearly seen. ×500, reduced by 35%.

and secondary culture for cells derived from restenotic and primary atherosclerotic lesions were similar. This concept is supported by the previous demonstration of a metabolically active subpopulation of smooth muscle cells in arterial intima and media (28) and in human peripheral arterial plaques, where the active subpopulation was more dominant in restenotic lesions and grew more rapidly in secondary culture (15). Also, experimental studies have shown accelerated smooth muscle cell proliferation after balloon injury (4,29) and in relation to early but not late atherosclerotic lesions in animals and humans (9,11,30,31). It might be proposed that increased growth of restenotic smooth muscle cells in secondary culture actually reflects senescence and attenuated growth of cells from chronic primary atherosclerotic lesions (3). However, this seems unlikely because the growth pattern of primary atherosclerotic cells in the present study was very similar to that of healthy umbilical artery medial cells that have undergone far fewer in vivo population doublings. Of interest, the population-doubling times that we report for primary atherosclerotic and umbilical artery cells are identical to those previously described for smooth muscle cells cultivated from adult human atherosclerotic aortic plaques and control aortic media (9).

Identification and phenotype of coronary smooth muscle cells in culture. For all three cell sources, the combination of culture morphology, electron microscopy and immunostaining for smooth muscle cell alpha-actin was consistent with vascular smooth muscle cell identity. Vascular smooth muscle cells placed in cell culture alter from contractile to synthetic phenotype within a few days, and this change becomes irreversible beyond five cumulative population doublings (32). The precise in vitro age of our cultured coronary cells is not known because a tissue explant culture method was used, but in these early passage cells, the ultrastructural features and the attenuated expression of smooth muscle cell alpha-actin (33,34), as evidenced by immunostaining, indicated that the coronary cells in culture were of synthetic phenotype. The morphology and behavior of the cells might therefore be attributed to the culture process. However, the differences in proliferation between restenotic and primary smooth muscle cells shown in this

Figure 3. Growth curves in secondary culture of smooth muscle cells derived from restenotic, primary atherosclerotic and umbilical artery media. Each **point** represents the mean value (± SEM) of a minimum of four observations from at least two passages of different cell strains. Restenotic cell numbers significantly exceeded primary atherosclerotic cell numbers at the times indicated.

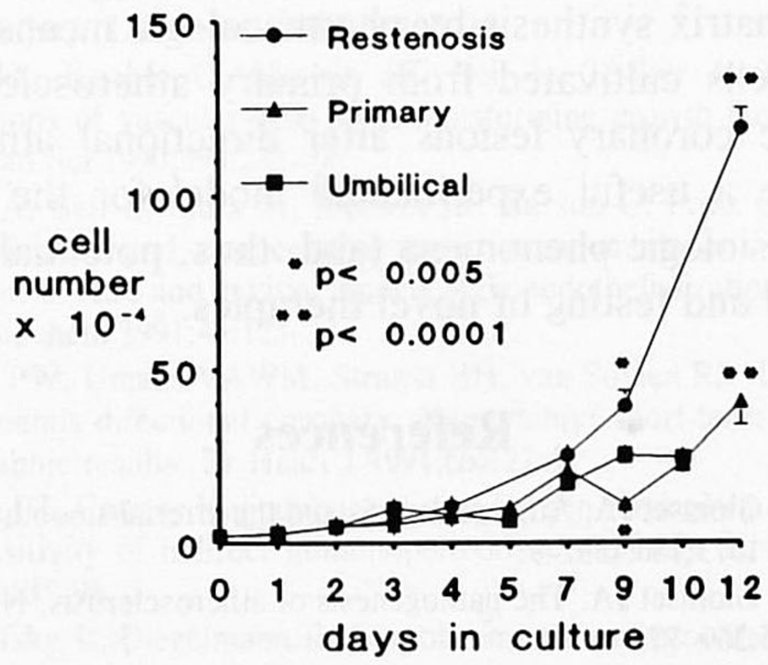

Table 2. Production of Extracellular Matrix Collagen and Sulfated Glycosaminoglycans

Cell Source	Collagen	Glycosaminoglycans
Restenotic lesion	0.034 ± 0.004 (n = 6)*	11.47 ± 1.07 (n = 6)*
Primary atherosclerotic lesion	0.033 ± 0.004 (n = 5)*	15.37 ± 3.10 (n = 4)†
Umbilical artery	0.019 ± 0.004 (n = 3)*	5.43 ± 1.00 (n = 4)*†

*$p < 0.05$. †$p < 0.005$. Values are mean value ± SEM, expressed as nmol isotope·μg total cell protein^{-1}, n = mean result of triplicate assay.

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study and in others (15–17) suggest that this is not the case. Cells from comparable subjects would be expected to react similarly to cultivation. Further, a number of histopathologic studies have revealed that smooth muscle cells in primary atherosclerotic and restenotic lesions are frequently of synthetic phenotype (3,11,35,36). Also, human coronary smooth muscle cells in atherectomized tissue have been shown recently to express mRNA for nonmuscle myosin heavy-chain-Br, associated with smooth muscle cell synthetic phenotype (37). Expression was greater in restenotic than primary atherosclerotic tissue. In experimental atherosclerosis, too, the modulation of smooth muscle cell phenotype from contractile to synthetic has been reported (38). We believe therefore that the nature of the coronary cells in vitro reflected their nature in vivo.

Smooth muscle cell outgrowth from coronary tissue obtained during atherectomy: the influence of thrombus. There is much current interest in the stimulation of smooth muscle cell proliferation and extracellular matrix synthesis in vivo by diverse growth factors, particularly in relation to restenosis. It has been suggested that endogenous mediators, such as platelet-derived growth factor, for example, may influence the initial outgrowth of smooth muscle cells from cultured atherosclerotic plaque tissue (17). In our study, the success of primary culture was enhanced in specimens containing organized thrombus. Despite the low prevalence of thrombus in the specimens (10 [9%] of 93), this finding supports the role of thrombus, recently reviewed by Schwartz et al. (39), as a fertile matrix fostering the growth of smooth muscle cells, perhaps by being a rich source of growth factors and chemoattractants.

Matrix synthesis of smooth muscle cells cultivated from coronary lesions. Coronary intimal smooth muscle cells from both restenotic and primary lesions synthesized more extracellular matrix protein than did human umbilical artery media cells. Two explanations may be offered. Either the difference arose from the obvious discrepancy in in vivo age between the coronary and umbilical artery cells, or the increased extracellular matrix synthesis of the coronary cells was a manifestation of disease. The total arterial content of glycosaminoglycans, particularly sulfated glycosaminoglycans, certainly increases with age (40), but this increase in total content is heavily influenced by the increase in vascular intimal and medial thickness that occurs with age. The relative tissue concentration of sulfated glycosaminoglycans, a better source of reference for the measurement made in the present study, varies to a lesser extent. Relevant to our findings is the evidence that glycosaminoglycans are increased in atherosclerosis, in coronary lesions in humans (40,41) and in lesions induced by diet or genetic predisposition in animals (38,42). Arterial collagen appears to alter little with age (43) but is associated with more severe or chronic lesions (19,41) and has also been shown to be elevated in experimental atherosclerosis (44). Thus, the increased matrix synthesis of the coronary cells is likely to be related to vascular disease as opposed to in vivo age.

Having observed a difference in the proliferative behavior of restenotic smooth muscle cells compared with primary atherosclerotic cells, reasons for not detecting a difference in matrix synthesis merit consideration. Although collagen synthesis appears to be unaffected by cell proliferation rate (45), glycosaminoglycan synthesis in cell culture has been shown to decrease during log phase growth (46) and with increasing cell density (47). Accordingly, the more rapid proliferation of the restenotic cells may have led to a reduction in ^{35}S-sulfate uptake, obscuring any potential difference from the primary atherosclerotic cells. This is speculative because we did not estimate the glycosaminoglycan synthesis of restenotic cells under other conditions, such as growth arrest.

Limitations of the study. First, the small amount of tissue retrieved during directional coronary atherectomy, typically 15 to 20 mg (16,48), particularly when of low cellularity, limits the success of secondary culture, and primary culture success rates decrease to 60% of those with tissue from peripheral atherectomy (16). A quarter of our procedures were for restenotic lesions that provided only four secondary cultures. Although our proliferation results are wholly consistent with other recent studies, they may only be extrapolated to the general population with caution. Second, in view of the proliferation findings, timing and cell density may have affected the estimation of sulfated glycosaminoglycan synthesis. New studies are under way to address this question. Third, with regard to the influence of vascular disease as opposed to in vivo cell age on extracellular matrix synthesis, it would be advantageous to study adult arterial smooth muscle cells cultivated from ostensibly healthy vessels, such as the internal mammary artery. This is also a focus of current experimental work.

Conclusions. The more rapid proliferation rate of restenotic lesion smooth muscle cells subcultured in vitro attests to a mechanism likely to be important in the vascular response to injury and, thus, highly relevant to the clinical problem of restenosis after angioplasty. The increased ability of adult coronary smooth muscle cells in secondary culture to synthesize collagen and sulfated glycosaminoglycans supports the putative role of the extracellular matrix in lesion formation and consolidation. Further research into this aspect of vascular smooth muscle cell (dys)function is required to explore the possibility of manipulating extracellular matrix synthesis by pharmacologic means. Smooth muscle cells cultivated from primary atherosclerotic and restenotic coronary lesions after directional atherectomy constitute a useful experimental model for the study of pathophysiologic phenomena (and, thus, potentially for the selection) and testing of novel therapies.

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