

Clinical and histological determinants of smooth-muscle cell outgrowth in cultured atherectomy specimens: Importance of thrombus organization

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Background: Coronary atherectomy provides a unique opportunity to obtain plaque tissue from a wide variety of clinical syndromes. We investigated the relation between the clinical status and histopathological substrate of tissue retrieved during directional coronary atherectomy and the proliferative and migratory potential of smooth-muscle cells judged from successful outgrowth during cell culture.

Methods: After directional coronary atherectomy, tissue samples were examined macroscopically, divided into two equal pieces, and separately subjected to cell culture and histopathological study. Cell culture was performed using an explant technique. In-vitro smooth-muscle cell outgrowth was related to clinical and histological variables.

Results: Atherosclerotic tissue was obtained from 98 consecutive atherectomy procedures. Histological examination revealed a broad spectrum of appearances, ranging from complex atheroma containing dense fibrous tissue, calcium deposits, macrophages, and necrotic debris to neointimal proliferation and organized thrombi. Smooth-muscle cell outgrowth was observed in 43 of the 98 samples (44%). Although not affected by any of the clinical variables, cell outgrowth was influenced by histological variables, in particular the presence of organizing thrombi. Outgrowth was successful in eight out of 10 samples with thrombus (80%) and in only 35 out of 88 (40%) without ($P=0.03$).

Conclusion: The presence of organizing thrombi in the retrieved tissue facilitates smooth-muscle cell outgrowth and suggests an enhanced proliferative and migratory potential. These findings may be relevant to the understanding of neointimal proliferation in coronary syndromes where mural thrombosis is likely to occur.

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Smooth-muscle cell proliferation plays a key role in the development of typical and accelerated forms of atherosclerosis [1]. Cell culture of atheromatous tissue retrieved during directional coronary atherectomy allows us to study smooth-muscle cells from the pathological substrate of a variety of human coronary syndromes. In studying the pathobiology of coronary atheroma, the use of coronary atherectomy specimens for cell culture offers several distinct methodological advantages over other sources of atheromatous material, such as peripheral atherectomy, carotid endarterectomy, or animal models of atherosclerosis. Vascular smooth-muscle cells are embryologically derived from local mesoderm [2,3]. Those found in the coronary arteries are likely to have biological differences from those located in other vessels. Likewise, the development of the atheromatous plaque is strongly affected by local factors [4] that may influence cell populations, extracellular matrix composition, and plaque architecture. The use of human coronary material also makes it possible to avoid some of the pitfalls associated with animal models of atherosclerosis [5,6].

Nevertheless, cell culture studies using human coronary smooth-muscle cells have some technical limitations. Once in culture, these cells undergo a progressive phenotypic modulation that is time-dependent and enhanced by successive cell passages [7-9]. This process may facilitate the selection of cell clones with a higher proliferative capacity. Furthermore, although isolated cell studies provide information regarding cellular function and pathophysiological conditions, their extrapolation to the clinical situation is limited because they ignore the complex cell-cell and cell-extracellular-matrix interactions that modulate smooth-muscle cell growth *in vivo* [10].

In order to minimize these limitations, in the present study we used an explant culture technique [11,12]. This maintains a representative section of the atherosclerotic plaque in the culture medium, thus retaining the intrinsic distribution and anatomical relations of the participating cells, cell-cell interactions, the correct chemical configuration of the extracellular matrix, and the general *in-vivo* milieu of the atherosclerotic plaque. Only coronary material was used. The initial outgrowth of smooth-muscle cells was then used as a surrogate index for their *in-vivo* migratory and proliferative potential while still under the influence of other histological and humoral variables that were present in the atheromatous plaque at the time of atherectomy.

Methods

Percutaneous directional coronary atherectomy was performed on 98 lesions in 98 patients. Informed consent was obtained from all patients before the procedure, in accordance with the protocol approved by the Thoraxcenter Institutional Review Board. Atherectomy was performed

using Simpson's Atherocath (Devices for Cardiovascular Intervention, Redwood City, CA, USA) and a conventional technique [13]. Multiple cuts in different sectors of the vessel were routinely performed. Under sterile conditions, the specimens were removed from the housing of the atherocatheter, washed with 0.9% saline, and placed in M199 HEPES buffered culture medium (Gibco Laboratories, USA) with antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin). They were immediately transferred to the laboratory where they were flushed with fresh culture medium and examined with a dissecting microscope. A representative section was then fixed in 3.6% buffered formalin for histopathological examination and the remainder placed in culture.

Clinical variables

A number of clinical variables were recorded for each patient. These included age, sex, previous myocardial infarction, stable or unstable angina pectoris, previous coronary intervention, and risk factors for coronary artery disease (hypercholesterolaemia, diabetes mellitus, cigarette smoking, hypertension, and family history of coronary artery disease). Unstable angina was defined as continuous or intermittent chest pain at rest requiring hospitalization, associated with electrocardiographic evidence of myocardial ischaemia and no increase in cardiac enzyme levels.

Tissue analysis

Specimens for histopathological study were routinely processed for light microscopy and stained with haematoxylin-azophloxin and Verhoeff-van Giesson stains. All specimens were reviewed independently by two observers, who were blinded to the clinical data. If they disagreed, the opinion of a third pathologist was sought and a consensus reached.

For the analysis of intimal constituents, the recommendations of the American Heart Association Medical/Scientific statement on the definitions of the intima of human arteries and of its atherosclerosis-prone regions were followed [14]. Fibrous tissue was classified as 'dense' when composed of acellular or poorly cellular connective tissue formed predominantly by dense collagen, and 'loose' when the tissue fragments showed moderate cellularity and collagen bundles separated by accumulations of extracellular matrix. Fibromuscular hyperplasia was defined as fibrous connective tissue showing a random orientation of spindle-shaped and stellate cells embedded in abundant extracellular matrix. Thrombus was identified as amorphous material, often in close apposition with atheromatous material, and frequently showing collections of leukocytes between layers of fibrin. Discrimination between fibrin and dense collagen was achieved using Verhoeff-van Giesson staining. The thrombus was regarded as organizing when infiltrated by cellular elements such as smooth-muscle cells or fibroblasts. Cholesterol crystal clefts, necrotic debris, and calcium deposits were recorded independently. The presence of macrophages was recorded only when these formed clusters or when they were present in unusually high numbers. Medial tissue was identified on the basis of a parallel arrangement of smooth-muscle cells, embedded in collagen, and frequently associated with a fragment of the internal or external elastic lamina. The adventitia was recognized by the presence of coarse bundles of dense collagen intermingled with elastin fibres, sometimes in association with fragments of the external elastic lamina and media.

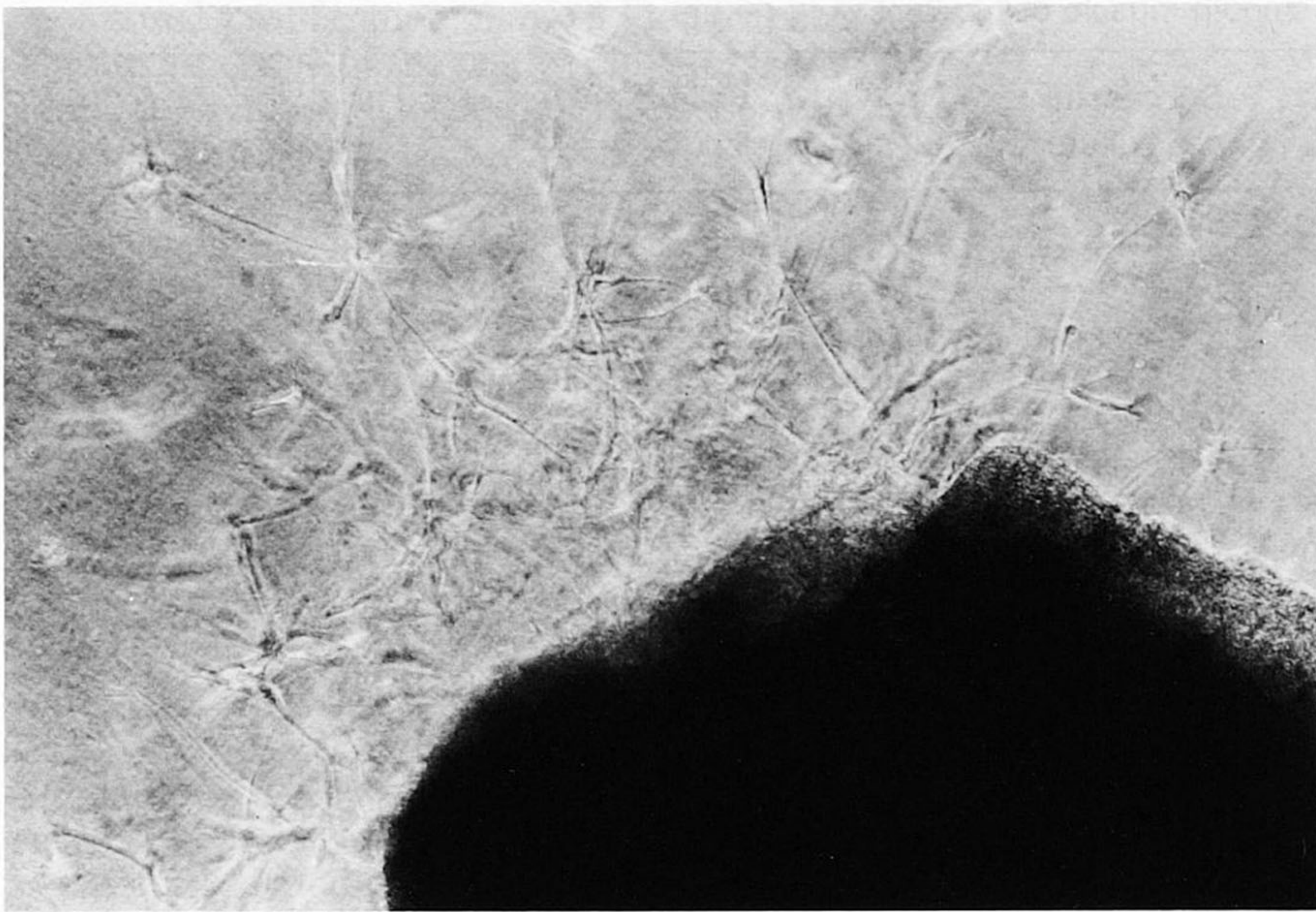


Fig. 1. Fragment of retrieved tissue examined under phase-contrast microscopy after 12 days of culture. A dense population of cells migrating out of the atherectomy explant is evident. These cells fulfil the morphological criteria of myofibroblasts discussed in detail in the text. (Original magnification $\times 60$.)

Cell culture

The atheromatous tissue was cultured by a cell biologist (MdJ) blinded to the clinical data. An explant technique was used. Tissue explants were placed on human fibronectin-coated ($10 \mu\text{g}/\text{cm}^2$) glass cover slips in 2 cm^2 wells (Four-well plates, Nunc) and cultured in $300 \mu\text{l}$ culture medium [M199 with NaHCO_3 (Gibco Laboratories) supplemented with glutamine, 10% human serum, 10% fetal calf serum, 100 IU/ml penicillin and 0.1 mg/ml streptomycin, and mixed in a ratio of 1:1 with conditioned medium from established smooth-muscle cell lines actively growing in our laboratory]. Cultures were maintained in a CO_2 incubator at 37°C in a humidified atmosphere equilibrated with 5% (vol/vol) CO_2 in air. The culture medium was changed every 3–4 days. Smooth-muscle cell outgrowth was identified using inverted light microscopy and morphological criteria. These included a characteristic growth pattern of multiple layers of spindle-shaped or stellate cells showing stress fibres and lamellipodia (Fig. 1). These morphological criteria were reinforced by positive immunostaining for smooth-muscle-cell specific α -actin (DAKO, Denmark) with human skin fibroblasts as negative controls.

Statistical analysis

Mean values and standard deviations were calculated for all continuous variables. Comparison of mean values was made using the two-tailed unpaired t-test. Discrete variables were compared using the chi-squared test, and continuity correction was applied when indicated. $P < 0.05$ was considered statistically significant.

Results

Clinical

Of the 98 patients in the study, 49 presented with stable and 47 with unstable angina pectoris. The remaining two were post-cardiac transplantation patients with cardiac allograft vasculopathy (Table 1). Twenty-four of the patients had a previous history of coronary intervention (14 of balloon angioplasty,

six of stent implantation, three of atherectomy procedures, and one of excimer laser angioplasty) and had restenosis at the site of this intervention. The mean time interval between the previous revascularization procedure and the atherectomy was 147 ± 108 days. The target lesion was located in the left anterior descending coronary artery in 62 patients, in the circumflex in 12, in the right coronary in 20, and in saphenous vein grafts in four. An average of 6 ± 3 passes in different directions were made across each lesion.

In the study population, seven patients had a history of hypercholesterolaemia (serum cholesterol level $\geq 8 \text{ mmol}/\text{dl}$), 27 had systemic hypertension, 36 were smokers, and 18 had a family history of coronary artery disease; none of the patients had a history of diabetes mellitus. None of these risk factors appeared to influence smooth-muscle cell outgrowth (Table 1). Likewise, none of the other clinical variables discussed above could be related to enhanced cell outgrowth.

Histological

Thrombus was present in 10 out of 97 sections examined, predominantly in unstable angina patients: nine out of 49 (18%), compared with one out of 49 (2%) stable patients, $P = 0.019$. Some degree of organization was present in all of the thrombotic specimens examined (Fig. 2). This ranged from the presence of endothelial-like cells in lacunar spaces or capillaries and the presence of scarce myofibroblasts infiltrating the thrombotic mass from the adjacent fibrous tissue, to infiltration by high numbers of myofibroblasts with the production of extracellular matrix. The thrombotic material appeared to be embedded in the fibrocellular tissue, suggesting that areas of fibrin and platelets derived from an episode of thrombosis or plaque haemorrhage were being integrated into the atheromatous plaque. In four cases,

Table 1. Clinical variables and outcome of smooth muscle cell culture.

	Successful cell culture	Failed cell culture	Significance (<i>P</i>)
Clinical variables			
Age (years, mean \pm SD)	57 \pm 10	57 \pm 11	NS
Previous MI	14/43	21/55	NS
Syndrome			
Stable angina	20/49 (41%)	29/49 (59%)	NS
Unstable angina	23/47 (49%)	24/47 (51%)	NS
Transplant vasculopathy	0/2	2/2	—
Risk factors for CAD			
Male sex	34/43 (79%)	46/55 (84%)	NS
Hypercholesterolemia	3/39 (8%)	4/59 (7%)	NS
Hypertension	10/40 (25%)	17/58 (29%)	NS
Smoking	17/42 (40%)	19/56 (34%)	NS
Family history of CAD	8/43 (19%)	10/55 (18%)	NS
Previous intervention	11/43 (26%)	13/55 (24%)	NS
Histological variables			
Neointimal hyperplasia	14/30 (47%)	29/68 (43%)	NS
Thrombus (organizing)	8/10 (80%)	35/88 (40%)	0.03
Dense fibrous tissue	32/79 (41%)	11/19 (58%)	NS
Loose fibrous tissue	10/16 (62%)	33/82 (40%)	NS
Cholesterol clefts	4/8 (50%)	39/90 (43%)	NS
Calcium deposits	13/27 (48%)	30/71 (42%)	NS
Necrotic debris	6/9 (67%)	37/89 (42%)	NS
Macrophage clusters	7/15 (47%)	36/83 (43%)	NS
Media	11/23 (48%)	32/75 (43%)	NS

MI, myocardial infarction; CAD, coronary artery disease.

this process was seen in conjunction with extensive fibromuscular proliferation.

Neointimal hyperplasia was observed in 31 samples, predominantly in restenotic (17 out of 24, 71%) rather than de-novo (14 out of 69, 20%) lesions ($P = 0.0001$).

In seven of the 31 samples, (22%), a neovascularization network was found in the interphase between neointimal hyperplasia and surrounding dense fibrous or loose fibrous tissue (four in primary and three in restenotic lesions). In secondary lesions,

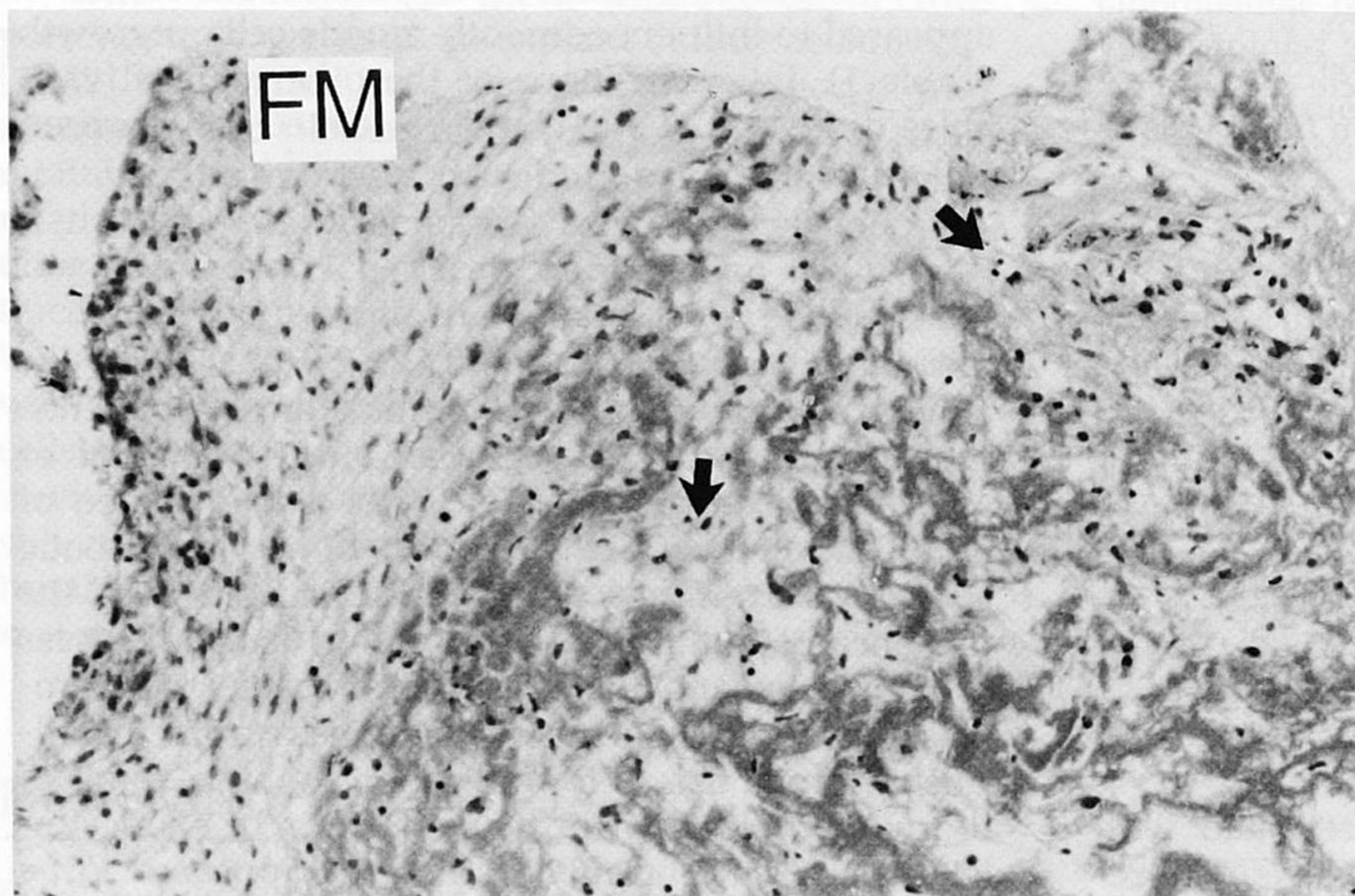


Fig. 2. Histological cross-section showing thrombus partially infiltrated by myofibroblasts (arrows) in close association with newly formed fibromuscular tissue (FM). (Original magnification $\times 30$.)

neointimal proliferation showed identical characteristics irrespective of the nature of the previous intervention. Dense and loose fibrous tissue was found in 79 and 17 samples, respectively. Calcium deposits were observed in 27 samples. Macrophages were identified in 15 samples, predominantly in those with necrotic debris. Media and adventitia were found in 23 (23%) and 7 (7%) specimens, respectively.

Smooth-muscle cell outgrowth

Depending on the volume of the retrieved tissue, an average of 4.5 fragments (range 2–8), each measuring approximately 1 mm³, were placed in culture. Cells started to grow out from explants after 4–14 days (Fig. 1), reaching a steady state within 4–6 weeks. The samples were discarded if no outgrowth was observed after 3–4 weeks. Despite the use of antibiotics in the culture medium, six of the cultured specimens developed infections and were discarded. The infections tended to develop after 3–4 weeks, by which time cell outgrowth should have occurred but did not.

Primary cell outgrowth was observed in 43 out of 98 samples (44%). When primary outgrowth occurred, light microscopy showed that the majority of cells tended to form multiple layers and were polygonal or spindle-shaped, with multiple stress fibres extending to lamellipodia. These appearances are characteristic of smooth-muscle cells. The cells were confirmed to be smooth-muscle cells using positive immunocytochemical staining with an α -actin-specific monoclonal antibody. In addition to smooth-muscle cells, a second cell type, oval in shape with eccentrically placed small indented nuclei, was identified in six cultures. Immunoperoxidase staining with macrophage-specific HAM 56 confirmed these cells to be macrophages; typically, they disappeared after 10–14 days of culture.

Cell outgrowth was not significantly influenced by any of the clinical variables recorded (Table 1), including the age or sex of the patient, coronary syndrome (stable or unstable angina), type of coronary lesion (de-novo or secondary), or risk factors for coronary artery disease (hypercholesterolaemia, hypertension, smoking, family history). Although cell culture failed in samples obtained from the two cardiac transplant patients, the small number involved precludes any conclusions about the statistical significance of this finding. Smooth-muscle cell outgrowth was significantly influenced, however, by the presence of organizing thrombus documented during histological examination. Smooth-muscle cell outgrowth was documented in eight out of 10 (80%) samples with and only 35 out of 88 (40%) without thrombus ($P=0.03$, Table 1). None of the other histological variables analysed, including the presence of neointimal hyperplasia, fibrous tissue (dense or loose), lipid deposits, necrotic debris, macrophages, media, or adventitia, influenced

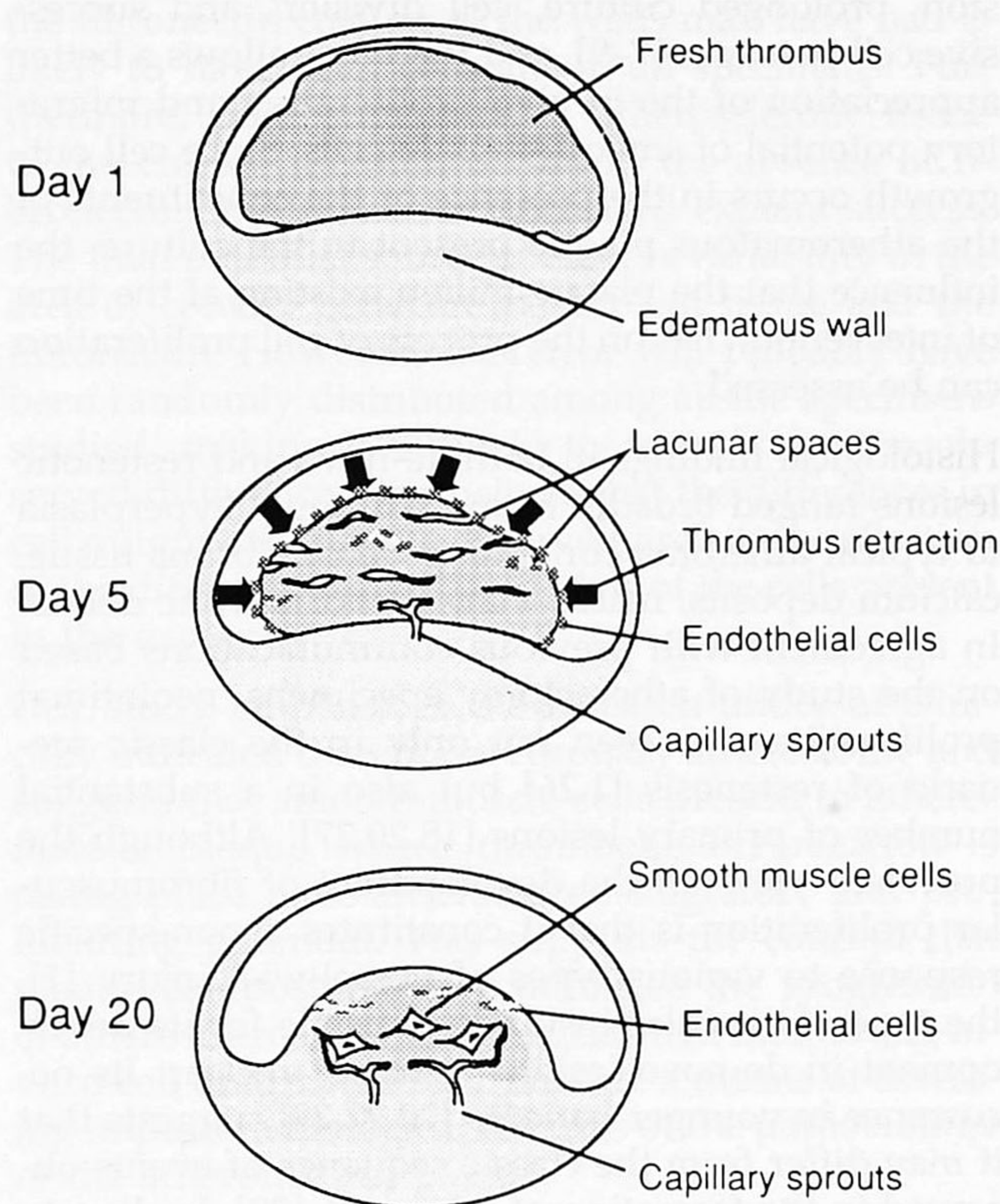


Fig. 3. The sequence of events leading to thrombus organization, based on observations in experimental animal models [15–17]. After the initial episode of thrombosis, a significant retraction of the thrombotic mass occurs, with the formation of lacunar spaces. These, like the thrombotic surface, become quickly endothelized. A granulation reaction with capillary ingrowth from the vessel wall occurs simultaneously. By day 20, smooth-muscle cells that may have migrated from the vascular wall during the granulation reaction or that are derived from circulating cells that have infiltrated the thrombus are seen along newly formed capillaries. Some of the endothelized lacunar spaces may give origin to multiluminal channels, which constitute a classic histopathological landmark of previous thrombotic recanalization.

cell outgrowth. Finally, there was no correlation between the number of explants used in each case and the outgrowth of smooth-muscle cells (4.5 ± 1.6 and 4.5 ± 1.9 explants in cases with successful and failed culture, respectively; NS).

Discussion

Atherectomy has facilitated not only the study of the histological constitution of the atheromatous plaque [13,18–20] but also the culture of smooth-muscle cells present in human atheroma [11,12,21–25]. Several groups [22,24], including ours [21], have reported improved cell outgrowth rates when an explant cell culture technique is used. The advantage of this method is that it minimizes the modifications of cell phenotype associated with enzymatic disper-

sion, prolonged culture, cell division, and successive cell passages [7-9], and therefore allows a better appreciation of the in-vivo proliferative and migratory potential of smooth-muscle cells. Since cell outgrowth occurs in the presence of the constituents of the atheromatous plaque present in the culture, the influence that the plaque milieu existing at the time of intervention has on the process of cell proliferation can be assessed.

Histological findings in both de-novo and restenotic lesions ranged broadly from neointimal hyperplasia to typical atheroma containing dense fibrous tissue, calcium deposits, macrophages, and necrotic debris. In agreement with previous communications based on the study of atherectomy specimens, neointimal proliferation was seen not only in the classic scenario of restenosis [1,26] but also in a substantial number of primary lesions [18,20,27]. Although the prevalent view on the development of fibromuscular proliferation is that it constitutes a non-specific response to various types of vessel-wall injury [1], the type of vascular insult responsible for its development in de-novo lesions remains unclear. Its occurrence in younger patients [20,27,28] suggests that it may differ from the classic sequence of events observed in the formation of atheroma [29], leading to a more aggressive form of atherosclerotic disease. Finally, we have found that organization is common in thrombotic atherectomy samples from patients with unstable angina [20], in agreement with recent work by Isner *et al.* [30], who used atherectomy specimens, but not with post-mortem studies, emphasizing the distinct advantages derived from the use of atherectomy samples in the in-vivo study of coronary syndromes.

Our explant culture success rate (44%) is low in comparison with other studies [12,24], although this must be the result of the sole use of coronary material. Cell culture from coronary atherectomy specimens is difficult, however, because of the small amounts of tissue involved, and yields significantly lower outgrowth success rates than peripheral tissue. When culture of both coronary and peripheral tissue has been attempted, a lower success rate and a longer time span until outgrowth has been observed in the coronary samples [12]. This observation was explained on the grounds that there were fewer coronary specimens and that their wet weight was lower [12]. However, it must be remembered that there are major differences in the histopathological characteristics of coronary and peripheral artery samples. The prevalence of thrombus in peripheral artery specimens obtained during directional atherectomy is as high as 61% [13], significantly higher than that found in the present and previous studies in the coronary arteries [19,20]. Although the relevance of this fact for cell culture is highlighted by the conclusions of the present study, it is unfortunate that none of the previous studies reported on the histological characteristics of the material used for culture, a limitation

recently acknowledged in a report by Pickering *et al.* [24].

Our study is the first to consider the influence of a broad spectrum of histopathological features of retrieved tissue in addition to clinical features on cell outgrowth. Like Bauriedel and colleagues [12], we found that no clinical variables, including unstable angina and drug therapy, influence the outcome of plaque cultivation. This is reflected in clinical experience, which has shown little evidence that clinical factors influence the restenosis rate, and in which all therapeutic strategies have been singularly unsuccessful. Common sense dictates that clinical factors must operate through the histological milieu of the atherosclerotic plaque. In spite of the fact that human smooth-muscle cells cultured from restenotic lesions appear to migrate more rapidly than those from primary atheroma [12] and show accelerated growth curves [25], we did not find significant differences between the primary outgrowth of smooth-muscle cells from explants of restenotic and primary lesions. A possible reason for this discrepancy is that some of these studies used isolated smooth-muscle cells, obtained after several passages, and were free of the complex cell-cell and cell-extracellular-matrix interactions which modulate smooth-muscle cell proliferation and migration in the atherosclerotic plaque *in vivo* [10].

Evidence in the literature suggests an enhanced proliferative potential of smooth-muscle cells present in restenotic lesions. An improved smooth-muscle cell outgrowth from the injured vascular wall has been demonstrated by Grunwald *et al.* [31] in a rat model. Smooth-muscle cell outgrowth has been found to occur more rapidly in restenotic than in de-novo atherectomy specimens obtained in peripheral vessels [12,24], although the initial outgrowth was similar. In our study, cell outgrowth was not significantly different in explants from de-novo or restenotic lesions. There are a number of possible reasons for this. Our experiments were performed using only coronary atherectomy specimens, which, as discussed before, may differ substantially in their histological substrate from those obtained in peripheral vessels. The time interval from the former percutaneous intervention may also be of importance because evidence suggests that smooth-muscle cells experience a process of senescence during their migration to the neointima [32] and that their proliferation rate decreases after a period of time [33].

We found that smooth-muscle cells present in coronary atheroma where thrombotic organization is taking place had an enhanced proliferating potential. This may be related to three major factors. First, mural thrombus is rich in circulating elements, such as platelets, monocytes, and lymphocytes, which can secrete a number of vascular growth factors [1], promoting smooth-muscle cell proliferation. Thrombin and fibrin have both been shown to have chemotactic and mitogenic activity on vascular smooth-muscle cells [34], an effect that may be prolonged

after the incorporation of thrombus into the plaque. Thrombin may also act as a competence factor, stimulating the expression of growth factors, including platelet-derived growth factor, and their receptors [35,36], and thus help to perpetuate the activation of smooth-muscle cells. Any or all of these mechanisms may have been operating in our study, resulting in the increased migratory and proliferative activity of the smooth-muscle cells when surrounded by thrombus.

Second, the process of thrombus organization may have played a key role in the observed outgrowth of cells; since organization was taking place in all of our thrombotic specimens, we believe that our conclusions should be restricted to the presence of organizing thrombus. Growing evidence suggests that thrombus organization plays a key role in the development of neointimal hyperplasia after vascular injury [37–39,15]. It has been suggested that the smooth-muscle cells involved in this process are derived from circulating mononuclear cells rather than being of intimal or medial origin [15,16] (Fig. 3). Thrombus would serve as a biodegradable fibrin matrix colonized by circulating mononuclear cells that heal the injury site from the lumen side inwards, towards deeper vascular layers [15]. In this scenario the mononuclear cells that have colonized and started organizing the thrombus are self selected for their migratory and proliferative ability, providing an unexpected explanation for a previous report showing that smooth-muscle cells in organizing thrombi have a monoclonal origin [40]. Although the smooth-muscle cells cultured from thrombotic and non-thrombotic origin were morphologically indistinguishable, the above tentative scenario remains plausible.

Finally, a third explanation for our findings is that organizing thrombus may have facilitated cell outgrowth by optimizing cell transfer to the culture medium.

In our study we tried to reach a compromise between obtaining information from histopathology and from cell culture. As discussed above, meticulous inspection of the samples under the dissecting microscope was performed to ensure that the tissue fragments dedicated to the histological examination and cell culture samples were equally representative of the whole specimen. However, the possibility that the two pieces were significantly different cannot be ruled out. Atherosclerosis is a segmental disease process; in this regard our study shares the limitations of all histopathological studies using atherectomy specimens, in which conclusions are reached using fragmented samples of the arterial wall [26]. A second limitation is that pretreatment of the culture wells with fibronectin may have facilitated the transformation of smooth-muscle cells from the contractile to the synthetic phenotype [41], affecting explant outgrowth. We believe that this is unlikely to have occurred, however, because fibronectin is already present in serum and any additional effect that

the fibronectin coating of the wells may have had is likely to have been constant in all specimens. Furthermore, poor adherence of atherosclerotic tissue to the culture medium occurs in the absence of fibronectin, and results in decreased explant success. The final potential source of error is variability in the area of contact between the explant tissue and the fibronectin. However, this error will probably have been randomly distributed among all the specimens studied, making it unlikely to account for the observed differences. We believe that the differences in cell outgrowth that we observed are a true reflection of the differing growth potentials of the cells present in the explants.

Our study emphasizes the research utility of clinically indicated directional coronary atherectomy and suggests that smooth-muscle cells present in atheromatous plaque where thrombotic organization is taking place have an enhanced migratory and proliferating potential. This supports the concept that plaque composition may influence the progression of atherosclerosis. It also suggests that monitoring in-vitro cell outgrowth may provide a means of assessing important biological features of the pathobiology of the atheromatous plaque.

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