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Intimal Proliferation in an Organ Culture of Human Saphenous Vein

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This study investigated whether intimal proliferation, the characteristic feature of the response of human saphenous vein to arterial implantation, also occurs in organ culture. Vein segments were maintained for 14 days in medium supplemented with 30% fetal bovine serum. Tissue viability (measured by adenosine triphosphate [ATP] concentration) decreased only 20% from 280 ± 20 to 220 ± 20 nmol/g wet weight. In veins prepared for culturing, endothelial loss (approximately 20%) was confined to near the cut edges. Cultured veins retained an endothelial layer in the initially undamaged areas, while the initially injured areas became covered by a mixture of endothelial and vascular smooth muscle cells. Autoradiography in conjunction with scanning electron microscopy showed the presence of proliferating cells on the intimal surface. Transverse sections of cultured veins showed the development of a new intima containing vascular smooth muscle cells identified by immunocytochemistry with anti-α-actin. There were also endothelial cells identified with Ulex europaeus lectin arranged in capillarylike structures. Pulse or continuous labeling of cultures with [^{3}H]thymidine showed that proliferating cells were confined to the new intima and suggested that the smooth muscle cells in this layer arose from both immigration and proliferation. The results demonstrate that intimal proliferation occurs in organ culture of human saphenous veins. (Am J Pathol 1990, 137:1401–1410)

Intimal smooth muscle proliferation is an important feature of arteriosclerosis[1] and occurs after intimal injury in experimental models[2,3] and in the clinical context of angioplasty.[4] It is also a characteristic feature of the response of human saphenous vein to implantation as an arterial bypass graft.

Aortocoronary bypass grafting, in particular, has become popular in the management of atherosclerotic coronary artery disease because it relieves symptoms in most patients and improves survival in those with the most severe disease.[5,6] Reversed autologous saphenous vein remains the most commonly used conduit[7] because of its availability in sufficient quantity for multiple grafts and its ease of surgical preparation. However as many as 50% of saphenous vein grafts become occluded by 10 years after operation,[8,9] and graft occlusion is associated with recurrence of angina.[9,10] The factors responsible for vein graft occlusion have been reviewed.[7,11–13] Early occlusions occur from thrombosis promoted by exposure of subendothelial collagen at sites of endothelial damage.[12] Later, thrombotic occlusions are increasingly secondary to narrowing of the graft lumen by excessive intimal smooth muscle cell proliferation. This may occur initially as a result of release of growth factors at sites of endothelial damage caused during surgical preparation and grafting.[14,15] Its progression beyond the time (1 to 12 weeks) taken to regenerate a morphologically intact and nonthrombogenic endothelium suggests, however, the subsequent contribution of growth factors derived from other sources.[7]

Intimal smooth muscle cell proliferation is difficult to study in humans because its progression can only be inferred from pathologic specimens obtained after death. Therefore we tried to establish whether intimal proliferation occurred in an organ culture of human saphenous vein. Our experimental strategy was first to determine whether the tissue remained viable in culture and then to detect, localize, and identify any proliferating cells.

Materials and Methods

Patients

Surplus segments of saphenous vein harvested for therapeutic reasons were obtained from 42 patients (32 men,

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mean age 58 years; range, 41 to 71 years) who were undergoing coronary artery bypass graft. Ethical permission was obtained from the relevant authority. Veins were used regardless of preoperative drug therapy. Routine premedication, anesthesia, and intraoperative heparinization were performed as described previously.[14]

Preparation and Tissue Culture of Vein Segments

Segments of saphenous vein (measuring approximately 3 cm) were dissected carefully, as described previously,[14] and immediately transported to the laboratory at room temperature (23°C) in sterile RPMI 1640 tissue culture medium containing 20 mmol/l (millimolar) Hepes buffer (Flow Labs, High Wycombe, UK), 4 IU/ml of sodium heparin (CP Pharmaceuticals, Wrexham, UK), 0.2 mg/ml of papaverine hydrochloride (McCarthy Medical, Essex, UK), and 5 μg/ml of amphotericin B (Flow Labs).

The tissue preparation and culture method were modifications of the procedure of.[16] Briefly, the segment of vein was extended to approximately its *in situ* length by pinning it to set sylgard resin (Dow Corning, Seneffe, Belgium) under a sterile washing medium consisting of RPMI 1640 medium containing 20 mmol/l Hepes buffer (pH 7.4), 2.5 μg/ml of gentamicin (David Bull Laboratory, Warwick, UK), 100 μg/ml of penicillin: streptomycin (Flow Labs) and 0.8 mmol/l glutamine (Flow Labs). After gently removing excess fat and adventitial tissue, the vein was cut with scissors along its upper aspect and pinned open with its endothelial surface uppermost using minuten pins (size A1, Watkins & Doncaster, Kent, UK). After careful rinsing with washing medium, the vein was cut transversely at about 5-mm intervals with a scalpel blade. Vein segments analyzed at this stage are referred to throughout as freshly isolated vein.

Some segments of vein then were incubated for 1 to 2 minutes at room temperature in Dulbecco's phosphate-buffered saline, pH 7.4 (DPBS) containing 0.01% trypan blue (Flow Labs) to visually assess endothelial integrity.[16,17] Such veins were not subsequently used for measurement of purine metabolite concentrations or for organ culture.

Vein segments were cultured over a layer of set sylgard resin on top of a 2 cm × 1 cm square of polyester cloth (P500, Henry Simon, Cheshire, UK) in 6 ml of culture medium. This consisted of washing medium in which 2 g/l of sodium bicarbonate substituted for the 20 mmol/l Hepes and supplemented with 30% (v/v) of fetal bovine serum (Gibco, Paisley, UK). Cultures were conducted at 37°C in a humidified chamber equilibrated with 5% (v/v) CO_2 in air. The medium was replaced every 2 or 3 days. For autoradiography, the culture medium was supplemented with [^{3}H]-thymidine (1 μCi/ml, 15.6 Ci/mmol; NEN) either throughout or for the last 24 hours only as specified in the text.

Measurement of Purine Metabolites, DNA, and Total Thymidine Incorporation

Freshly isolated or cultured veins were rinsed with DPBS, gently blotted, snap frozen in liquid N_2, and stored for up to 4 weeks until required for analysis. Frozen veins were crushed under liquid N_2, the powder was extracted with perchloric acid, as previously described,[14] and the mixture was then centrifuged at 1500 g for 10 minutes at 4°C. Nucleotide concentrations were measured in the supernatants, as described previously.[14] The perchloric acid insoluble fractions were further homogenized to form a smooth suspension in 1 ml of 0.3 mol/l [molar] perchloric acid. The DNA concentration in aliquots (100 μl) of this suspension was measured using calf thymus DNA (Sigma, Poole, UK) dissolved in 1 mol/l NH_4OH as a standard.[18] For measurements of total thymidine incorporation, a further aliquot (100 μl) of this suspension was solubilized with 0.5 ml of soluene-350 (Canberra Packard) for 1 hour at 60°C, cooled, mixed with 10 ml of Dimilume-30 (Canberra Packard) scintillant, and the radioactivity was determined in a scintillation counter. Radioactivity was corrected for quenching using the channels ratio method.

Light Microscopic Studies

Vein segments were fixed in 0.1 mol/l phosphate buffer (pH 7.2) containing 10% formaldehyde, dehydrated through 70% to absolute alcohol series over 24 hours, cleared in chloroform, and embedded in paraffin wax. Sections (5-μ thick) were stained with Harris' hematoxylin and eosin[19] or Miller's elastic and van Gieson.[19] Intimal thickness was measured with a calibrated graticule (Agar Aids, Stanstead, UK) using Miller's stained sections at three points on each of three serial sections per vein.

For autoradiography, unstained sections were deparaffinized at 60°C for 30 minutes, rehydrated through alcohol to water, coated with K2 nuclear emulsion (Ilford, Cheshire, UK), and exposed for 2 weeks at 4°C. Sections then were developed and fixed, washed in distilled water, poststained with hematoxylin and eosin, and dehydrated through alcohols to xylene.

For identification of vascular smooth muscle cells by immunocytochemistry, sections were mounted on slides coated with 3-aminopropyl triethoxy silane, which improved their adherence (H. Navabi and B. Jasani, unpublished observations). Then they were deparaffinized, rehydrated, and digested with 0.1% trypsin/EDTA (Flow Labs) in DPBS at 37°C for 10 minutes, rinsed in DPBS,

and then incubated with fetal bovine serum for 30 minutes in a humidified chamber at 37°C to block nonspecific binding sites. Sections were incubated in DPBS with a 1/1000 dilution of the monoclonal anti-smooth-muscle actin (HHF 35, a gift from Dr. Allen Gown, Department of Pathology, University of Seattle, Washington) or a 1/200 dilution of the monoclonal anti-α-smooth muscle actin (clone 1A4, Sigma Chemical Co.) for 1 to 2 hours. Sections were then incubated with biotin-conjugated goat anti-mouse IgG (1:400 dilution, Sigma Chemical Co.) for 30 minutes, Extr Avidin horse radish peroxidase (1:200 dilution, Sigma Chemical Co.) for 30 minutes more, washed three times with DPBS, and incubated with a 0.05% solution of 3,3'-diaminobenzidine (Sigma Chemical Co.) containing 0.03% (v/v) hydrogen peroxide in 0.05 mol/l TRIS/HCl buffer (pH 7.3) for color development. Sections were washed and lightly counterstained with 0.5% aqueous methyl green. In negative control sections, the first antibody was substituted with control mouse ascites fluid (Sigma Chemical Co.).

Staining with Ulex europaeus lectin was used for identification of endothelium.[20] Deparaffinized, rehydrated sections were treated with 3% hydrogen peroxide for 30 minutes to inhibit endogenous peroxidase. They were then incubated with 10 μg/ml of Ulex europaeus agglutinin-I (UEA-I) peroxidase conjugate (Sigma Chemical Co.) for 30 minutes at 23°C. Control sections were incubated with 10% FCS in PBS. Sections then were washed, the color was developed, and the tissues were counterstained, as detailed above.

Scanning Electron Microscopic Studies

Vein segments were fixed in 0.1 mol/l Sorensen's phosphate buffer (pH 7.3) containing 2.5% glutaraldehyde (Agar Aids) for 24 to 36 hours at room temperature, rinsed in distilled water, and dehydrated through 30% to absolute alcohol. Dehydrated specimens were critical-point dried with three flushes of liquid CO_2 (CPD 750, EMSCOPE, Ashford, UK) and sputter coated with gold in a sputtering device (EMSCOPE). Specimens then were examined in a Jeol 840A scanning electron microscope (Welwyn, Garden City, UK).

For *en face* scanning electron autoradiography, glutaraldehyde-fixed vein segments were washed several times in distilled water, coated with K2 nuclear emulsion (Ilford, Cheshire, UK), and exposed for 1 week at 4°C. Segments then were treated with photographic developer and fixative, washed in distilled water, and dehydrated in alcohols, as described above. Specimens were critical-point dried, sputter coated, and examined, as described above.

Results

Intimal Surface Morphology and Cell Proliferation

Staining with trypan blue and scanning electron microscopy were used to investigate the integrity of the intimal surface cells after preparation for culturing, and after 14 days in culture. The intimal surface of segments of freshly isolated vein stained with trypan blue only close to the cut edges. Scanning electron micrographs of the central areas of freshly isolated vein segments (n = 20) showed a morphologically intact and almost continuous endothelial monolayer (Figure 1a). There were occasional gaps (G), much smaller than the dimensions of one cell, and a few adhering leukocytes (not shown). The edges of vein segments (about 20% of the total surface) had a more variable morphology, including areas of intact endothelium, areas showing endothelial cells with condensed nuclei, and areas of extensive endothelial denudation with exposure of the subendothelium (SE; Figure 1b). The intimal surface of vein segments that had been cultured for 14 days did not stain with trypan blue. In scanning electron micrographs, cells in the central region of vein segments (n = 20) were similar although less flattened than in freshly isolated vein (Figure 1c). Close to the cut edges of segments (Figure 1d), cells appeared larger, some appeared less cuboid, and there were frequent gaps of about cellular dimensions, spanned by fine filamentous projections, that have been called lamellipodia.[21]

Scanning electron autoradiographs of the intimal surface of three vein segments cultured for 1 day showed no nuclear localization of silver grains (not shown) but autoradiographs from all of the six vein segments examined after 14 days in culture showed silver grains over the nuclear region of cells (eg, Figure 1e). Most but not all of the replicating cells were confined to the intimal surface near the edges of vein segments.

To identify further and localize endothelial cells, transverse sections were subjected to cytochemistry with Ulex europeaus lectin. Sections of freshly isolated vein (n = 6; Figure 2b) showed an endothelial monolayer over an unstained medial layer. This was continuous in the central portion of segments but there were gaps at the edges of segments (not shown). Similar sections from cultured veins (n = 6; Figure 2c) showed that the intimal surface consisted mainly of endothelial cells with a few intervening unstained cells (X in Figure 2c). Beneath the intimal surface was a highly cellular neointimal layer. This new layer contained many unstained cells but there were also heavily stained profiles suggesting capillary lumeni (arrows in Figure 2c).

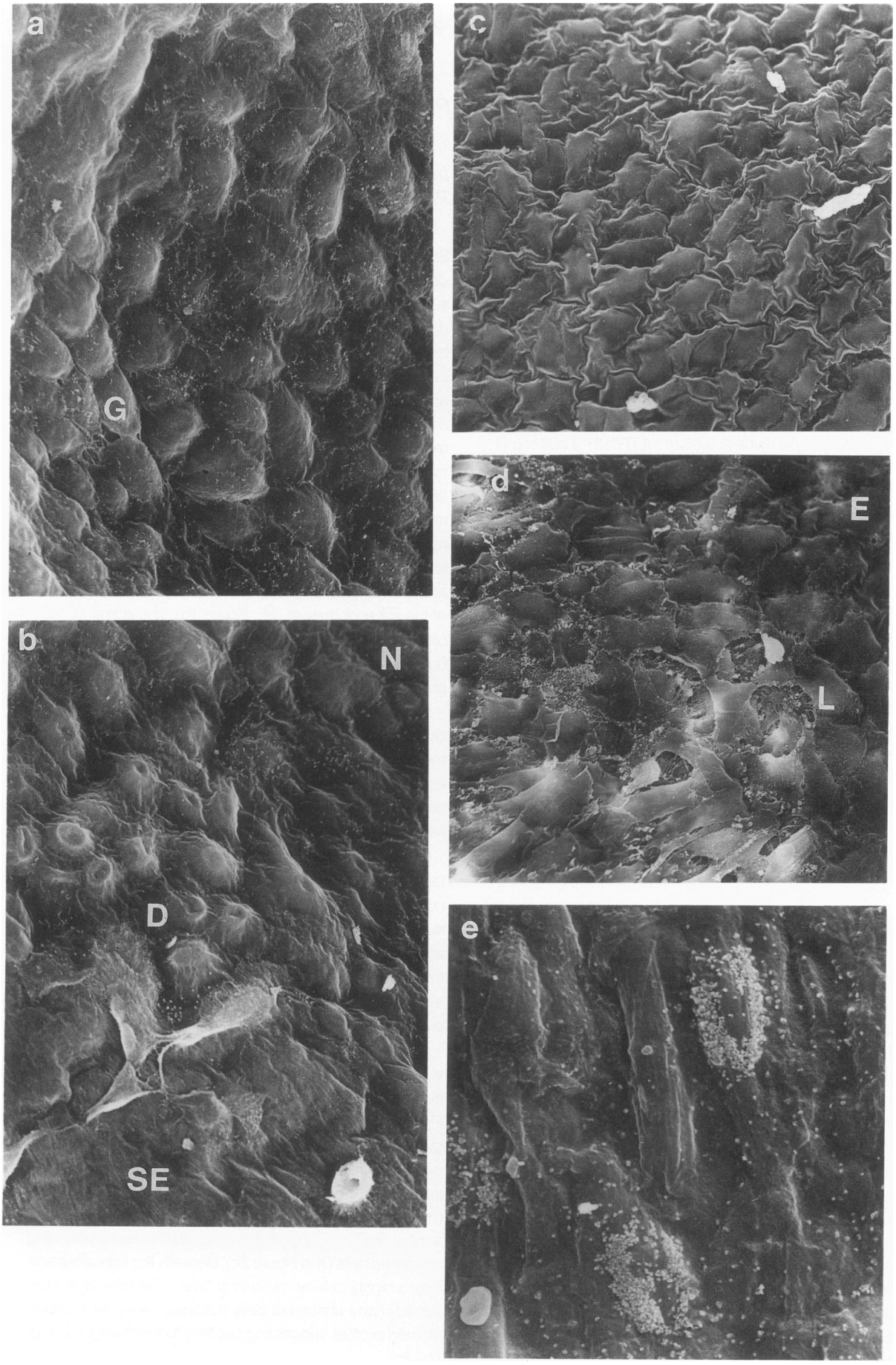
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Figure 1. *Scanning electron micrographs of veins before and after culturing for 14 days.* **a:** *The central area of a representative of 20 freshly isolated veins examined, showing well preserved endothelium save for occasional gaps (G) between cells (×1000).* **b:** *The intimal surface close to the cut edges of freshly isolated vein, showing a gradation from normal (N) to damaged (D) to denuded endothelium exposing the subendothelium (SE) (×1000).* **c:** *The central area of a representative of 20 cultured veins, showing a less flattened endothelium than in* **a** *(×800).* **d:** *The intimal surface close to the cut edge of another cultured vein, showing cells with typical endothelial morphology (E) but also cells with atypical morphology with large gaps spanned by fine filamentous projections, which have been called lamellipodia (L) (×800).* **e:** *A scanning electron autoradiograph of the intimal surface close to the cut edge of a representative of six cultured veins showing bulging nuclei surrounded by silver grains (×2500).*

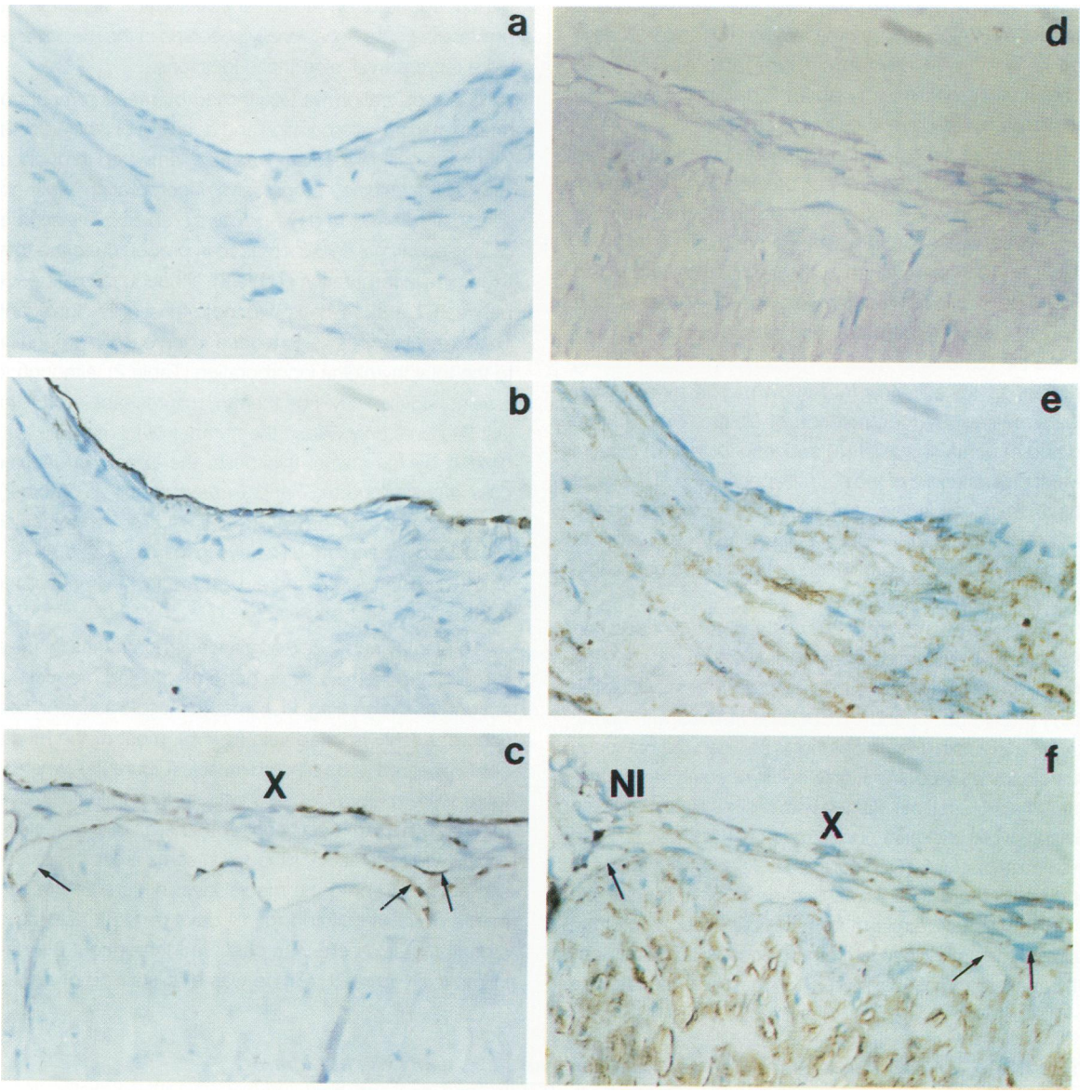

Figure 2. *Representative transverse sections of veins before (n = 6) and after culturing for 14 days (n = 6) stained with Ulex europaeus lectin (***b** *and* **c***) or antismooth muscle actin (***e** *and* **f***). Sections were counterstained with 0.5% aqueous methyl green. (Original magnification ×280.)* **a:** *The intima and part of the media of a freshly isolated segment. Negative control section for Ulex europaeus lectin.* **b:** *A serial section of* **(a)** *incubated with Ulex europaeus lectin to identify endothelial cells.* **c:** *A section from a cultured vein, showing a surface layer of endothelial cells but also an unstained cell (X). Note the highly cellular subendothelial neointimal layer, containing mostly unstained cells but with microlumeni bordered by endothelial cells (arrows).* **d:** *The intima and part of the media of a vein cultured for 14 days. Negative control section for anti-smooth muscle α- and γ-actins.* **e:** *A serial section to* **b**, *showing granular staining of the medial smooth muscle with no staining of the endothelial layer.* **f:** *A serial section to* **c** *showing granular staining of the medial layer and of most cells in the new intima (NI). Note the presence of a stained cell (X) at the intimal surface which was unstained with Ulex europaeus lectin in* **c**. *Note also the absence of staining of surface endothelial cells and of the microlumeni (arrows).*

Smooth Muscle Viability and Proliferation

The total tissue adenosine triphosphate (ATP) and adenosine diphosphate (ADP) concentrations were measured to quantify viability of the predominant cell type, the smooth muscle cells. The DNA concentration gave a measure of total cell numbers. Adenosine triphosphate concentration in segments of cultured veins was lower than in freshly isolated vein by approximately 30% after 7 days, and by approximately 20% after 14 days (Table 1). The ATP/ADP ratio increased by 25% after 7 days and 45% after 14 days in culture (Table 1). The DNA concentration declined by about 20% after 7 days but recovered by 14 days in culture (Table 1).

Transverse sections were examined by light microscopy to detect and localize cell proliferation. Sections of freshly isolated vein segments (n = 10; Figure 3a and d) showed the presence of an unthickened intima that was visible in sections stained with hematoxylin and eosin (Figure 3a) but invisible after staining with Miller's elastic and van Gieson (Figure 3d). Transverse sectioning confirmed the removal of most of the adventitial layer during preparation for culturing (not shown). The medial layer clearly defined by the presence of elastic fibres (Figure 3d and e) contained axial (A) and longitudinal (L) muscle layers, characteristic of the normal appearance of the large human veins, including saphenous vein.[12] Autoradiography of sections of freshly isolated vein exposed to [^{3}H]thymidine for 24 hours (Figure 3a) showed only background silver grain labeling.

In vein segments (n = 20) cultured for 14 days and exposed to [^{3}H]thymidine for the last 24 hours only, the medial morphology was similar to that of freshly isolated veins (Figure 3b, c, and e). Sections stained with hematoxylin and eosin (Figure 3b and c) showed the presence of a clearly defined and highly cellular neointima with frequent halos. Sections stained with Miller's elastic and van Gieson, which lacked a nuclear counterstain, also showed this neointimal layer (NI; Figure 3e) defined by the absence of staining for elastin. Intimal thickness (53 ± 5 μm thick, n = 13) was fairly uniform from the central portion of the vein segments to close to the cut edges. Autoradiography of transverse sections from 10 vein segments showed cells that had incorporated thymidine (Figure 3b and c). These were localized to the new intima (with only occasional labeled cells detectable in the medial layer) and were distributed both near the internal elastic lamina (Figure 3b) and near the endothelium (Figure 3c). Most of the labeled cells were clearly separated from the halos (Figure 3b and c) and appeared equally prevalent in the central portion of segments and close to the cut edges. There was no clearly defined layer of cell proliferation either along the cut edges or on the adventitial surface, although the proliferating cells occasionally observed in the medial layer were more prevalent in these locations.

Cell proliferation was quantified by measurements of total thymidine incorporation and counting of labeled cells. When the culture medium was supplemented throughout with [^{3}H]thymidine, progressive incorporation was observed after 7 and 14 days in culture (Table 2). The number of labeled intimal cells increased in proportion to the total thymidine incorporation (Table 2), although the difference between 7 and 14 days was not statistically significant. The intimal thickness also tended to increase in proportion to the total thymidine incorporation (Table 2), although to a value significantly less than in cultures labeled for the last 24 hours only. When the number of intimal cells was divided by the intimal thickness, the density of labeled cells was similar after 7 and 14 days in culture (Table 2). Approximately one half of the intimal cells were labeled with [^{3}H]thymidine after 14 days in culture (Table 2). At 1 and 7 days in culture, there were too few intimal cells to make a valid analysis.

To identify and localize vascular smooth muscle cells, immunocytochemistry was performed using monoclonal antibodies directed against smooth muscle actins. In sections of freshly isolated vein (n = 6), most of the medial cells appeared to be smooth muscle (Figure 2e), whereas there were many unlabeled cells in the adventitial layer (not shown). An unstained endothelial cell monolayer clearly was detectable, although in some areas it is closely superimposed onto the medial layer (Figure 2e). In segments of veins cultured for 14 days (n = 6), staining of smooth muscle cells was observed throughout the new intima (NI; Figure 2f). There was also staining of cells at

Table 1. *Variation in Purine Metabolite and DNA Concentrations with Days in Culture*

Days in culture	0	7	14
ATP concentration (nmol/g wet wt)	280 ± 20 (20)	190 ± 30* (8)	220 ± 20* (16)
ATP/ADP ratio	2.45 ± 0.15 (20)	3.05 ± 0.10* (8)	3.70 ± 0.20*† (16)
DNA concentration (μg/mg wet wt)	0.54 ± 0.05 (25)	0.44 ± 0.06 (10)	0.55 ± 0.04† (20)

* $P < 0.05$ *vs.* freshly isolated vein.
† $P < 0.05$ *vs.* 7 days in culture.
Values are means ± SEM for the number of veins shown in parenthesis.

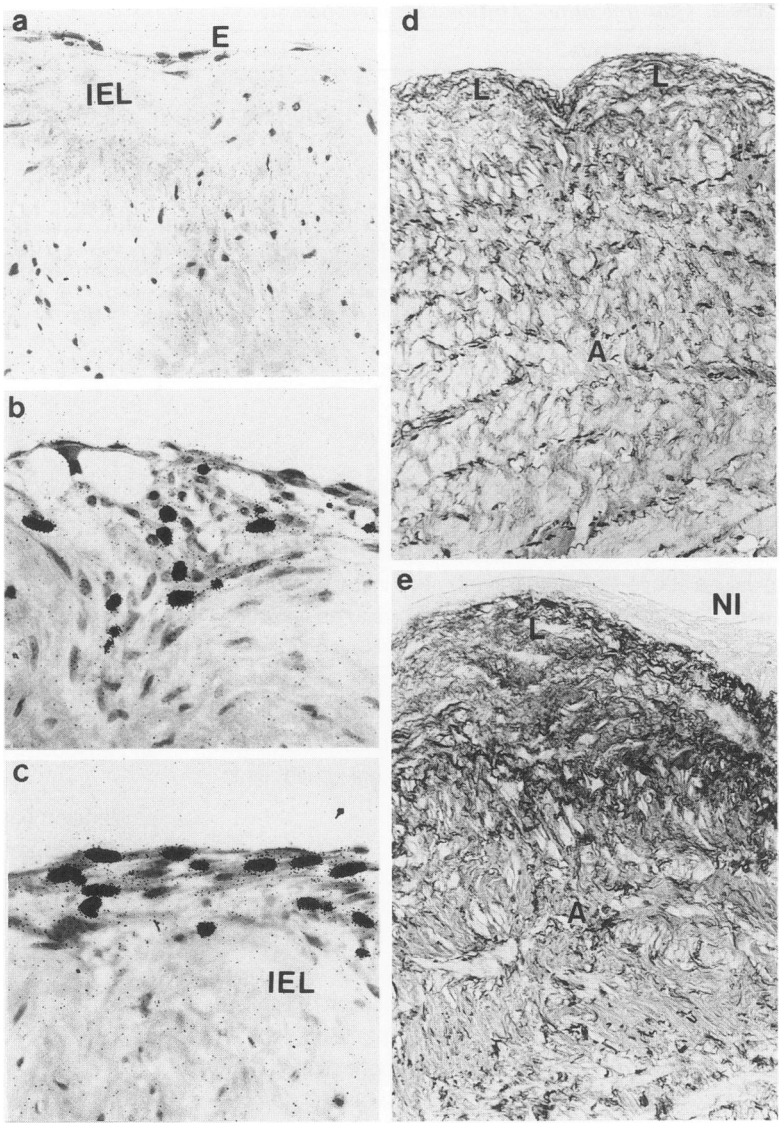

Figure 3. *Autoradiographs of transverse sections of veins cultured for 24 hours and 14 days. Sections were counterstained with haematoxylin and eosin (Original magnification ×900).* **a:** *The central portion of a representative of 6 veins cultured and exposed to [^3H]thymidine for 24 hours, showing only background silver grains over the endothelium (E), internal elastic lamina (IEL) and medial cell layers.* **b and c:** *Similar autoradiographs of 2 representatives of 20 cultured veins exposed for the last 24 hours to [^3H]-thymidine, showing silver grain labeling mainly in subendothelial nuclei* **(b)**, *but also at the intimal surface* **(c).** **d and e:** *Transverse section stained with Miller's elastic and van Gieson of veins before and after culturing for 14 days. (Original magnification ×170.)* **(d)** *The central portion of a representative of 10 freshly isolated veins showing elastic staining throughout the axial (A) and longitudinal (L) medial smooth muscle layers extending up to the intima (×170).* **(e)** *The central portion of a representative of 20 cultured veins showing the lightly stained and thickened new intima (NI) lacking elastic fibers (×170).*

the intimal surface (X in Figure 2f) that were unstained with Ulex europaeus lectin (X in Figure 2c).

Discussion

Cell Viability

Our first concern was to determine whether intimal and medial cells remained viable during preparation and tissue culture. Examination of intimal surface morphology showed that endothelial cell disruption occurred during preparation of veins for culturing, but only near the cut edges. The central areas of cultured veins appeared to retain a confluent endothelium both from scanning electron microscopy and lectin staining. The intimal surface at edges of cultured veins showed gaps between cells in scanning electron micrographs despite lack of trypan blue staining. Some cells had morphology atypical of endothelium in scanning electron micrographs. Transverse sections also showed cells at the intimal surface that failed to stain with Ulex europaeus lectin.

As we have previously shown,[14,22] tissue purine metabolite concentrations in human saphenous vein can be used to quantify the viability of the predominant cell type, namely the vascular smooth muscle cells. The concentrations of ATP and ATP/ADP ratio observed here were similar to values we found previously in human saphenous vein immediately after surgical removal and rinsing in physiologic saline,[22] showing that the preparative procedure caused little additional injury. The 30% decrease in ATP concentration observed during the first 7 days in culture and the reduction in DNA concentration indicated some smooth muscle cell loss. The decline in ATP con-

Table 2. *Quantification of Intimal Proliferation*

Days in culture	1	7	14
Thymidine incorporation (dpm/mg wet wt)	270 ± 60	1560 ± 220*	3020 ± 480*†
Labeled nuclei/mm	0	6 ± 2*	13 ± 4*
Intimal thickness (μm)	0	13 ± 2*	22 ± 5*
Labeled nuclei/mm^2	0	340 ± 110*	480 ± 120*
Thymidine index (%)	ND	ND	49 ± 8

Vein segments were continuously exposed to [^{3}H]-thymidine. Labeled nuclei contained at least 20 silver grains. The number of labeled nuclei was expressed per mm of intimal length. Labeled nuclei/mm^2 of cross section was calculated dividing the number of labeled nuclei/mm of intimal length by intimal thickness averaged over three measurements at equal intervals over the length available. The thymidine index was defined as the number of labeled cells in the intima divided by the total number of intimal cells (excluding surface endothelial cells). Values are mean ± SEM of observations on 10 to 12 veins.

* $P < 0.01$ *vs.* day 1.

† $P < 0.05$ *vs.* day 7.

ND, not determined.

centration appeared, however, to be halted between 7 and 14 days, and the DNA concentration actually increased. The ATP/ADP ratio also increased during culture to values above those consistently seen in freshly isolated veins. This may be characteristic of cultured vascular smooth muscle cells because this ratio is also higher in cultured rabbit aortic smooth muscle cells than in the intact aorta.[23] It might reflect transformation of some cells from the contractile into the synthetic phenotype.[24] There were, however, no detectable medial morphologic changes in light micrographs during culture.

Intimal Proliferation

The most interesting finding of this study was that a new intima was formed during culture. The location of labeled cells in transverse sections of veins exposed to [^{3}H]thymidine for the last 24 hours or throughout the culture period was similar to that of neointimal vascular smooth muscle cells. There were also labeled cells at the intimal surface, and this was confirmed by scanning electron autoradiography. Intimal surface labeling could have resulted from proliferation of endothelial or smooth muscle cells. Cultures continuously exposed to [^{3}H]thymidine showed a progressive increase in total thymidine incorporation and the number of labeled cells in the intima. Only approximately one half of the intimal cells were labeled after 14 days in culture, which suggests that the remaining cells probably migrated from the media.[3] The intimal thickening in such veins was, however, less than in veins exposed to thymidine for the last 24 hours only, which suggests that cell proliferation was inhibited somewhat by the continuous presence of [^{3}H]thymidine. This labeling index thus represents a minimal estimate of the proportion of cells that might arise by cell proliferation. There were no similarly defined areas of smooth muscle cell proliferation either at the cut edges or on the adventitial surface, although occasional proliferating cells were seen in these locations. A number of different hypotheses might account for this intimal-directed proliferation:

1. The distribution is unlikely to be explained entirely by a concentration gradient of exogenous growth factor because segments were completely immersed in culture medium and any gradient from the culture medium into the tissue would be equal at the intimal surface, cut edges, and adventitial surface.

2. A requirement for intimal injury in stimulating intimal proliferation has been suggested by studies *in vivo*[25,26] and in organ culture.[27,28] Thus a gradient of injury might theoretically account for intimal-directed proliferation. In our model, however, intimal proliferation occurred even in the central portion of segments where there were only slight initial changes in endothelial morphology and no evidence of subendothelial injury in scanning electron micrographs.

3. A gradient of cell viability also seems unlikely to explain intimal proliferation because a high overall degree of cell viability apparently was maintained.

4. There are known constraints on the proliferation of the medial cells due to contact between cells or with extracellular matrix components.[24] If these constraints are less at the intimal surface, it also might account for directed proliferation.

5. Finally, in other experimental models, fully confluent endothelial cells have been shown to inhibit smooth muscle cell proliferation by secreting glycosaminoglycans.[24] If production of such agents were reduced under our culture conditions, this also might contribute to the intimal-directed proliferation we observed. Intimally directed smooth muscle cell proliferation in organ cultures of pig aorta has been reported to depend on the presence of endothelium,[28,29] which suggests that an endothelium-derived chemoattractant may be released during culture. A similar phenomenon also might account for our observations, although this remains to be demonstrated directly.

Comparison of Organ Culture with Intimal Proliferation in Arteriovenous Bypass Grafts

Migration of proliferating smooth muscle cells was directed in our organ culture system toward the intima, as it is in vein grafts and in atherosclerotic arteries. Most of the cells in the new intima were identified by immunocytochemistry as vascular smooth muscle cells, as is the case in arteriovenous bypass grafts. The neointima was clearly visible as an orange-colored layer in sections counterstained with van Gieson (Figure 3e). This indicated a relatively low level of elastin staining of the neointimal cells. Proliferating vascular smooth muscle cells previously were shown to synthesize greatly increased amounts of collagen as their extracellular matrix[24] and to increase expression of specific collagen messenger RNAs.[30] On the other hand, the neointima of human saphenous vein arteriovenous bypass grafts stain histologically for elastin, despite the absence of organized elastic fibres.[7,11,12] The organ culture, therefore, does not mimic this aspect of the intimal changes in arteriovenous bypass grafts. The neointima in our organ culture model also contained microlumeni bounded by cells identified as endothelial cells with Ulex europaeus lectin. Further studies are needed to determine whether connections exist between the surface endothelial cells and these microlumenal cells. The formation of endothelial tubules is a characteristic feature of capillary growth that occurs both *in vivo*, in some advanced atherosclerotic lesions,[31] and *in vitro*.[32,33] It has not, however, been previously noted in organ culture. Neither has it been specifically referred to as a feature of the intimal changes that occur after arteriovenous bypass grafting. However transverse sections of rabbit jugular vein transplanted into the carotid arterial circulation stained with anti-human factor VIII-related antigen (Figure 3a of Zwolak et al[34]) apparently show immunoreactive cells deep in the neointima. This phenomenon deserves further investigation, given the potential importance of endothelium-derived angiogenic and mitogenic substances.

Because intimal proliferation is detectable in our organ culture system after periods as short as 14 days, this system promises to be useful for studying its molecular basis in human vein and to identify therapeutically useful inhibitors. The experimental model described here also may be readily adapted to simulate the effects of exposure to arterial pressure and flow or to investigate factors such as lipoprotein concentration, which are known to influence the patency of coronary artery bypass grafts.

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