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Jennichjen Slomp · Adriana C. Gittenberger-de Groot
J. Conny van Munsteren · Hans A. Huysmans
J. Hajo van Bockel · Victor W.M. van Hinsbergh
Robert E. Poelmann

Nature and origin of the neointima in whole vessel wall organ culture of the human saphenous vein

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Abstract Intimal proliferation is a characteristic feature of arteriosclerosis. Whole vessel wall organ culture systems have been developed to study the early stages of neointima formation. We have cultured a large number of explants of human saphenous vein specimens for several weeks, and have identified the nature of the cells in the newly formed intima by a panel of monoclonal antibodies recognizing endothelial cells (von Willebrand factor, platelet endothelial cell adhesion molecule-1 and EN-4 antigen), smooth muscle cells (monoclonal antibodies HHF35 and CGA-7) and fibroblasts (5B5 antibody). In addition we determined the uptake of fluorescently labelled acetylated low density lipoprotein by the surface cells of the explants. We found that an apparent neointima was formed in the vein organ system, the cells of which were predominantly smooth muscle cells and originated from the cut edges and from the adventitia of the vein segment. The endothelial cells originally lining the luminal surface of the vessel segments became overgrown by these cells. They remained at the base of the newly formed neointima and a number of them reorganized into capillary-like structures. Our data suggest that explant culture of saphenous vein does not reflect the classical concept of neointima formation, in which intimal smooth muscle cells migrate through the internal elastic lamina and accumulate in the intima. Although it has this limitation, the model may serve well to study specific aspects of cell migration, smooth muscle cell differentiation and angiogenesis, and may reflect aspects of intimal thickening at surgical suture sites.

Key words Organ culture · Saphenous vein · Neointima formation · Endothelial cells · Smooth muscle cells

J. Slomp · A.C. Gittenberger-de Groot (✉) · J.C. van Munsteren
R.E. Poelmann
Department of Anatomy and Embryology, University of Leiden,
Wassenaarseweg 62, P.O. Box 9602,
NL-2300 RC Leiden, The Netherlands
Tel.: (31) 71-5276691; Fax: (31) 71-5276680

H.A. Huysmans
Department of Cardiothoracic Surgery,
University Hospital Leiden, Leiden, The Netherlands

J.H. van Bockel
Department of Surgery, University Hospital Leiden,
Leiden, The Netherlands

V.W.M. van Hinsbergh
Gaubius Laboratory, TNO-PG, Leiden, The Netherlands

Introduction

Proliferation and migration of intimal smooth muscle cells play important roles in several forms of pathological intimal thickening: in arteriosclerosis [31, 32], in pulmonary hypertension [14], in restenosis of bypass grafts [22] and after percutaneous transluminal coronary balloon angioplasty [24]. In physiological intimal thickening, as seen in the last phase of the development of the ductus arteriosus, proliferation and migration of smooth muscle cells are also important. In this vessel, the develoment of intimal thickening starts with lifting of endothelial cells from the underlying layer [11, 33]. High levels of glycosaminoglycans and particularly hyaluronan are present directly underneath the endothelium [7, 33]. The increased synthesis of hyaluronan arises from increased transforming growth factor-β (TBG-β) levels present in the ductus arteriosus just prior to intimal cushion formation [5].

In pathological intimal thickening in man it is difficult to study the early stages. Clinical symptoms of the intimal thickening are often not noted until the lumen of the vessel is largely occluded. To study early intimal thickening formation, an in vitro system has been developed in which the structure of the vessel wall remained intact. In this type of vessel wall culture, neointima formation has been observed in segments of pig aorta [12, 20], human saphenous vein [1, 34, 35] and human mammary artery [15, 16].

The initial purpose of the present study was to identify whether neointima formation in the cultured vessels is identical to the formation of intimal thickening in the ductus arteriosus. We cultured human saphenous veins

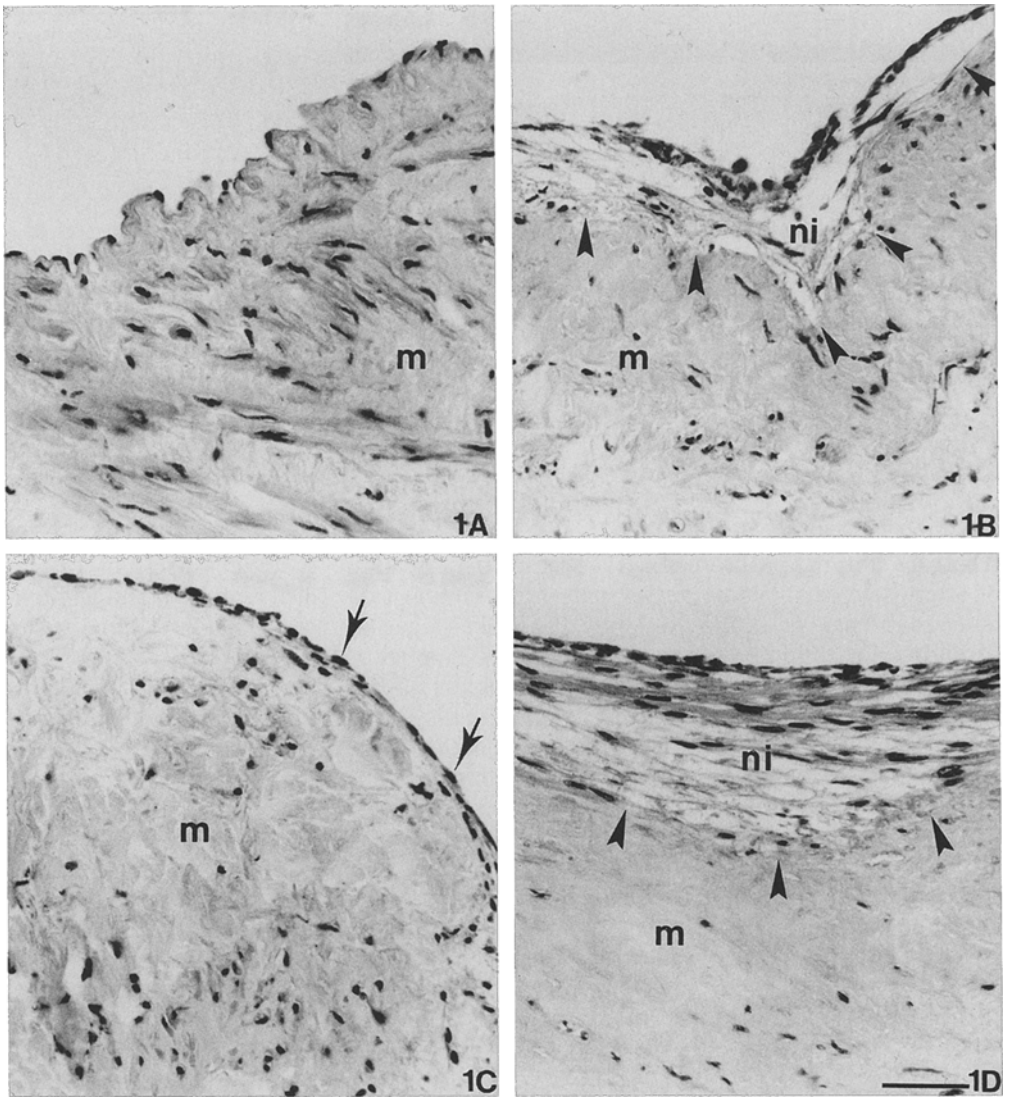

Fig. 1 Haematoxylin and eosin (H&E) staining of a cross-section of a segment of saphenous vein before culture (**A**). The vessel wall is intact and not damaged. **B** Segment of saphenous vein after 2 weeks of culture. A loose neointima is found on top of the vessel wall (*arrowhead* shows bottom of neointima). **C** Same segment as in **B**, showing the cells lining the cut edges of the specimen (*arrows*), which form a continuity between the surface cells and the adventitia. **D** Segment of saphenous vein cultured for 4 weeks. Note the highly cellular neointima (*ni*). (*m* Media, each ×80)

up to 5 weeks, according to the method of Soyombo and colleagues [34]. Preliminary histochemical studies indicated that the surface of the neointima was lined by cells that were not recognized by anti-endothelial cell antibodies. Subsequently, we have identified the lining and neointimal cells in the vein segment explants, using a panel of monoclonal antibodies that specifically recognize endothelial cells, smooth muscle cells and fibroblasts. In addition, the presence of hyaluronan was studied using the hyaluronectin-anti-hyaluronectin complex as a probe.

Table 1 Number of specimens of human saphenous vein cultured for different time periods with various serum concentrations (*ND* not determined)

Serum concentrations in culture medium	Culture period in weeks				
	1	2	3	4	5
30% Fetal bovine serum	2	37	2	6	1
20% Fetal bovine serum	ND	8	ND	1	ND
10% Fetal bovine serum	ND	5	ND	ND	ND

Materials and methods

Preparation and culture of freshly-isolated vein

Segments of saphenous vein were obtained from 52 patients (43 male, 9 female; mean age 63 years, range 39–79 years), who were undergoing coronary bypass graft operation, according to the guidelines of the Institutional Review Board of the University Hospital Leiden, The Netherlands. Damage to the vessel was prevented by immediate immersion in culture medium and using a no touch technique. The culture of the veins was done as described by Soyombo and colleagues [34]. Briefly, segments were collected in sterile RPMI 1640 tissue culture medium supplemented with 20 mmol/l HEPES buffer (Gibco, Paisley, Scotland), 4 IU/ml sodium heparin (Organon, Boxtel, The Netherlands), 2.5 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l L-glutamine and 2.5 μg/ml amphotericin B (Gibco), and transported to the laboratory at room temperature. Excess fat and adventitial connective tissue were removed gently. The for a vein typical muscular adventitial layer remained intact. The vessel was opened along its upper aspect. Two small pieces were cut off. The first was fixed

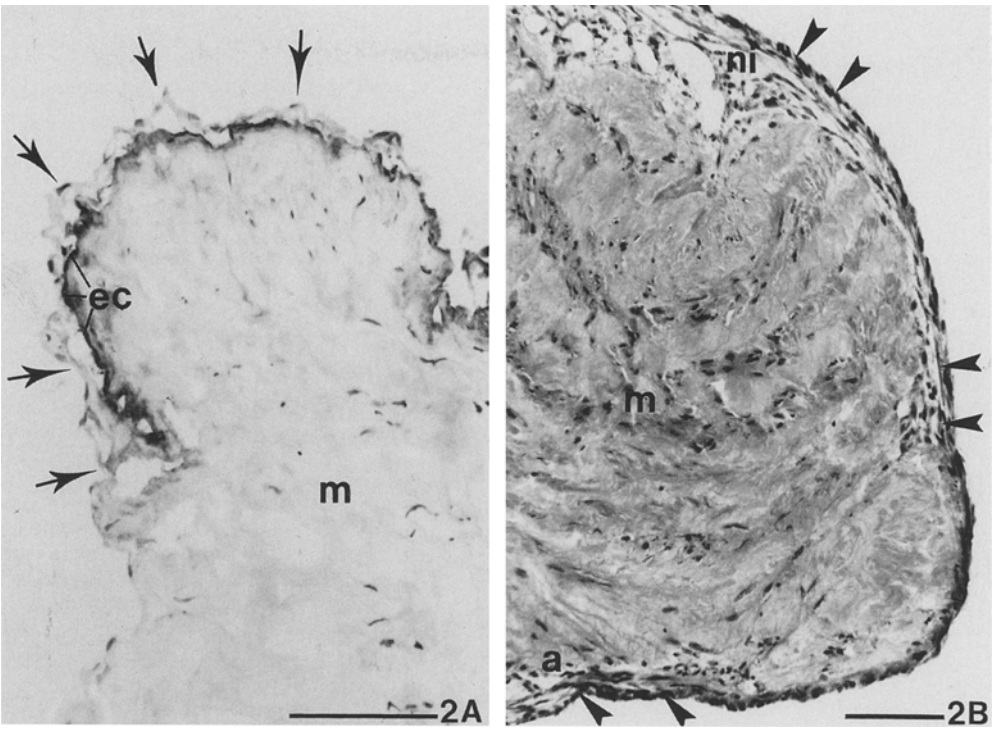

Fig. 2 **A** Initial stages of apparent neointima formation. Cells negative for α-von Willebrand factor (vWF) staining (*arrows*) grow over the edges and lay on top of the endothelial cells (*ec*), which are stained by α-vWF (×68). **B** Newly formed proliferations (*arrowheads*) at the luminal side, the cut edge side and the adventitial (*a*) side of the explant shown by H&E staining. (×43.5)

in 2% acetic acid in ethanol and embedded in paraffin, the second submerged in OCT compound (Tissue Tek; Miles Laboratories, Naperville, USA) and quickly frozen in liquid nitrogen-chilled isopentane (−196° C). The remaining part of the vein, which was between 0.5 and 1 cm in length, was fixed by needles in a petri dish coated with Sylgard 184 silicone elastomer (Down Corning, Seneffe, Belgium), with its endothelial surface upward and washed with the described medium lacking the heparin. Vein segments were cultured for a period up to 5 weeks at 37° C under 5% (v/v) carbon dioxide in air in RPMI 1640 medium supplemented with 30% heat-inactivated fetal calf serum (Gibco), 2 mmol/l L-glutamine and the above given antibodies. The medium was replaced every 2–3 days. To study the progression of the formation of the neointima, we ended the culture at different time points. After culture, the vein was divided into two pieces and fixed as described above. The effect of serum concentration, 30, 20 or 10% heat-inactivated fetal calf serum or 10% heat-inactivated newborn calf serum (Gibco) and heparin concentration (5, 10 or 15 IU/ml) were evaluated separately. In addition to fixed vessel explants, floating segments and tubular pieces of vein were cultured and evaluated.

Light microscopy

Paraffin embedded tissue was sectioned at 5 μm. Before use the sections were rehydrated. Frozen tissue was sectioned at 5 μm at −12° C and stored at −20° C. Before staining the sections were fixed in cold acetone (−20° C), air dried for 1 h at room temperature and rehydrated. Sections used for immunohistochemistry were preincubated with 0.3% hydrogen peroxide (H_2O_2) and 0.1% sodium azide in phosphate buffered saline (PBS) for 15 min to eliminate endogenous peroxidase activity, after which they were rinsed in PBS.

To study the morphology of the vein, haematoxylin and eosin (H&E) staining was used. Immunohistochemistry was performed for identification of the various cell types. Sections were incubated overnight at room temperature with various monoclonal antibodies. To identify endothelial cells, we used EN-4 (1:5, Sera-Lab, Sussex, UK), a monoclonal antibody recognizing all types of human endothelial cells, and the monoclonal antibody RUU-P1 7E8, which recognized human platelet endothelial cell adhesion molecule-1 (PECAM-1; 1:3,000) [23] (a gift from Dr. H.K. Nieuwenhuis, University of Utrecht, Utrecht, The Netherlands) and a rabbit polyclonal antibody recognizing human von Willebrand factor (vWF; 1:40,000; Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands).

For the identification of smooth muscle cells the monoclonal antibody CGA-7 was used, which recognizes smooth muscle actin isozymes (1:1,000) [13] and HHF35 a monoclonal antibody against isotypes of muscle specific actins (1:1,000) [36]. Both antibodies were a gift from Dr. A. Gown, University of Washington, Seattle, USA.

To see whether there were any fibroblasts in the culture we used the fibroblast 5B5 monoclonal antibody (1:500, DAKO, Glostrup, Denmark), which recognized human prolyl-4-hydroxylase.

Bound monoclonal antibodies were detected using horseradish peroxidase (HRP) conjugated rabbit anti-mouse antibodies (1:300, DAKO) and bound polyclonal antibodies with HRP conjugated swine anti-rabbit antibodies (1:200, DAKO). All antibodies were diluted in PBS with 0.1% bovine serum albumin (Sigma, St. Louis, Mo., USA). The sections were exposed for 8 min to 0.04% diaminobenzidine tetrahydrochloride (DAB) in 0.05 mol/l TRIS-maleate buffer (pH 7.6) with 0.006% H_2O_2. The reaction was stopped by tap water. Sections were counterstained with haematoxylin.

To study the distribution of the glycosaminoglycan hyaluronan, we used a hyaluronectin-anti-hyaluronectin complex as a probe. The hyaluronectin was isolated from lamb brain by affinity chromatography [9], lyophilized and stored at 4° C until use. The hyaluronectin antibody was obtained by immunizing rabbits. The first immunization was performed using 100 μg of lamb hyaluronectin in complete Freund's adjuvant. Boosters were made every fortnight with 100 μg hyaluronectin in incomplete adjuvant [8]. The antiserum was purified by adsorption to a hyaluronectin column. The hyaluronectin-anti-hyaluronectin complex was made by mixing the antibody with a slight excess of hyaluronectin [10]. Sections were incubated for 3 h at 37° C in a moisture chamber. The complex was detected by using HRP conjugated swine anti-rabbit antibodies (1:200, DAKO) followed by an incubation with the above described DAB solution and counterstained. All sections were dehydrated in graded ethanol and mounted in Entellan (Merck, Darmstadt, Germany).

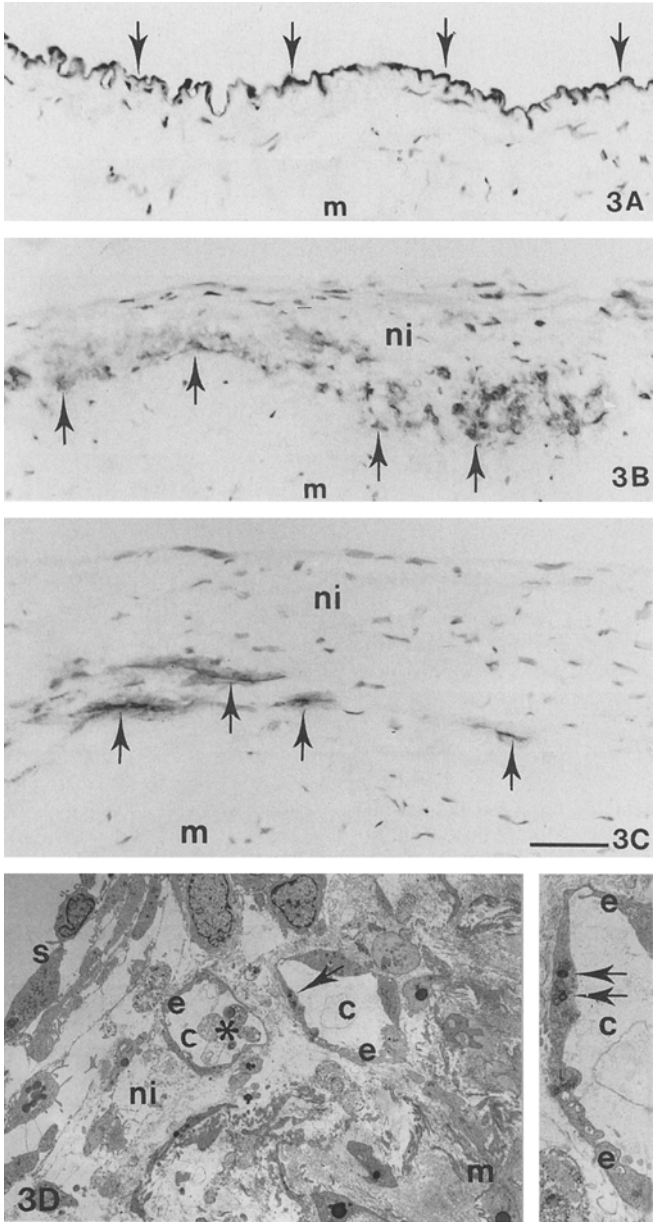

Fig. 3 Anti-endothelial staining (EN-4) of saphenous vein before culture (**A**). The endothelial layer (*arrows*) is intact. **B** Saphenous vein after 2 weeks of culture. Surface cells of the neointima are negative, while cells and remnants of cells are stained at the base of the neointima (*arrows*). **C** Segment of vein cultured for 4 weeks showing a similar picture as after 2 weeks of culture, with the endothelial cells (*arrows*) at the base of the apparent neointima (×80). **D** Electron micograph of neointima showing endothelial cells (*e*) arranged in capillary-like structures (*c*; ×2,200). Note that the lumen contains cell debris (*asterisk*) indicating that it is probably not in contact with the surface (*s*) of the explant. *Inset* shows detail with Weibel-Pallade bodies (*arrows*) in the endothelial cells (×5,000)

Electron microscopy

To verify the endothelial cells within the neointima electron microscopic (EM) studies were performed on parts of cultured vessels. Tissues were fixed in a mixture of 2% paraformaldehyde and 1% glutaraldehyde for 4–16 h. Postfixation in 1% osmium tetroxide was followed by dehydration through a graded series of ethanol and embedding in Epon.

Ultrathin sections were contrasted with lead hydroxide and uranyl acetate and examined in Philips 200 electron microscope.

Acetylated low-density lipoprotein incorporation

Cultures were studied for their ability to incorporate diI-labelled acetylated low-density lipoprotein (diI-ac-LDL; a generous gift from Dr. Theo van Berkel, University of Leiden, Leiden, The Netherlands), which is often used as a marker for endothelial cells and macrophages [20, 28]. At day 0 and day 14 of culture, organ cultured segments of vein were incubated with RPMI 1640 medium containing 20 mmol/l HEPES buffer, 2.5 μg/ml gentamicin, 100 μg/ml penicillin, 100 U/ml streptomycin, 2 mmol/l glutamine, 2.5 μg/ml amphotericin B and 10 μg/ml diI-ac-LDL for 6 h. The vein segments were washed three times in the probe-free medium and then fixed in 4% paraformaldehyde in PBS for 10 min. The cultures were mounted in glycerol: PBS (1:1) on a slide and the surface covered with a glass coverslip. The surface was studied under rhodamine light on a Leitz dialux 2Q EB photomicroscope. Afterwards the specimens were postfixed in 4% paraformaldehyde in PBS, paraffin embedded, sectioned, H&E stained and controlled for presence of the neointima.

Results

Segments of human saphenous vein, pinned down and cultured for 2 weeks, formed a loosely structured neointima (Fig. 1B). When the culture time was prolonged, the neointima became thickened and highly cellular (Fig. 1D). A continuity (Fig. 1C) between the neointima and cut edges of the segment was always present, raising the possibility that cells from the cut edges contributed to the apparent neointima.

To evaluate the possibility that cells from the cut edges contributed to the neointima, we harvested segments of vein in early stages at different time points (Table 1). The time course showed that cells coming from the sides of the explant migrated over the endothelium and formed a neointima (Fig. 2A). This overgrowth was inhibited when the serum concentration was reduced to 10% and 5–15 IU heparin was added simultaneously (13 cases). Low serum concentrations, however, reduced the viability of the cells in the media of the vein as seen with H&E staining. Development of a neointima in our organ culture supplemented with 20% serum was comparable to neointima formation in a culture supplemented with 30% serum. When the segments were not pinned down properly or when they were cultured floating in the medium (4 cases), an apparent neointima was found and in addition a proliferative layer formed on the cut edges of the segment and on the adventitial side (Fig. 2B). The latter phenomenon was also evident when tubular segments were cultured (4 cases). In tubular segments, however, a neointima was only found at the cut edges of the specimen and not in the middle. In none of the vessel explants did we find a neointima without a connection between the surface cells and the adventitia.

Before explant culture, endothelial cells lined the lumen of the veins (Fig. 1A). This was verified by immunohistochemical staining with endothelial cell recognizing

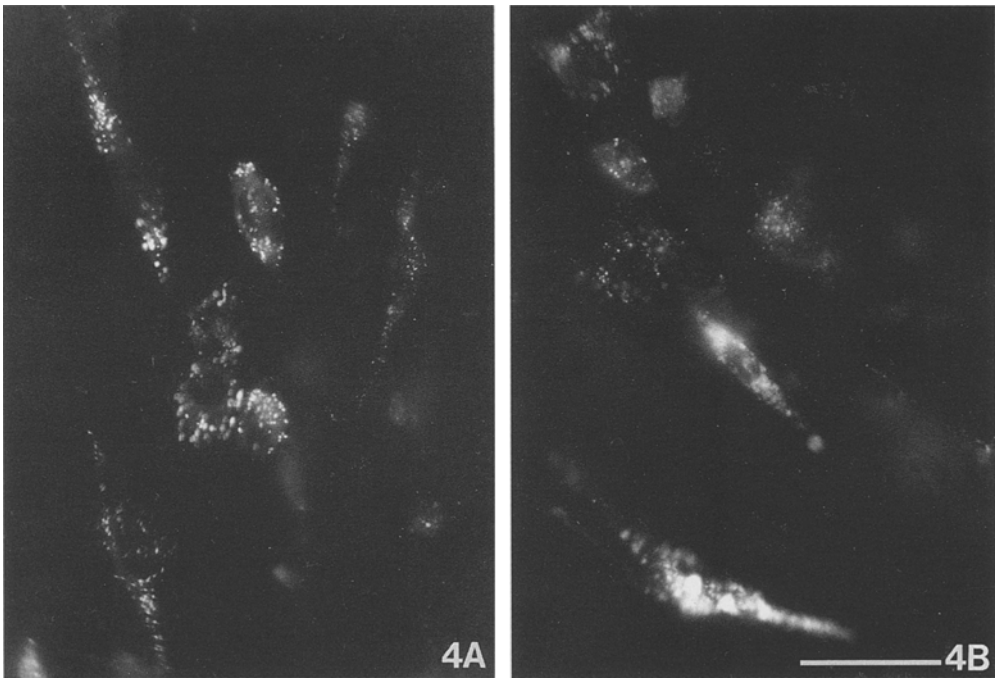

Fig. 4 En face view of vein segment explants demonstrating diI labelled acetylated low density lipoprotein incorporation by the surface cells of the vessel vein segment (**A**) before culture and (**B**) after 2 weeks of culture. Both segments show a similar staining pattern. Autofluorescence of the surface cells was very weak (×128)

antibodies EN-4 (Fig. 3A), anti-PECAM-1 and anti-vWF (not shown). Incorporation of diI-ac-LDL was shown in endothelial cells in day 0 cultured veins by a strong fluorescence of the surface cells (Fig. 4A; four cases).

During initial stages of neointima formation the endothelial cells became overgrown by cells, which were not recognized by any of the endothelial specific antibodies. The endothelial cells and/or remnants of these cells were found at the base of the neointima (Fig. 3B, C) being the the transition zone between the original vessel and the neointima. The endothelial cells were often reorganized into capillary-like structures (Fig. 3B). These tubular structures which stained for EN-4 were confirmed to be lined by endothelial cells at the EM level (Fig. 3D). Weibel-Pallade bodies are found in the endothelial cells of the capillary like structures (Fig. 3D, inset) but not in the cells lining the surface of the explant. Fluorescence of the surface layer after diI-ac-LDL incorporation was similar to day 0 cultured veins (Fig. 4B; four cases).

Smooth muscle cells in the intima, media and inner adventitia of freshly obtained veins were strongly positive for the antigens recognized by the monoclonal antibodies HHF35 and CGA-7 (Fig. 5A). After explant culture, the staining for these antigens became less intense in the smooth muscle cells of these areas (Fig. 5B, C). The cells in the apparent neointima were only slightly positive during the early stages of neointima formation (Fig. 5B). There was, however, an increased expression of antigens recognized by HHF35 and CGA-7 when the neointima had expanded and contained more cells (Fig. 5C). The upper layer stained less than the base of the neointima.

In both early and late stages of neointima formation, neointimal cells were positive for the fibroblast 5B5 antibody (Fig. 5E, F), which has been reported to recognize fibroblasts. Before explant culture the fibroblast 5B5 antibody did not stain any cell of the vessel wall including the remaining part of the adventitia.

The apparent neointima of veins maintained in culture for 2 weeks was rich in extracellular matrix, as was deduced from the loose structure observed with H&E staining. Hyaluronan was detected throughout the vessel wall, being markedly present in the neointima (Fig. 6B, C). Before explant culture the highest concentration of hyaluronan was found directly underneath the endothelium (Fig. 6A).

Discussion

Various organ culture systems of whole vessel walls, such as human saphenous vein, mammary artery and pig aorta, have been developed to study the phenomenon of neointima formation [1, 12, 15, 16, 20, 34, 35]. In this study we observed that the apparent neointima formed in a whole vessel wall organ culture system originates from cells of the adventitia growing over/from the cut edges to the surface area (Fig. 7). Under our experimental conditions, these cells overgrow the original lining endothelial cells. Simultaneously, some of the endothelial cells positioned at the base of the apparent neointima reorganize into capillary-like structures. Subendothelial and medial smooth muscle cells may also contribute to the neointima, but the formation of the neointima does not fully resemble the early stages of intimal proliferation in vivo. When intimal proliferation is seen in vivo, as in the early development of arteriosclerosis [31], spontaneous changes in the coronary arteries of infants [26, 30] and (pulmonary) hypertension [14, 21, 25, 32], endothelial cells line the lumen [11, 31–33].

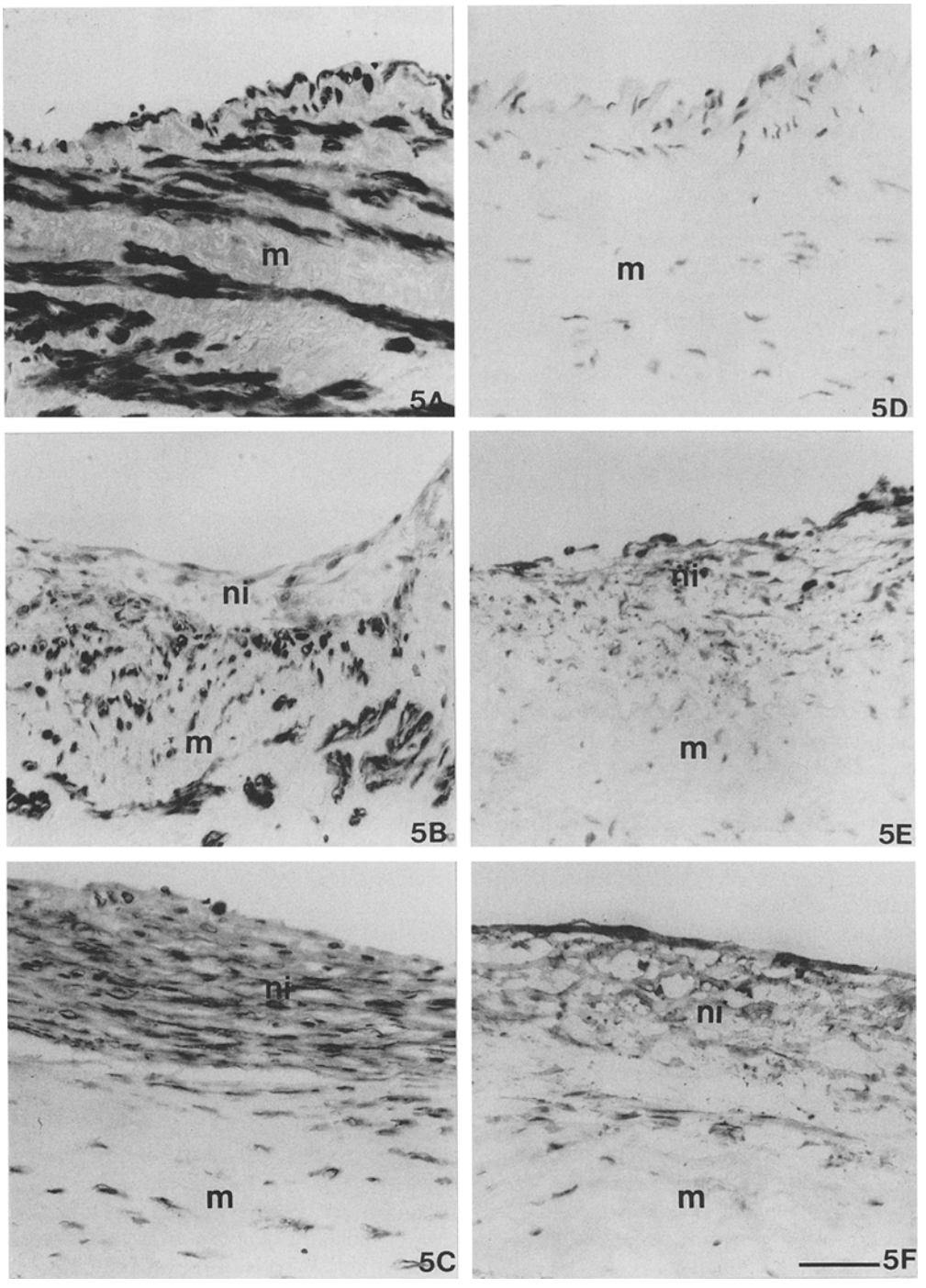

Fig. 5A–F Presence of smooth muscle actin isozymes demonstrated by monoclonal antibody CGA-7. **A** A segment of human saphenous vein before culture. Medial smooth muscle cells are stained strongly. **B** Neointimal cells of the segment of saphenous vein after 2 weeks of culture are only faintly stained by the CGA-7 antibody. **C** After 4 weeks of culture staining of smooth muscle actin in the apparent neointima is more prominent. **D** The anti-fibroblast 5B5 antibody did not react with the vessel wall before culture, but reacted with neointimal cells (**E**) after 2 weeks of culture and (**F**) after 4 weeks of culture. (×80)

We found that the cells at the surface of the apparent neointima were identical to the other neointimal cells and most probably are smooth muscle cells. Whether they are differentiated or undifferentiated is not known, but the weak actin staining pattern in the first 2 weeks of culture suggests that they are not fully differentiated. The migration of the smooth muscle cells from the sides of the segments to the surface may be enhanced by hyaluronan; before culture large amounts of hyaluronan were found at the surface of the explant. In the ductus arteriosus hyaluronan is known to be a chemoattractant for smooth muscle cells in the process of intimal cushion formation [4, 7]. Production of hyaluronan by smooth muscle cells is endothelium-dependent and is partly regulated by TGF-β, which has been shown to be elevated in the ductus arteriosus just prior to intimal thickening [3–5, 7]. In tubular segments and segments that were not pinned down properly into the culture dish we found proliferation on the adventitial side of the segment. De Mey and colleagues and Boonen and colleagues found similar

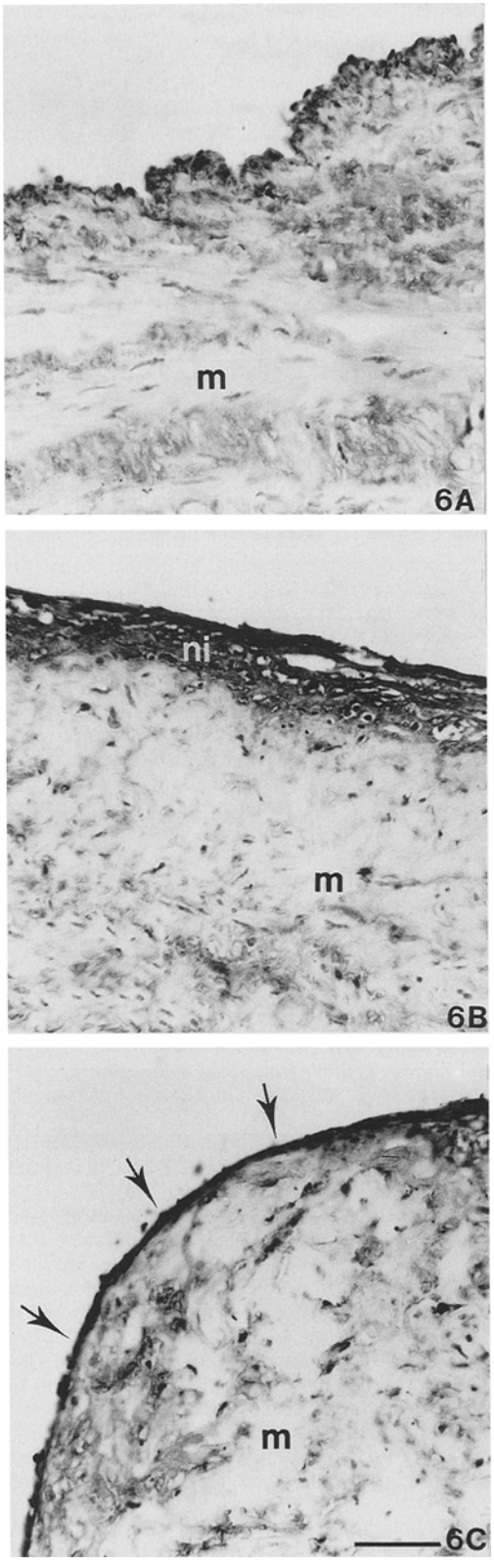

Fig. 6 **A** Presence of hyaluronan in human saphenous vein before culture. Staining is predominantly seen in and directly underneath the endothelium. **B** After 14 days of culture the apparent neointima contains large amounts of hyaluronan. **C** The cells along the sides of the specimen are also highly positive for hyaluronan (*arrows*). (×80)

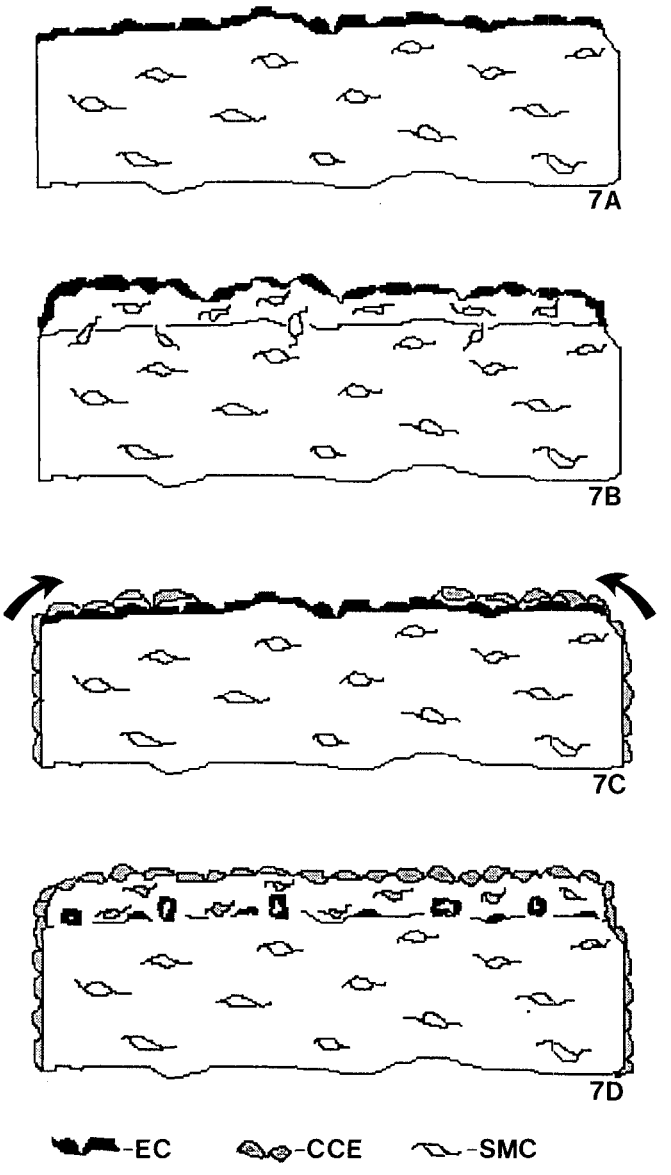

Fig. 7A–D Schematic drawing of a human saphenous vein segment in culture. **A** Vein segment before culture. **B** Concept of a cultured vein after 2 weeks of culturing as described by Soyombo and colleagues [34]. Note the high similarity with initial stages of intimal thickening, in which cells from the media contribute to the intima. **C** A few days after the start of the culture according to our results. Cells from the cut edges of the explant overgrow the endothelial cells. **D** A vein cultured for 2–4 weeks. The intima is lined and formed by the cells coming from the sides of the explant. The endothelial cells are found at the bottom of the neointima, often reorganized into capillary-like structures. (*EC* Endothelial cells, *CCE* cells from the cut edges of the explant, *SMC* smooth muscle cells)

smooth muscle cell proliferations on the adventitial side when they cultured rat arteries [2, 6].

The absence of antigens recognized by several endothelial cell specific antibodies suggests that the neointimal lining cells were not endothelial cells. The latter were found at the base of the neointima. We observed, however, that the surface cells were able to take up diI-ac-LDL, which is considered to be specific for endotheli-

al cells and macrophages [20, 28]. This is not conclusive for endothelial cell detection as smooth muscle cells and fibroblasts may also display dil-ac-LDL receptors and dil-ac-LDL uptake under certain conditions [17–19, 27, 29]. The results found with dil-ac-LDL suggest that the surface neointimal cells have some features in common with endothelial cells and may represent pseudoendothelial cells. This may also explain why Soyombo and colleagues [34] found that *Ulex europaeus* lectin-1, a fucose-binding lectin that is often used as an endothelial marker, bound to the luminal surface of human saphenous vein segments after organ culture. In their study some of the luminal cells did not stain, however, and a number of cells at the base of the neointima were positive, which fits with our findings.

Our data suggest that the whole vessel wall organ culture system has its limitations when studying the formation of early intimal proliferations as it proceeds in vivo. However, the model may reflect part of the often observed intimal thickening at surgical sutures of grafted vessels. Furthermore, cultured vessel explants can be used to study cell migration and differentiation characteristics of migrating smooth muscle cells. In addition, the reorganization of endothelial cells into capillary-like structures [34, this paper] after lateral overgrowth by smooth muscle cells can provide mechanistic information about angiogenic and remodelling processes.

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