Role of endothelial cell denudation and smooth muscle cell dedifferentiation in neointimal formation of human vein grafts after coronary artery bypass grafting: therapeutic implications

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Abstract

Objective—To provide better insights into the genesis of neointimal thickening in human vein grafts early after surgery.

Design—Retrospective study.

Setting—Tertiary referral centre.

Subjects—18 distal anastomotic sites of patent grafts, obtained at necropsy from eight patients who died over differing periods (ranging from two days to nine months) after the procedure.

Main outcome measures—Immunohistochemical evaluation of smooth muscle cell phenotype modulation in relation to proliferative activity.

Results—The earliest changes are characterised by loss of surface lining endothelial cells and insudation of blood corpuscular elements admixed with fibrin-platelet thrombus. At sites of injury vimentin positive and actin negative spindle shaped cells appear in the intima, while the related pre-existent media shows focal absence of actin positive smooth muscle cells. Proliferative activity colocalises at these sites. With time distinct neointimal thickening occurs, associated with disappearance of proliferative activity and a phenotypic shift of the smooth muscle cells.

Conclusions—The observation that luminal endothelial cell denudation, with insudation of the intima with blood elements, occurs in the very early stages suggests that these phenomena are responsible for the observed dedifferentiation of pre-existent smooth muscle cells, known to be a prerequisite for cell proliferation and the evolution of intimal thickening. It is likely, therefore, that platelet released growth factors play a pivotal role, which thus may provide a target for preventive pharmacological intervention.

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Late (> 1 year) obstruction of human vein grafts following coronary artery bypass grafting (CABG) has been extensively documented and is caused by either exuberant neointimal thickening or florid atherosclerosis.[1][2] However, little is known about the early (< 1 year) changes, despite the fact that one may anticipate that the scene for late obstruction is set soon after the surgical procedure.[3–6] Unni and colleagues[4] documented insudation of blood constituents into the vein intima as the earliest change, whereas Kockx and colleagues[6] showed that less than 10 days after grafting, veins contained smooth muscle cells (SMCs) in the media with reduced expression of α smooth muscle actin, suggesting a synthetic phenotype. These are intriguing observations which need further evaluation, since they may provide a better insight into the genesis of neointimal thickening and, hence, may open gateways to novel therapeutic interferences. This was the main purpose of our study.

Methods

PATIENTS

Hearts were obtained at necropsy from eight patients who had died within one year

Table 1   Relevant clinical data

Patient	Age (years)	Sex	Reason for CABG	Anastomotic sites examined*	Interval CABG/death	Cause of death	Risk factors
1	74	Female	Unstable AP	RCA (3) OM (12)	2 days	Abdominal aortic aneurysm rupture	Hypercholesterolaemia
2	66	Male	AMI	LAD (8) RCA (3) OM (12)	3 days	Perioperative myocardial infarction	Diabetes mellitus
3	82	Male	Unstable AP	LAD (8) OM (12)	6 days	Renal failure	Hypertension
4	64	Male	Unstable AP	RCA (3) DX (9)	7 days	Mediastinitis	
5	55	Male	OMI + CHF	LAD (7)	9 days	Perioperative myocardial infarction	Diabetes mellitus; hypercholesterolaemia; hyperuricaemia; smoking
6	61	Male	AMI	LAD (7) OM (12)	33 days	Cardiac failure	Diabetes mellitus; hypertension; smoking
7	77	Female	Unstable AP	LAD (7) RCA (3) DX (9) PL (14)	4 months	Cerebral infarction	Hypertension; hypercholesterolaemia
8	82	Female	Unstable AP	RCA (3) PL (14)	9 months	Cardiac failure	Hypertension

*Segments of the coronary arteries are shown in parentheses (according to American Heart Association committee report[7]).
AMI, acute myocardial infarction; AP, angina pectoris; CABG, coronary artery bypass grafting; CHF, congestive heart failure; DX, diagonal branch; LAD, left anterior descending artery; OM, obtuse marginal branch; OMI, old myocardial infarction; PL, posterolateral branch; RCA, right coronary artery.

Table 2   Monoclonal antibodies used in the study

Antibody	Specificity and reactivity	Reference	Source	Working dilution
HHF-35	Muscle actin	Gown et al[8]	Dako	1:50
CGA-7	Smooth muscle cell actin	Gown et al[8 9]	Enzo	1:20
Anti- vimentin	Smooth muscle cells, fibroblasts, macrophages, endothelial cells		Dako	1:100
HAM-56	Macrophages, some endothelial cells	Gown et al[8]	Dako	1:70
PGM-1	Macrophages		Dako	1:100
Anti-vWf	Endothelial cells		Dako	1:50
UCHL-1	T lymphocytes		Dako	1:150
L-26	B lymphocytes		Dako	1:250
Anti-PCNA	Proliferating cells		Dako	1:50

Antibodies were obtained from: Dako, Dako Laboratory, Glostrup Denmark; Enzo, Enzo Biochemicals, New York, USA.

following CABG surgery. The interval between operation and death ranged from two days to nine months. From these eight patients a total of 18 distal anastomotic sites of the patent grafts were used for pathological analyses and immunocytochemical investigations. The relevant clinical data are summarised in table 1.

All hearts were fixed in methanol-Carnoy's fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid).

Five non-implanted saphenous vein segments, harvested at surgery and fixed in methanol-Carnoy's fixative, were used as controls. The vein grafts, together with the coronary arteries which contained the sites of anastomoses, were removed from the epicardial surface. The anastomotic sites were cut serially at 1 mm intervals, perpendicular to the long axis, and four slices from each anastomotic site were obtained. A total of 72 slices from the 18 anastomotic sites were examined. All slices, from the anastomotic sites and from normal saphenous vein segments, were processed
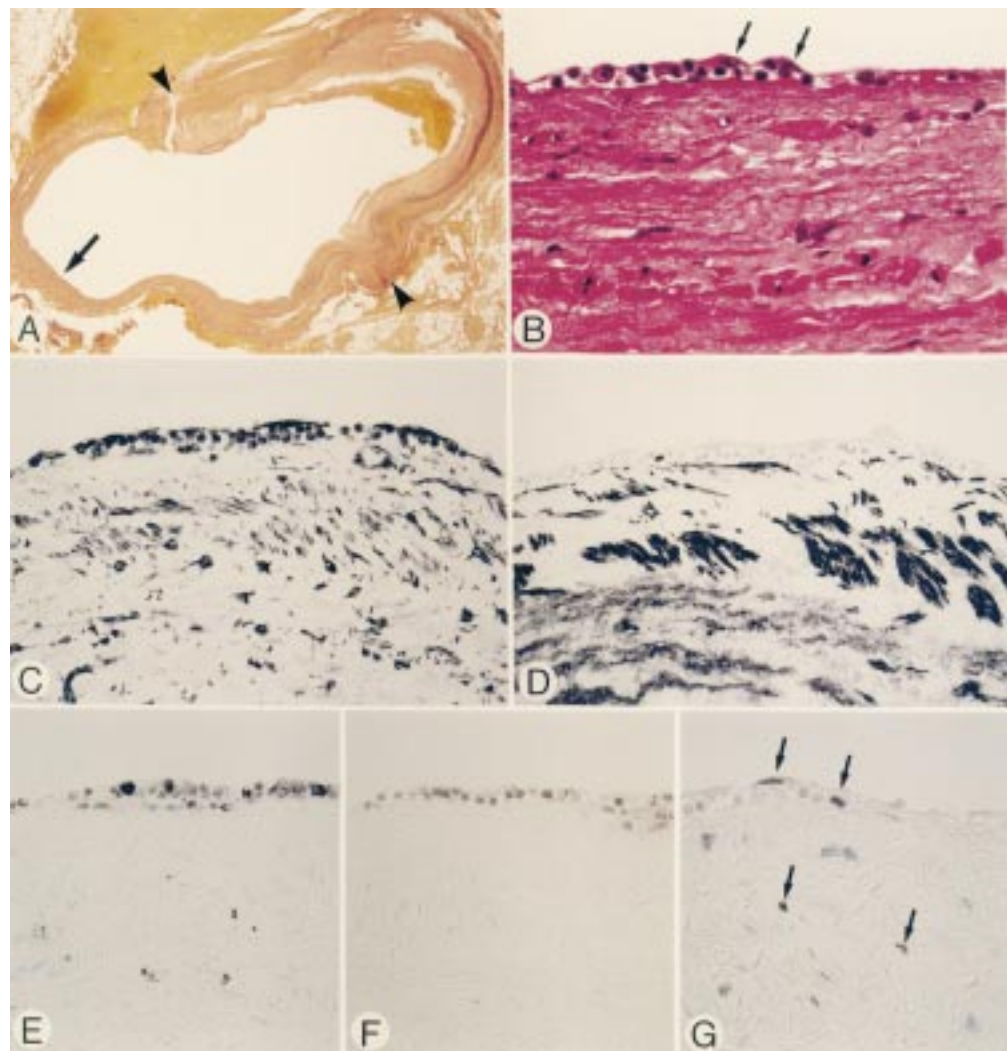

Figure 1   Micrographs of an anastomotic site, taken two days after grafting (patient 1). Panels A–G are serial sections. (A) Elastic tissue stain. The site of anastomosis, indicated by arrowheads, with the right coronary artery. The luminal surface of the vein graft shows a cellular response. Details of the cellular response, indicated by the arrow, are shown in panels B–G. (B) Haematoxylin and eosin stain. At the luminal surface endothelial cells have been denuded. The earliest cellular response of the grafts is demonstrated by an accumulation of polymorphonuclear leucocytes and mononuclear round cells, amid a fibrin-platelet thrombus, partially covered by spindle shaped cells (arrows). (C) Vimentin stain. Spindle shaped cells and round cells at the response site are positive. (D) HHF-35. The spindle shaped cells and round cells are negative. (E) HAM-56. Some round cells stain with this macrophage marker. None of the spindle shaped cells at the luminal site stain positive. (F) UCHL-1. Some small round cells stain with this T lymphocyte marker. (G) PCNA. Positive cells are seen at the site of cellular response and in the adjacent pre-existent media. Original magnification: (A) × 18; (B) × 580; (C–G) × 360.
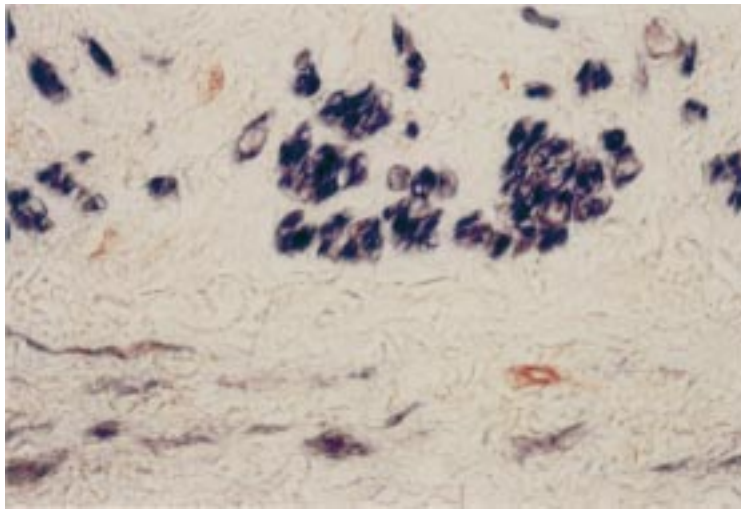
Figure 2   Double immunostaining with HHF-35 (blue) and PCNA (red) of an anastomotic site, two days after grafting (patient 1). The media contains a few PCNA positive cells (red), which lack staining for actin and most likely represent dedifferentiated SMCs. Most SMCs are actin positive (blue). Original magnification × 720.

lymphocytes, and proliferating cell nuclear antigen (PCNA). PCNA is expressed in the late G_1 (presynthetic), S (DNA synthetic), and G_2 (premitotic) phase of the cell cycle. Anti-PCNA thus serves as a marker for proliferating cells. The source, specification, and working dilution of the antibodies used are shown in table 2.[8][9]

For the evaluation of the phenotypic characteristic of SMCs in relation to PCNA positivity, we performed double immunostaining for HHF-35 and PCNA in patients 1 and 2, according to the procedure previously reported.[10] Alkaline phosphatase was visualised with fast blue BB (blue, HHF-35) and the peroxidase with 3-amino-9-ethylcarbazole development (red, PCNA).

routinely and embedded in paraffin. From each slice 20 sections were cut serially at a thickness of 5 μm. Every first and second section was stained with haematoxylin and eosin and with Weigert's elastic van Gieson's stain, respectively; the other sections were used for immunocytochemical staining.

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The monoclonal antibodies used were directed against α isotypes of muscle specific actin common to all muscle cells (HHF-35), and the γ actin isotypes specific only for SMCs (CGA-7), vimentin, endothelial cells (vWf), macrophages (HAM-56 and PGM-1), T lymphocytes, B

MORPHOMETRY

Morphometric analysis was performed using two sections through a midportion of the anastomotic area from each site. The thickness of the neointimal tissue was measured using computer aided planimetry. The number of macrophages within the surface area of the neointimal thickening was quantified, again using computer aided planimetry, and expressed as the absolute number of macrophages per 0.01 mm² surface area.

STATISTICAL ANALYSIS

Statistical comparisons of the thickness of the neointimal tissue were performed by multiple comparisons test, using Scheffé's method. Student's *t* test was used for the comparisons of the number of macrophages.
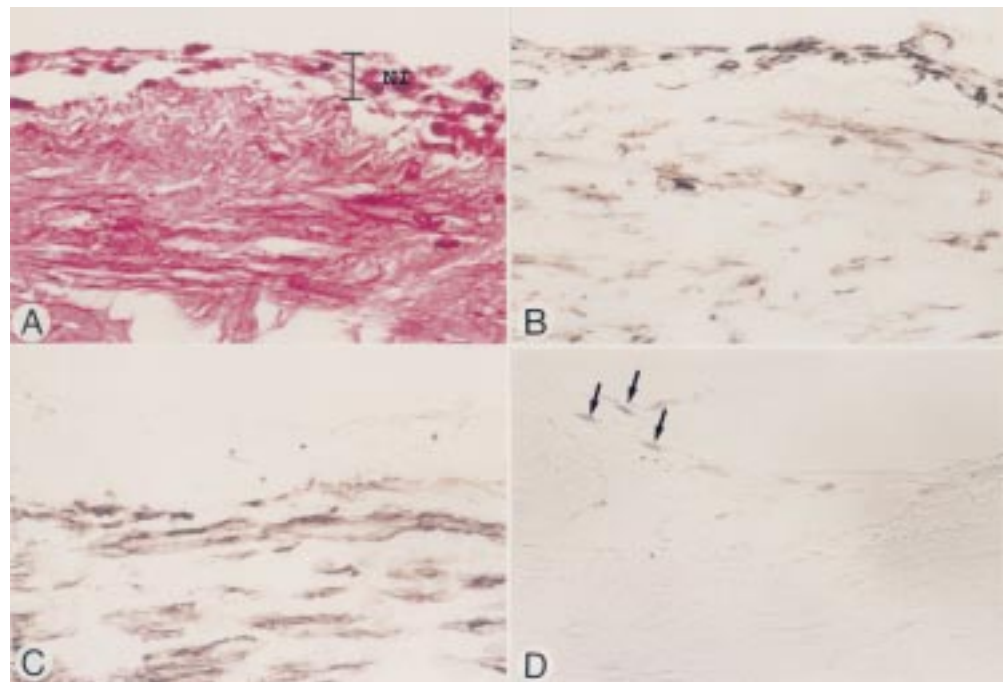

Figure 3   Micrographs of a vein graft at an anastomotic site, nine days after grafting (patient 5). Panels A–D represent serial sections. (A) Haematoxylin and eosin stain. Endothelial cells have been denuded. Early neointimal tissue (NI) is seen at the luminal surface of the graft. (B) Vimentin stain. Both spindle shaped cells and round cells in the neointima are positive. (C) HHF-35. The cells in the neointima do not stain. (D) PCNA. Some of the HHF-35 negative spindle shaped cells (arrows) stain positive. Original magnification × 580.
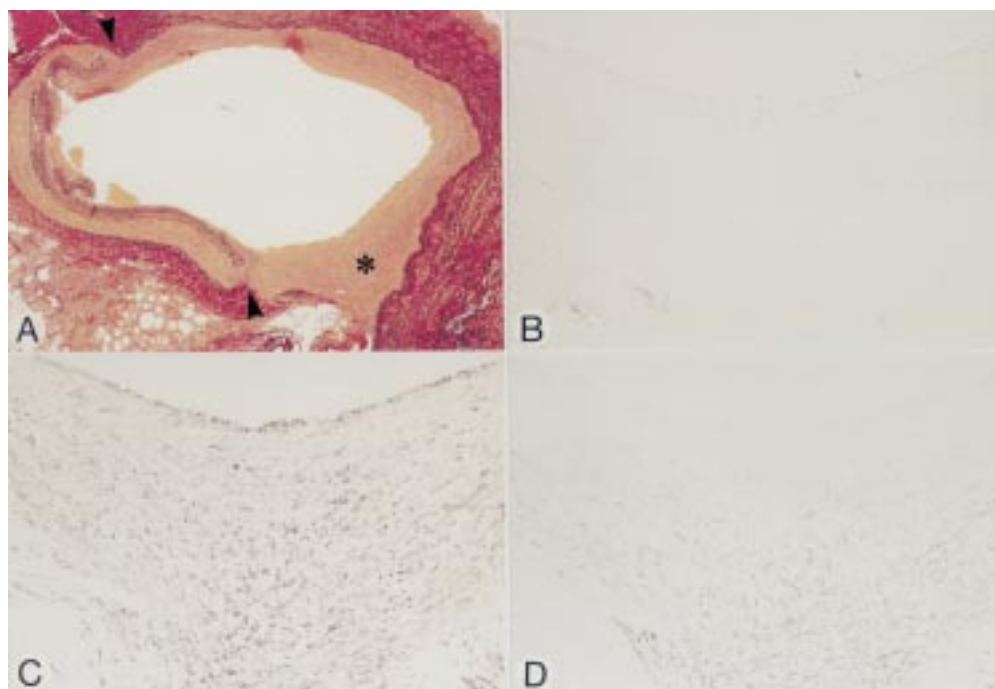
Figure 4   Micrographs of an anastomotic site, 33 days after grafting (patient 6). Panels A–D represent serial sections. (A) Elastic tissue stain. The anastomotic site (arrowheads) to the obtuse marginal branch shows distinct neointimal proliferation of the vein graft (asterisk). (B) HAM-56. No positivity for macrophages in the neointima. (C) HHF-35. Spindle shaped cells in the neointima stain positive. (D) CGA-7. Spindle shaped cells within the deeper layers of the neointima stain positive, but those closer to the luminal site are negative suggesting that these cells have not yet fully differentiated. The anti-vWf antibody was negative at the luminal side (not shown). Original magnification: (A) × 36; (B–D) × 145.

Results

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In normal saphenous veins used as controls, almost all SMCs within the media were positive for both HHF-35 and CGA-7. In the normal saphenous vein the intima was hardly discernible, but when distinctly present contained HHF-35 and CGA-7 positive SMCs. Some macrophages were scattered in the media. T lymphocytes were not found in these specimens. There were no PCNA positive cells in either media or intima.

Although all saphenous vein grafts harvested were patent, all 72 slices obtained from anastomotic sites showed neointimal tissue, albeit to varying degrees.

The earliest cellular response was encountered at five anastomotic sites (20 slices) in patients 1 and 2, each with an interval of < 3 days. At these sites the luminal surface of the vein showed an accumulation of polymorphonuclear leucocytes and mononuclear round cells with occasional spindle shaped cells, amid a fibrin-platelet thrombus. Immunocytochemically, the mononuclear round cells were identified as macrophages and T lymphocytes. The spindle shaped cells, which had a basophilic cytoplasm and a pale euchromatic nucleus with a large nucleolus, were positive for vimentin, but none of these cells stained for the actin markers HHF-35 and CGA-7. Moreover, these spindle shaped cells were negative for antimacrophage antibodies. At the luminal surface there were no cells that stained positive for the anti-vWf antibody. PCNA positivity was seen at the site of cellular response, both in round cells and in spindle shaped cells, as well as in the pre-existent media (fig 1). The media,

moreover, contained scattered areas which did not stain for the two actin markers HHF-35 and CGA-7. Double immunostaining for PCNA and HHF-35 revealed that the PCNA positive cells in these areas were actin negative (fig 2). Moreover, these PCNA positive areas in the media did not co-localise with the sites where macrophages were present.

The five anastomotic sites ranging from six to nine days after CABG (patients 3, 4, and 5) showed more prominent neointimal tissue at the luminal surface of the venous side. At this stage, the neointimal tissue was composed of macrophages and spindle shaped cells, intermingling with a fibrin-platelet thrombus. There were no polymorphonuclear leucocytes or T lymphocytes. The number of spindle shaped cells appeared to be increased compared to the number at two days after grafting. These cells were positive for vimentin, but negative for both actin markers HHF-35 and CGA-7. PCNA positivity in the actin negative spindle shaped cells was more evident (fig 3), while PCNA positive macrophages were observed only occasionally. The anti-vWf antibody was negative at the luminal side.

The two anastomotic sites at 33 days (patient 6) showed a distinct layer of neointimal tissue. Most neointimal cells stained positive with HHF-35; macrophages and T lymphocytes were found scarcely within the neointima. SMCs in the deeper layers of the neointima also stained positive for CGA-7, whereas those close to the luminal surface were negative for CGA-7 (fig 4). Anti-vWf revealed no positivity at the luminal surface of the neointima. PCNA positivity in the neointima was observed only in
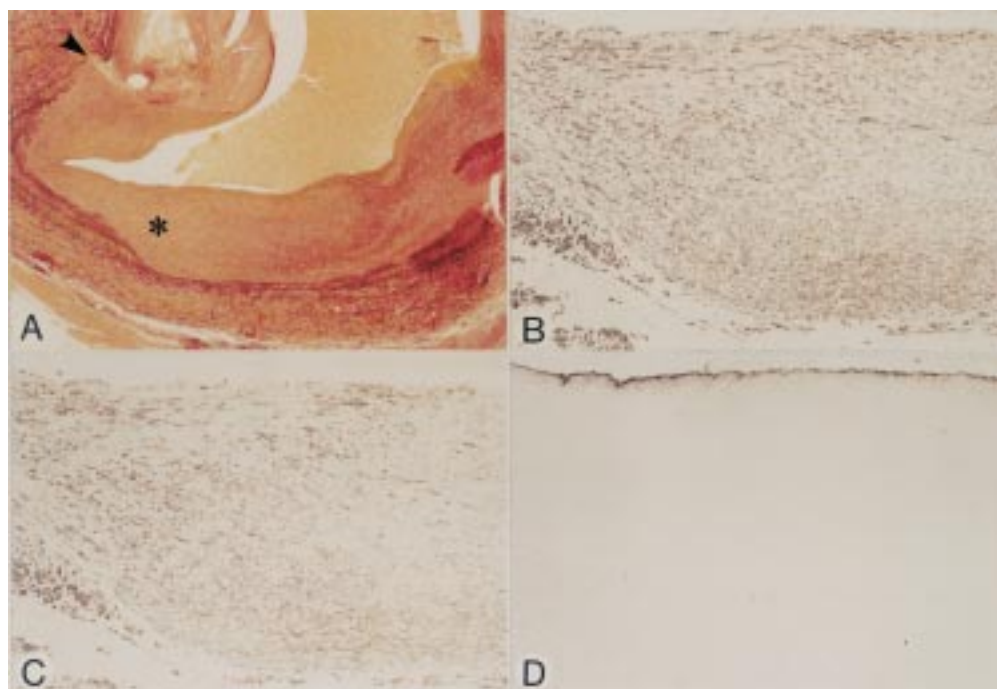
Figure 5   Micrographs of an anastomotic site, nine months after grafting (patient 8). Panels A–D represent serial sections. (A) Elastic tissue stain. Note distinct neointimal tissue at the anastomotic site (arrowhead). The neointima at the site of the asterisk is shown at higher magnification in panels B–D. (B) HHF-35. Spindle shaped cells in the neointima are positive. (C) CGA-7. Most spindle shaped cells are positive suggesting that most cells have the phenotype of fully differentiated SMCs (compare to panel B). (D) Anti-vWf. Regenerated endothelial cells line the luminal surface. Original magnification: (A) × 30; (B–D) × 90.

a few spindle shaped cells among those closest to the lumen.

At four months after CABG (patient 7), the four anastomotic sites presented a more prominent neointima, but otherwise the find-

Table 3   Results of immunophenotypic expression of neointimal smooth muscle cells

Patient	Interval CABG/death	Immunophenotype		
		HHF-35	CGA-7	Vimentin
1	2 days	−	−	+++
2	3 days	−	−	+++
3	6 days	−	−	+++
4	7 days	−	−	+++
5	9 days	−	−	+++
6	33 days	+++	+	+++
7	4 months	+++	++	+++
8	9 months	+++	+++	+++

+++, almost all cells positive; ++, about two thirds of cells positive; +, approximately half of all cells positive; −, negative.
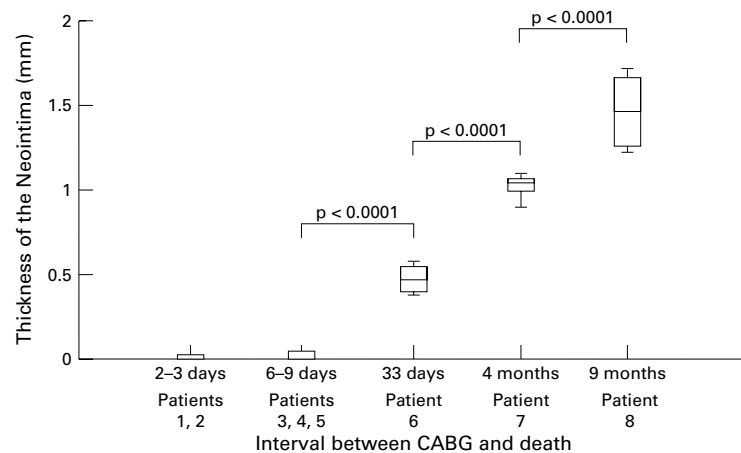

Figure 6   Thickness of the neointima.

ings were similar to those at 33 days. Approximately two thirds of the SMCs at the deeper side of the neointima stained positive for both HHF-35 and CGA-7, but the remaining SMCs on the luminal side of the neointima were positive only for HHF-35 and not for CGA-7. The majority of the luminal surface was covered by regenerated endothelial cells; there were no PCNA positive cells within the neointima.

The two anastomotic sites at nine months after CABG (patient 8) showed distinct neointimal thickening, which consisted almost exclusively of SMCs with positive staining for both HHF-35 and CGA-7. The difference between the deeper and more luminal layers, with respect to CGA-7 positivity of neointimal SMCs, had almost vanished. Macrophages were scarcely found within the neointima and a complete layer of regenerated endothelial cells lined the luminal surface. No PCNA positive cells were observed in the neointima (fig 5). B lymphocytes were not detected in the neointima in any of the cases.

A survey of the cytoskeletal changes of the spindle shaped cells is presented in table 3.

MORPHOMETRY

The morphometric findings regarding neointimal thickness related to the time interval between CABG and death are shown in fig 6. There was no significant difference ($p = 0.998$) between the thickness of the neointima at 2–3 days and at 6–9 days. However, there were significant differences ($p < 0.0001$) in thickness of the neointima between 6–9 days and 33 days, between 33 days and four months, and between four months and nine months.
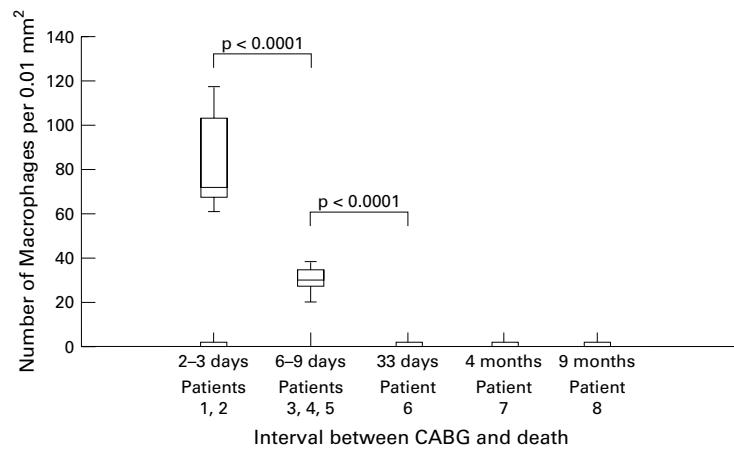
Figure 7   Number of macrophages within the neointima.

Morphometric analysis of the number of macrophages within the neointima showed a significantly ($p < 0.0001$) higher number in cases with an interval of < 3 days than in those 6–9 days after grafting (fig 7).

Discussion

This investigation reveals that human vein grafts very soon after CABG show distinct changes. These changes are characterised by loss of surface lining endothelial cells, insudation of blood corpuscular elements such as polymorphonuclear leucocytes and monocytes, admixed with a fibrin-platelet thrombus, the appearance of vimentin positive, actin negative spindle shaped cells, and absence of actin positive SMCs in the pre-existent media topographically related to the sites of neointimal reaction. These observations allow speculations as to the mechanisms involved in neointimal thickening.

Denudation of the surface endothelial cell lining appears to be a crucial factor, and its extent and severity may eventually determine graft patency. Loss of endothelial cells, as observed in veins 2–9 days after grafting, is accompanied by insudation with polymorphonuclear leucocytes, blood monocytes, and T lymphocytes. To this end our observations fit with previous experimental works. Brody and colleagues[11] observed endothelial cell desquamation with inflammatory cell infiltration in vein grafts of dogs at three days after grafting, and Hoch and colleagues[12] reported that early inflammatory cell infiltrates in rat vein grafts were composed primarily of monocytes/ macrophages with a lesser number of T lymphocytes. The importance of these observations relates to the fact that macrophages and T lymphocytes may secrete a variety of cytokines and growth factors, shown in vitro to cause SMC proliferation and chemotaxis.[13 14] Moreover, the adherence of a fibrin-platelet thrombus at the site of injury makes it likely that the potent platelet derived growth factor (PDGF) is a key factor involved. The role of PDGF in promoting SMC growth and, hence, neointimal thickening has been shown in animal experiments using angioplasty models.[15 16] Our own in situ hybridisation and immunohistochemical studies of human cor-

onary arteries after percutaneous transluminal coronary angioplasty (PTCA) have shown that PDGF A and B chain mRNA, PDGF B protein, and PDGF β receptor protein are expressed in the neointima in the very early stages after injury.[17 18] Whether similar phenomena occur in the process of neointimal thickening in human vein grafts is as yet unknown but seems likely, given the circumstances alluded to above. It seems worthwhile, therefore, to direct future studies to this particular facet, which requires use of frozen sections of human vein grafts.

Our study also documents that the media related to the site of intimal injury contained areas in which SMCs of the pre-existent media showed no staining for α actin, thus confirming the findings of Kockx and colleagues[6] in vein grafts of < 10 days. This observation suggests strongly that dedifferentiation of medial SMCs at the site of intimal injury had occurred and that this process is associated with an increase in proliferative activity. Previous studies have shown that phenotypic modulation of SMCs is a prerequisite for the evolution of intimal thickening, both in experimentally induced intimal injury and in human atherosclerotic plaques,[19 20] while our own studies in human coronary arteries after PTCA revealed that dedifferentiated SMCs preceded the intimal repair process.[21 22] The significance of these phenomena in human vein grafts is also supported by the observation that a shift in phenotype of SMCs, contributing to neointimal thickening, occurred with time. Amano and colleagues[5] have documented a similar phenotypic shift in human vein grafts within six months of CABG surgery.

Our study provides novel observations with respect to the proliferative activity related to injury. We observed PCNA positivity of both inflammatory cells within the reactive tissues at the site of intimal injury, as well as in the dedifferentiated areas within the pre-existent media. Moreover, with an increase in the time interval between CABG and death a decrease in PCNA activity was noted. These findings support previous experimental studies of neointimal cellular kinetics and phenotypic changes of SMCs,[23 24] suggesting that the decline in cell proliferative activity is part of the evolution of the neointima and relates to the state of neointimal SMC differentiation. However, in humans the role of cell proliferation in the setting of neointimal formation remains controversial. O'Brien and colleagues[25] reported that PCNA positivity occurred infrequently and at low levels in atherectomy specimens obtained from human coronary restenotic lesions after PTCA. The discrepancy may relate to differences in tissue characteristics, but it appears much more likely that the difference relates to time factors and, to this end, the state of differentiation of the lesions studied. PCNA positivity in our study was seen frequently only in the very early stages and it is unlikely that this material bears any resemblance to the restenotic lesions observed by O'Brien and colleagues.[25]

CLINICAL IMPLICATIONS

The observation that luminal endothelial cell denudation, with insudation of the intima with blood corpuscular elements and adherence of a fibrin-platelet thrombus at the site of injury, are the most prominent changes in the early stages of human vein grafts after CABG, suggests that these phenomena trigger the repair process; eventually this may lead to neointimal formation which has been shown to be the most common underlying pathology of obstructed human vein grafts. This then could provide a target for pharmacological intervention at the stage of the CABG procedure itself, for instance by suppressing the irreversible attachment of platelets to the sites of injury using inhibitors of receptor glycoprotein IIb/IIIa. A suppressed or mitigated SMC response may also have a beneficial effect on the eventual development of atherosclerosis in these vein grafts, since SMCs also play an important role under these circumstances. At present we are not aware of any studies documenting the effects of preventive measures as alluded to above in patients undergoing CABG.

STUDY LIMITATIONS

Obviously, the present study does not allow for a time related sequence of events in human vein grafts after CABG. The number of observations is too small to allow further statistical analysis. Nevertheless, the observations are intriguing, not least since they relate very well to observations made at other sites, such as lesion formation following interventional procedures in arteries.

The present study also falls short in the more detailed study of the molecular mechanisms involved. However, for that purpose one has to handle material that comes available in a different manner. For this a strict protocol should be employed and basically fresh material should be sampled. This is extremely difficult, if not almost impossible, in the setting of patients undergoing a CABG procedure.

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