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

Y Sasaki, S Suehiro, A E Becker, H Kinoshita, M Ueda

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

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.

(Heart 2000;83:69–75)

Keywords: smooth muscle cell proliferation; vein graft stenosis; platelet derived growth factor; platelet receptor inhibitors

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 colleagues7 documented insudation of blood constituents into the vein intima as the earliest change, whereas Kockx and colleagues8 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 interventions. This was the main purpose of our study.

Methods

PATIENTS

Hearts were obtained at necropsy from eight patients who had died within one year of CABG/death Cause of death Risk factors

Hypercholesterolaemia

Diabetes mellitus

Hypertension

Diabetes mellitus; hypercholesterolaemia; hyperuricaemia; smoking

Hypertension; smoking

Hypertension

Table 1 Relevant clinical data

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

*Segments of the coronary arteries are shown in parentheses (according to American Heart Association committee report).
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.

### Table 2  Monoclonal antibodies used in the study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity and reactivity</th>
<th>Reference</th>
<th>Source</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHF-35</td>
<td>Muscle actin</td>
<td>Gown et al</td>
<td>Dako</td>
<td>1:50</td>
</tr>
<tr>
<td>CGA-7</td>
<td>Smooth muscle cell actin</td>
<td>Gown et al</td>
<td>Enzo</td>
<td>1:20</td>
</tr>
<tr>
<td>Anti-vimentin</td>
<td>Smooth muscle cells, fibroblasts, macrophages, endothelial cells</td>
<td>Dako</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>HAM-56</td>
<td>Macrophages, some endothelial cells</td>
<td>Gown et al</td>
<td>Dako</td>
<td>1:70</td>
</tr>
<tr>
<td>PGM-1</td>
<td>Macrophages</td>
<td>Dako</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>Anti-vWF</td>
<td>Endothelial cells</td>
<td>Dako</td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>UCHL-1</td>
<td>T lymphocytes</td>
<td>Dako</td>
<td>1:150</td>
<td></td>
</tr>
<tr>
<td>L-26</td>
<td>B lymphocytes</td>
<td>Dako</td>
<td>1:250</td>
<td></td>
</tr>
<tr>
<td>Anti-PCNA</td>
<td>Proliferating cells</td>
<td>Dako</td>
<td>1:50</td>
<td></td>
</tr>
</tbody>
</table>

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

![Figure 1](link)  
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 leukocytes 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.
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 isoforms of muscle specific actin common to all muscle cells (HHF-35), and the γ actin isoforms specific only for SMCs (CGA-7), vimentin, endothelial cells (vWF), macrophages (HAM-56 and PGM-1), T lymphocytes, B lymphocytes, and proliferating cell nuclear antigen (PCNA). PCNA is expressed in the late G1 (presynthetic), S (DNA synthetic), and G2 (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.

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. Alkaline phosphatase was visualised with fast blue BB (blue, HHF-35) and the peroxidase with 3-amino-9-ethylcarbazole development (red, PCNA).

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 Scheffe’s method. Student’s t test was used for the comparisons of the number of macrophages.

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.

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 neo intim a 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.
Results

IMMUNOCYTOCHEMISTRY

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. 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-\(\alpha\)-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-\(\alpha\)-VWF revealed no positivity at the luminal surface of the neointima. PCNA positivity in the neointima was observed only in...
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 findings were similar to those at 33 days. Approximately two thirds of the SMCs at the deeper side of the neointima stained 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.

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.

Table 3 Results of immunophenotypic expression of neointimal smooth muscle cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>Interval CABG/death</th>
<th>HHF-35</th>
<th>CGA-7</th>
<th>Vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 days</td>
<td>−</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>3 days</td>
<td>−</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>6 days</td>
<td>−</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>7 days</td>
<td>−</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>9 days</td>
<td>−</td>
<td>−</td>
<td>+++</td>
</tr>
<tr>
<td>6</td>
<td>33 days</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>4 months</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>8</td>
<td>9 months</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

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

Figure 6 Thickness of the neointima.

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.
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, insula-
tion of blood corpuscular elements such as polymorphonuclear leukocytes and mono-
cytes, 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 neointi-
mal reaction. These observations allow specu-
lations 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 insula-tion with polymorpho-
nuclear leukocytes, blood monocytes, and T
lymphocytes. To this end our observations fit
with previous experimental works. Brody and
colleagues15 observed endothelial cell desqua-
mation with inflammatory cell infiltration in
vein grafts of dogs at three days after grafting,
and Hoch and colleagues16 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 obser-
vations 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,
zeintimal thickening has been shown in
animal experiments using angioplasty
models.15 16 Our own in situ hybridisation and
immunohistochemical studies of human cor-
ony 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 phe-
nomena occur in the process of neointimal
thickening in human vein grafts is as yet
unknown but seems likely, given the circum-
crances 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 colleagues1 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 neoini-
timal thickening, occurred with time. Amano
and colleagues23 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 de-
differentiated 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 neo-
zeintimal 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 prolifera-
tion in the setting of neointimal formation remains
controversial. O’Brien and colleagues25 re-
ported that PCNA positivity occurred infre-
cently and at low levels in atherecomy speci-
mens obtained from human coronary
erstenotic lesions after PTCA. The discrep-
ancy may relate to differences in tissue charac-
teristics, 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 resem-
blance to the restenotic lesions observed by
O’Brien and colleagues.25
Genesis of human vein graft neointimal thickening

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 CAGB, 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 CAGB 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 CAGB.

STUDY LIMITATIONS

Obviously, the present study does not allow for a time related sequence of events in human vein grafts after CAGB. 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 the phenomenon of cell differentiation is a fundamental change preceding wound healing after percutaneous transluminal coronary angioplasty in humans. Coron Artery Dis 1995;6:71–81.


1 Kalan JM, Roberts WC. Morphologic findings in saphenous veins used as coronary arterial bypass conduits for longer than 1 year: necropsy analysis of 53 patients, 123 saphenous veins, and the prospective study of 164 patients. 1993;51:25–33.


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