Migration of Smooth Muscle and Endothelial Cells
Critical Events in Restenosis

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Restenosis after percutaneous transluminal coronary angioplasty (PTCA) continues to present a challenge. Multiple therapies, including antiplatelet and anticoagulant agents, calcium antagonists and inhibitors of angiotensin converting enzyme, corticosteroids, endovascular prostheses, atherectomy, fish oil diets, and many other treatments have had only modest effects on the incidence of restenosis. These results highlight how little we know of the underlying mechanisms.

Pathology of Restenosis

Most reports indicate that early restenosis—hours to days after PTCA—is due to thrombosis, with some contribution by elastic recoil and vasospasm. In contrast, the histology of late restenosis is mainly fibrocellular proliferation with an extracellular matrix ranging from loose collagen fibrils and proteoglycans to dense collagen scar, with few inflammatory cells and little lipid in most cases. Fresh thrombus represents a minor component in the majority of specimens, but unlysed clot that has undergone hyaline change or been colonized by smooth muscle cells and macrophages is probably an important constituent in many cases. By light microscopy, most of the cells have a mesenchymal appearance, similar to that of fibroblasts. Electron microscopy reveals a variety of phenotypes. Some have substantial myofilament bundles and a basement membrane suggesting a smooth muscle cell (SMC) origin. This is supported by the presence of immunoreactivity for \( \alpha_c \)-smooth muscle actin and for vimentin and not desmin. Other cells lack these features and may represent a more dedifferentiated SMC phenotype similar to that of fibroblasts and macrophages.

This histological picture differs from that of stable atherosclerotic lesions. The latter, though variable in appearance, generally reveal more dense scar, more foam cells, necrotic debris and cholesterol clefts, old hemorrhage and thrombus—often with calcification—and sparse SMCs that are typically spindle-shaped rather than stellate. When atherectomy specimens from patients with post-PTCA restenosis have this appearance, the presumption has been that some primary atherosclerosis has been sampled. However, a substantial minority of atherectomy specimens from patients with primary atherosclerosis (and no PTCA) look like restenosis specimens.

Migration is Distinct from Proliferation

From 24 to 72 hours after balloon injury to the rat carotid, a subpopulation of medial SMCs (99.9% of which are not synthesizing DNA before injury) undergo DNA synthesis (Figure 1). These cells then migrate through breaks in the internal elastic membrane. Many of these neointimal cells continue to proliferate for several cycles and to generate an abundant and increasingly collagen-rich extracellular matrix. Nearly half of the migrating cells are not synthesizing DNA. Another feature that distinguishes migration from proliferation is that migration is unaffected by doses of radiation or drugs that inhibit cell division and utilizes different plasminogen activators. Most migrating cells express urokinase plasminogen activator (u-PA) and sometimes tissue-type plasminogen activator (t-PA), and the consequent activation of plasmin and collagenases is thought to facilitate migration by digesting away obstructing thrombus and extracellular matrix proteins. Plasmin is also needed to lyse cell substrate attachments, releasing the cell to allow it to advance. Cloves et al have reported that proliferating SMCs express u-PA while migrating SMCs express t-PA. The role of t-PA in proliferation is not clear. Another difference between migration and proliferation is that migration is...
an earlier response than proliferation to such stimuli as platelet-derived growth factor (PDGF) and is stimulated by lower concentrations than those needed for cell division. Moreover, there are a number of factors that stimulate migration but not proliferation.\textsuperscript{17-19}

Even if half of the migrating SMCs do not synthesize DNA, the SMCs that do divide eventually constitute a much larger percentage of the neointimal cells, suggesting that migration, although interesting, is less than critical. On the other hand, migration of SMCs may contribute to division of neighboring cells by releasing them from contact inhibition. Migration may play a similar role in endothelial cells, as discussed later.

Cell migration has been extensively studied because it is known to occur during embryogenesis, wound healing, tumor metastasis, and physiological angiogenesis. Although not fully understood, cell migration is known to involve regulated attachment, detachment, contraction of nonmuscle myosin and actin, cytoskeletal plasticity, and, with the exception of leukocytes, to require O\textsubscript{2} and protein synthesis.\textsuperscript{20-22} The extracellular signals include physical forces\textsuperscript{23,24} and soluble regulators such as vasoactive hormones,\textsuperscript{19,25} polypeptide growth factors,\textsuperscript{21,26} and probably [Ca\textsuperscript{2+}], [Mg\textsuperscript{2+}], pH, and P\textsubscript{O\textsubscript{2}}.\textsuperscript{27} Cell adhesion is controlled by a balance of proteoglycans, collagens, and glycoproteins of the extracellular matrix, particularly laminin and fibronectin,\textsuperscript{21,28} and by activators and inhibitors of plasmin, which is used to lyse cell substrate attachments so that the cell is not anchored in one spot\textsuperscript{14,15} (also unpublished observations: M.T. Jaklitsch, S. Biro, W. Casscells, D.A. Dichek, 1992).

Migration is often analyzed as chemokinesis (random migration) and chemotaxis (directed migration). Several factors have recently been shown to stimulate chemokines. For example, scatter factor (hepatocyte growth factor) stimulates chemokinesis of epithelial cells; autocrine motility factor stimulates migration of the malignant cells that produce it; and migration-stimulating factor—derived from malignant or fetal fibroblasts—causes migration of nonproducing adult fibroblasts.\textsuperscript{18} In contrast, acidic fibroblast growth factor (aFGF) stimulates chemotaxis more than chemokinesis, at least in endothelial cells.\textsuperscript{29}

The role of chemokinesis in restenosis is uncertain. After all, cells do not migrate through the medial layer into the adventitia but rather into the intima. Presumably, directed migration factors (chemoattractants) are involved. Nevertheless, chemokinesis may be important. Interestingly, in a recent issue of \textit{Circulation}, Bauriedel et al\textsuperscript{30} reported that, in primary culture, cells derived from atherectomy specimens of patients who developed restenosis exhibited more rapid chemokinesis (random migration) than cells derived from atherectomy specimens of primary atherosclerosis. This adds to previous reports that neointimal SMCs derived from balloon-injured rat aorta migrate and proliferate faster than those derived from the uninjured medial layer\textsuperscript{31} and that cultured cells derived from restenosing vessels grow faster than those from atherosclerotic vessels not previously subjected to PTCA.\textsuperscript{32} An advantage of the model used by Bauriedel et al is that the migration can be studied in primary culture before passing. With each round of cell division and with each passage, the cells are less and less likely to maintain the physiological distinctions caused by the balloon injury.

Factors That Regulate Smooth Muscle Cell Migration

\textbf{Plasma and Platelets}

Multiple factors have been shown to stimulate SMC migration, and others are candidates. Plasma contains norepinephrine, lipoprotein (a), angiotensin II, epidermal growth factor, and insulin-like growth factor. The last three are also made by endothelial cells (ECs) and SMCs.\textsuperscript{33,34} Thrombus, an invariable consequence of endothelial denudation, contains the fibrin and fibronectin necessary for smooth muscle attachment, and thrombin itself is mitogenic and stimulates PDGF expression and release.\textsuperscript{33,34} Moreover, platelets release at least five mitogens for SMCs,\textsuperscript{34} including serotonin, thrombospondin, and the three dimeric forms of PDGFs, and transforming growth factor β\textsubscript{1} (TGF-β\textsubscript{1}), which can stimulate or inhibit SMC migration and proliferation, depending on cell culture conditions.

The richness of thrombus notwithstanding, there is continuing debate about the magnitude of its role in SMC migration and proliferation.\textsuperscript{9,10,35,36} It has been pointed out that balloon injury generally results in only a thin layer of activated platelets, whereas gentle endothelial denudation often causes occlusive thrombosis but seven- to eightfold less SMC DNA synthesis than balloon injury.\textsuperscript{10} Moreover, in most cases of vascular injury, the thrombus has almost completely resolved by 48 hours, whereas cell proliferation continues for several weeks.\textsuperscript{10} Furthermore, anticoagulant and antiplatelet agents have not been very effective in inhibiting restenosis.\textsuperscript{1} Other studies have indicated that the migratory response of SMCs in vitro peaks at serum concentrations as low as 3%.\textsuperscript{37} Thus, thrombus formation may have to be curtailed very early and very thoroughly to prevent migration and proliferation. In fact, in experimental models, severe thrombocytopenia (produced by antiplatelet antibodies) does inhibit neointimal accumulation to a substantial degree, even though SMC DNA synthesis is virtually unaffected.\textsuperscript{10,38} This suggests that cell migration or elaboration of matrix (which eventually accounts for over half of the neointimal tissue) are more dependent on platelet products than is DNA synthesis. Moreover, these data indicate that the failure of aspirin and related agents to substantially reduce restenosis should be interpreted cautiously. It may be necessary to inhibit not only platelet aggregation but also attachment and release. Antibodies to the platelet glycoprotein IIb-IIIa or to other integrins may be helpful but are apt to be supplanted by nonpeptide inhibitors. Together with newer agents such as hirudin and argatroban, inhibitors based on the cloning of the thrombin receptor\textsuperscript{39} may sufficiently inhibit thrombosis to blunt restenosis without causing bleeding.

\textbf{Hemodynamics}

High pressure stimulates SMC DNA synthesis (in part via stretch-induced ion channels), whereas high flow and shear have the opposite effect, at least as inferred from in vivo studies (in vitro studies have been conflicting).\textsuperscript{34,40} Increasing flow may be one mechanism...
FIGURE 1. Photomicrographs of key events in restenosis as deduced from balloon injury to the rat carotid artery. Upper left panel: Four days after balloon injury; some smooth muscle cells (SMCs) in the media (M) have died (those with pyknotic or clear nuclei) while others are synthesizing DNA (shown by the black silver grains over the nucleus and by the arrowheads). Daily injections of tritiated thymidine label 10–15% of the medial cells. By this time, fibrin is no longer grossly visible in the lumen (L), even though it has not been reendothelialized. A, adventitial layer. Upper right panel: By day 5, SMCs have migrated into the neointima. This occurs rapidly. Only one cell (arrowhead) is caught in the act. A few inflammatory cells are noted at the luminal surface. Lower right panel: By day 5, autoradiography after daily injections of tritiated thymidine reveals that DNA synthesis (arrowheads) in medial SMCs is subsiding while neointimal SMCs continue to proliferate. However, many neointimal SMCs are known to migrate without synthesizing DNA,10 as suggested by the unlabeled neointimal cells shown here. E, internal elastic membrane. Lower left panel: Electron microscopy at 10 days reveals another migrating SMC, with lamellipodia (L) and extensive rough endoplasmic reticulum at the leading edge. Elastases, collagenases, and plasminogen activators are thought to be critical to the cells’ migration through the internal elastica (black diagonal) and collagen (C). Higher-power views (not shown) indicate that most of the medial SMCs retain the bundles of actin filaments that characterize contractile SMCs. In contrast, the neointimal cells have few filaments or lamellipodia and appear to be involved mainly in secretion of extracellular matrix.
by which stents can exert a modest reduction in rate of restenosis.

Taken together with the platelet studies, these data suggest the hypothesis that cell injury and thrombosis are additive stimuli of SMC division and/or migration. Balloon dilation is thus likely to serve several functions: 1) removal of the endothelial cells and their basement membrane, which serve as semipermeable barriers to plasma mitogens and contain growth inhibitors (see below); 2) exposure of thrombogenic subendothelial collagens; 3) rupture of internal elastic laminae, exposing SMCs to serum factors and monocytes; 4) stretch of SMCs (directly activating proto-oncogenes); 5) separation of SMCs, causing loss of contact inhibition and allowing permeation of serum mitogens; 6) rupture of ECs and SMCs with consequent release of mitogens to surviving SMCs; 7) recruitment of monocyte/macrophages, and 8) stimulation of SMCs to synthesize their own growth factors, as described below.

Paracrine Mechanisms: Macrophages

The likelihood that thrombosis accounts for no more than half of the stimulus to restenosis has focused attention on cells in the vessel wall. Although they constitute a minority population even in the injured vessel, macrophages synthesize a wide variety of growth factors, including PDGFs, basic FGF, TGF-β1, TGF-α, IL-1, and heparin-binding EGF. However, steroids, nonsteroidal anti-inflammatory agents, and cyclosporin-A have not been very effective at inhibiting neointimal accumulation clinically or in animal models to date. 

Autocrine and Paracrine Mechanisms: SMCs

Recently, growth factors in the SMCs themselves have attracted attention. Atheromatous SMCs may be oligoclonal transformants, as originally proposed by Benditt. Several groups have reported an upregulation of PDGF and PDGF receptor isoforms in response to vascular injury for review, see Reference 34. Recently, Ferns et al. reported that antibodies to PDGF could inhibit SMC migration and neointimal accumulation. From a clinical standpoint, however, the problem with such a therapy would be the generation by the patient, after several injections, of antibodies that neutralize the anti-PDGF antibodies, which is why Ferns et al used an immune-deficient rat. However, these data should stimulate the development of a synthetic antagonist to PDGF receptors.

Recent data also suggest a role for basic fibroblast growth factor (bFGF) in smooth muscle proliferation and migration. bFGF is the best understood member of a seven-gene family of heparin-binding polypeptides and appears to play important roles in induction of embryonic mesoderm, angiogenesis, and neuronal differentiation and survival. bFGF also regulates certain endocrine functions and is mitogenic for most cell types, including SMCs. It is not detectable in normal serum, but is stored in many cell types, including ECs and SMCs and in their extracellular matrix. Whether bFGF gene expression is increased by balloon injury in either endothelial cells or SMCs is not yet resolved, but it is clear that infusions of bFGF increase both endothelial and smooth muscle proliferation (and perhaps migration) in vivo. Anti-bFGF antibodies inhibit SMC DNA synthesis after balloon injury. Moreover, there is evidence that at least one of the several FGF receptors is upregulated after balloon injury.

The messenger RNAs for insulin-like growth factor-I, tumor necrosis factor-α, and TGF-β are also increased in abundance after experimental balloon injury. In addition, novel smooth muscle–derived growth and migration factors have been partially characterized.

Endothelial Cells Influence Growth of SMCs and Vice Versa

Proliferating or injured ECs release peptides and proteoglycans that stimulate growth of SMCs. In contrast, confluent, quiescent ECs inhibit SMC migration and proliferation, in large part by activating TGF-β1, by releasing growth-inhibiting types of heparan sulfate proteoglycans and by providing a thromboresistant barrier between SMCs and serum mitogens and chemoattractants.

Bauriedel et al found no ECs in their atherectomy specimens, which is consistent with the results of Dartsch et al and our group. Does this mean that reendothelialization has generally not occurred by 2–3 months after PTCA, or is it just that the catheter scraped off the EC? It probably means both. Many advanced plaques lack EC, even before PTCA, and in their place have a modified type of EC that is squamous and relatively nonthrombogenic. Most autopsy and atherectomy studies after PTCA have not mentioned ECs, probably because ECs are often not easy to distinguish from modified luminal SMCs on morphological grounds alone. However, in one study using antibodies to von Willebrand factor and Ulex Europeanus, there were no ECs in the 11 patients dying within 1 month of PTCA, but in the six late deaths, there was substantial reendothelialization. In the balloon-injured rat carotid artery, Lindner et al have shown that endothelial cell regrowth begins at about 2 mm per week and then slows, virtually ceasing at 10 weeks and leaving about a centimeter of nonendothelialized artery. In the aorta, which has branches every few millimeters that serve as sources of ECs, reendothelialization is more successful and proceeds at 0.4 mm per day. In the balloon-denuded canine circumflex, 81% of a 3-cm denuded area was recovered by 10 days. These variable results may reflect varying degrees of injury; gentle denudation results in complete regrowth of ECs, whereas balloon injury involving the medial layer does not. This may be due to the fact that SMCs migrate into the intima before the arrival of the ECs from more distal regions of the vessel. Because SMCs inhibit EC growth in vitro, the result may be an arrest of endothelial migration. Probably contributing to this are many factors that stimulate SMC migration and/or proliferation yet inhibit or fail to stimulate ECs: thrombospandin, angiostatin-II, serotonin, norepinephrine, interleukin-1 and (in some conditions) TGF-β. Furthermore, most large vessel ECs are unresponsive to PDGF. Cell senescence may also be a factor because chronically injured and replicating endothelium, especially in the older patient, may have used up its lifespan. Indeed, the nearly exclusive use of young animals in experimental models may limit their applicability.

Thus, migration of SMCs into the intima is likely to contribute to the failure of reendothelialization in many
cases. The lack of ECs, in turn, probably promotes SMC growth. Migration of SMCs from media to intima is also likely to contribute to SMC proliferation by 1) eliminating the contact inhibition of cells left behind and 2) placing SMCs in the lumen, where they are bathed by mitogens.

**Current and Future Therapy**

Several drugs in current clinical use were shown by Bell and Madri to influence SMC migration in vitro. For example, inhibitors of angiotensin converting enzyme inhibit SMC migration yet stimulate EC migration. Calcium antagonists inhibit SMC migration but have little or no effect on EC migration. Cimetidine inhibits histamine-induced SMC proliferation and migration, whereas diphenhydramine inhibited histamine-induced EC proliferation and histamine-induced reduction of EC migration. Ketanserin inhibited serotonin-induced SMC proliferation and migration, whereas imipramine inhibited the serotonin-induced decrease of EC migration and proliferation. Many vasodilators modestly inhibit SMC proliferation and could also inhibit migration. Clearly, none of these treatments is perfect, because patients taking these medications continue to present with restenosis.

Tissue plasminogen activator and streptokinase may also be having unsuspected effects, because proteolysis of cell-substratum contacts is essential for migration and inhibitors of plasmin inhibit migration. Whether the addition of plasminogen activators to those already produced by the cell would further stimulate either cell type is not known. Paradoxically, complete degradation of fibrin might inhibit cell attachment and so inhibit migration. Moreover, plasmin activates TGF-β1, releases matrix stores of bFGF, and may have still other effects, as it has a broad spectrum of action similar to trypsin.

Other agents in current use that are likely to influence SMC migration are antioxidants (such as vitamins or probucol) because minimal oxidation modifies low density lipoprotein and moderate oxidation inactivates FGF and inhibits cell growth. It is also interesting that prostaglandins, prostacyclin, and hydroxyeicosatetraenoic acids are reported to influence SMC proliferation in vitro. Several agents in current use affect these eicosanoids and could influence SMC growth directly and also indirectly, e.g., aspirin, by its antithrombotic effect, and fish oils, by inhibiting production of PDGF by endothelial cells.

Heparins, low-molecular-weight non–anticoagulant heparins, and heparin sulfate proteoglycans all inhibit cultured SMC migration and proliferation in vitro and in animal models. Multiple mechanisms are involved. Some heparins appear to stimulate migration or growth of some types of ECs in some culture conditions, and complex effects have been found in vivo as well. Unfortunately, preliminary clinical trials of heparins and of angiotensin-converting enzyme inhibitors have not shown much inhibition of restenosis. These data raise the possibility that the rat model (in which these therapies worked) may not be a good predictor of results in patients. However, larger trials with more patients and dosing regimens may show better effect. Meanwhile, more therapies should be sought.

From a therapeutic standpoint, it would be useful to know the key step(s) in migration. There may be multiple pathways that are to some extent redundant, such that depriving the cells of one growth factor might make little difference if multiple alternative chemoattractants and mitogens are available. However, antibodies against PDGF, like antibodies against bFGF, show significant inhibition of SMCs in vivo and anti-bFGF antibodies inhibit PDGF-stimulated SMC migration in vitro. Thus, the SMCs may respond to growth factors in an additive fashion or in sequence (such that breaking any link would sever the chain). It should also be possible to target the cell attachment factors, integrins, and related receptors required for smooth muscle migration.

Attempts are also being made to cripple the migration machinery (“downstream” from the signal pathways) using antiserum oligonucleotides to the particular isomers of myosin and actin involved in cell migration. Further even in the future is the possibility of using homologous recombination to knock out genes that are overexpressed by migrating cells. But even simpler goals like overexpressing t-PA by in vivo gene transfer are not yet practical.

A goal of several laboratories is the enhancement of reendothelialization. Candidates include bFGF and anti-bFGF (which may simultaneously stimulate SMCs) as well as the endothelial-specific mitogens VEGF (vascular endothelial growth factor) and PD-EGF (platelet-derived endothelial cell growth factor).

Another theoretically attractive approach is to selectively kill the migrating SMCs by 1) determining a receptor or antigen that is unique to those cells and 2) linking the corresponding ligand or antibody to a toxin or other cytoidal agent. Such immunotoxins and related Trojan horse approaches have not been particularly successful in cancer therapy, largely because of the genetic instability and resultant heterogeneity that results in resistant clones and because of the inability of antibodies to penetrate deep into the poorly vascularized tumor. Recent data indicate that neointimal SMCs are a more practical target. The potential diagnostic and prognostic utility of such antibodies (to unique markers on the surface of activated SMCs) is being explored in several laboratories.

In summary, the fact that therapies to date have been disappointing reinforces the need for more in vitro and animal research; second, the necessity of randomized controlled trials, as agents that are effective in vitro and in laboratory animals have not had dramatic clinical success. Molecular biology is thrilling, but to quote J. Madri, “in vivo veritas.”

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