Heparin Responsiveness In Vitro as a Prognostic Tool for Vascular Graft Stenosis
A Tale of Two Cell Types?

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Vascular graft stenosis after infrareguinal vein grafting or coronary artery bypass graft surgery is a significant cause of morbidity and suboptimal long-term clinical outcome of patients with vascular disease. Numerous animal and clinical studies have been undertaken to reveal the pathophysiological mechanisms accounting for this detrimental process. Stenotic lesions are dominated by neointima formation with migration and proliferation of SMCs and deposition of extracellular matrix. This appears to be a conserved response in the vasculature not only after vein grafting but also after various injurious stimuli such as angioplasty, endarterectomy, embolectomy, and arterial catheterization. However, the mechanisms of the process of stenosis are still not fully understood, and there is neither an effective treatment for prevention nor a diagnostic test for reliable prediction of patients at risk for developing graft stenosis. Most clinical and experimental studies have focused on the control of SMC proliferation with the intention of developing strategies for the prophylaxis of restenosis, whereas relatively little has been achieved in the area of early tests to define the prognosis and need for clinical monitoring of particular patients.

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The anticoagulant heparin has been used for many years in the therapy and prophylaxis of thrombotic conditions. It is also known for its antiproliferative effects on SMCs when applied in higher doses and has been studied extensively in animal models and clinical studies for the inhibition of neointima formation after vascular injury. As for many other compounds, encouraging results obtained in animal models have not been matched by successful clinical studies, perhaps because of dissimilar dosing levels or strategies in the patients or different contributions of SMC proliferation in stenotic lesions of animals and humans. Notwithstanding the failure of heparin to prevent graft stenosis, recent investigations, including that by Refson et al in this issue of Circulation,1 focus attention on a new potential utility of heparin in the management of patients with bypass graft surgery. This application of heparin is as a biological test reagent for stratifying patient populations with respect to their risk for chronic graft occlusion. The ability of heparin to contribute to such an assay is based on the observations that SMCs from given individuals may be categorized on the basis of their diversity of response in culture to the antiproliferative activity of heparin and that the heparin-resistant phenotype is associated with an increased risk for subsequent graft stenosis. Underlying this approach is a growing recognition of the phenotypic diversity of vascular SMCs among subjects as well as among distinct sites in particular individuals.

The findings of this and the other related studies, as with many emerging experimental observations, raise many more questions than they answer. These provocative questions have a number of implications for either vascular biology, clinical management, or both. Some of these issues may be summarized as follows.

What are the molecular transducers of the heparin response that are altered in heparin-resistant cells? Are the key abnormalities to be found at surface-receptor or intracellular levels?

It is known that the effects of heparin on cell proliferation are dissociable from the anticoagulant function of heparin, because nonanticoagulant fractions that retain antiproliferative activity have been described. It is also clear from clinical experience that patients differ widely in their responsiveness to heparin, even when the extent of thrombus and weight-adjusted dosing are considered. This raises the question, not addressed by the study of Refson et al, as to whether the observed heparin resistance correlates with an alteration in heparin anticoagulant function.

Are the interindividual differences in SMCs based at the genetic level, and thus heritable, or rather epigenetic, with a causal basis in the environment, diet, or other factors? Currently, no clearly described genetic syndromes of altered heparin sensitivity are known.

If the molecular mechanism for the variability in heparin response is due to an epigenetic alteration, is this an alteration that occurs throughout the vascular tree, or does it instead reflect local selection of a population characterized by heparin resistance and a concurrent “hyperstenotic” phenotype? In either case, what are the key factors that play a role in this selection? Are they classic risk factors of atherosclerosis or local hydraulic or mechanical forces that affect the vessels later to be used as grafts?

If the molecular basis of the variability in heparin response occurs at the genomic level, can molecular genetic testing be...
used to stratify patients with respect to their vascular smooth muscle responsiveness to heparin? In this case, it would be particularly important to pursue research to define the gene(s) whose allelic variants underlie heparin resistance. Successful identification of these genes would permit the development of comparatively straightforward diagnostic testing. Such tests might be based on polymerase chain reaction for the underlying altered allele, which could then be performed on any cellular material (such as leukocytes in standard blood samples) rather than requiring vascular tissue sampling at the time of surgical intervention, or could be based on immunoassay for an altered protein in tissue exhibiting its expression.

Will the information gained from molecular or cellular analysis truly find a place in clinical decision-making and management for patients with peripheral vascular disease? The widespread adoption of any such analysis in general patient care settings will be strongly influenced by the practicality of the specific test for physicians as well as patients in a nonresearch setting. Accordingly, the work necessary to translate these cellular findings into tests not based on tissue culture analyses will take priority, provided that the current data can be replicated in more extensive studies.

Does the described interindividual variability in smooth muscle behavior also predict the behavior of synthetic grafts with respect to their frequency of anastomotic stenosis, which is similarly dependent on smooth muscle proliferation? Also, does this phenomenon hold true for venous and arterial grafts in coronary positions? Furthermore, if relative heparin resistance is a characteristic of the entire vascular tree in particular individuals, does it form a basis for prognostic information with regard to angioplasty or stent restenosis?

What would be the optimum use of information concerning the heparin-related vascular cell genotype or phenotype? Possible effects on patient care might include (1) directing selection of endovascular versus surgical bypass therapy; (2) influencing the aggressiveness/timing of bypass surgery; (3) influencing the type of bypass to be used, ie, venous, arterial, or synthetic grafts; (4) helping to determine the frequency of postprocedural follow-up of patients; and (5) altering the heparin regimen according to heparin sensitivity. This latter might involve addition/substitution of alternative antiplatelet agents and postoperative anticoagulant medications or intensification of the level of heparinization in heparin-resistant individuals. This would be conceptually similar to the use of exogenous insulin in insulin-resistant states.

Might patients known to be heparin-resistant be treated systemically in ways that facilitate restoration of heparin sensitivity? This would be directly analogous to the use in diabetes of agents that promote peripheral insulin sensitivity. Would information concerning the heparin sensitivity of SMCs in given individuals be useful to direct selection of nonsurgical management options, such as the choice of heparin-coated versus standard stents or the decision to use local delivery of heparin in the context of angioplasty or stenting?

A clear identification of the mechanism(s) underlying the range of responses to heparin may be challenging because of complexity at several levels: (1) heparin preparations are themselves polydisperse polymers with subfractions possessing distinctive properties, so that antiproliferative and anticoagulant properties are clearly distinguishable; (2) mechanisms of heparin action are multiple, even when only proliferation effects are considered; and (3) SMC populations possess significant phenotypic diversity when viewed with respect to any of several parameters, not limited to heparin sensitivity.

We will now examine literature relevant to some of these issues and questions.

Molecular Bases of Heparin Effects on Proliferation

Among the spectrum of the effects of heparin on SMCs, a potential for differential influences on SMC proliferation, depending on the applied dose, environmental conditions, and treatment duration, has been noted in vitro and in vivo. Heparin in high concentrations (μg/mL) is typically an inhibitor of arterial SMC replication, whereas heparin in lesser quantities (ng/mL) may facilitate binding of bFGF to its receptor as well as expression of the elements of this system, thereby mediating a potential for growth stimulation. Further evidence for a paradoxical growth-stimulatory effect of heparin is provided by recent investigations on human saphenous veins, in which it has been shown that heparin can displace bFGF from binding sites at the luminal surface, with released bFGF available to stimulate SMC proliferation. Studies of rat SMCs showed that heparin inhibits MAPK in the presence of FCS but not in the presence of epidermal growth factor, thus suggesting heparin-sensitive and -insensitive pathways of MAPK activation. A study performed on baboon SMCs similarly revealed heparin inhibition of MAPK activity when stimulated by serum but not platelet-derived growth factor. When bFGF was used, heparin had a stimulatory effect on MAPK activity. Although it has been shown in the balloon injury model of the rat carotid artery that heparin inhibits neointima formation and that the injury causes the activation of MAPK, it could not be demonstrated that the antiproliferative effect of heparin is exerted by the inhibition of MAPK. Accordingly, the ability of heparin to block cell-cycle entry at the comparatively early stage of MAPK inhibition may reflect differences in growth factor sensitivity among discrete cell populations.

In addition to inducing arrest before G1 entry, presumably related to MAPK effects, heparin exhibits activity at several subsequent points of the cell cycle. Activity is also noted during the G1 phase, with reduction of cell-cycle regulatory factors such as cyclin D1 mRNA and protein, cdk2 mRNA and cdc2 protein, and traverse inhibited through the G1/M phase. It has also been described as blocking the expression of c-myc, an event that occurs in mid-G1 phase. At present,
Phenotypic Diversity Among Vascular SMCs

Several studies have demonstrated the existence of relatively heparin-sensitive and heparin-resistant populations of SMCs. Some of these investigations have involved prolonged in vitro exposure to heparin, in which case the finding either may reflect selection of a preexistent phenotype from among an inhomogeneous population or might represent the induction of a novel phenotype in culture under selection pressure. The phenomenon of cell clones developing a resistance to compounds is not heparin-specific, because similar effects are well known to occur on exposure to chemotherapeutic agents. For example, rat thoracic aortic SMCs exposed to long-term culture with 200 μg/mL heparin have exhibited a significant loss of sensitivity to growth inhibition by heparin. This heparin resistance was stable even after cells were grown for two passages in heparin-free medium, suggesting effective selection for this SMC phenotype. Characterization of the heparin-resistant SMCs compared with their nontreated controls revealed the treated cells to be smaller, to possess less smooth muscle α-actin and one half of the PKC activity, and to show a paradoxically greater contact inhibition. A marked decrease in heparin binding capacity was seen in the resistant cells. However, another group has shown that similar heparin-resistant and heparin-sensitive SMCs derived from rat abdominal aorta bound and internalized comparable amounts of heparin. The ability of heparin treatment to increase the percentage of cells that express smooth muscle α-actin was preserved in the heparin-resistant cells, suggesting that the antiproliferative and the differentiation-promoting effects of heparin were independent. With regard to extracellular matrix proteins, it has been shown that heparin-resistant SMCs differ from control cultures by low production of fibronectin, prevalent expression of laminin, and decreased cell-associated glycosaminoglycans. With each of these observations, the question remains whether the alterations represent primary characteristics of cells with the heparin-resistant phenotype or are secondary to a selection-induced SMC phenotypic shift.

These data have been extended by experiments not involving prolonged in vitro heparin exposure. SMCs derived from SHRs have been found to be less sensitive to the growth-inhibitory effect of heparin than controls from Wistar-Kyoto rats, in conjunction with a reduced capacity but comparable affinity for heparin binding to extracellular surface receptors. In addition, SHR-derived SMCs exhibited a greater capacity, but not affinity, for epidermal growth factor binding. Recent investigations of the rat carotid artery demonstrated that the typical inhibition of SMG replication by heparin acutely after a balloon injury was not present after a reinjury performed 28 days later. These results may be viewed in the context of an altered phenotype of SMCs to be found in vivo in neointima after vascular injury; it would be interesting to see whether the heparin resistance would return after a longer time interval subsequent to the initial injury, which might permit the SMCs to return to a contractile phenotype more similar to their baseline. A basis for variable heparin sensitivity in the clinical setting could thus reside in the intensity and timing of mechanical or biochemical injury, with resultant phenotypic shift.

Findings in Human Smooth Muscle

In some studies performed on human cells, aortic and saphenous vein SMCs have shown different sensitivities to heparin, with DNA synthesis in venous SMCs manifesting heparin dependence different from that found in corresponding aortic SMCs. This phenomenon could be demonstrated with paired aortic and venous samples obtained from the same individual as well as with pairs mixed from different patients. Another study design comparing heparin sensitivity among human aortic and saphenous vein SMCs in culture did not yield significant differences based on the site of origin but did show interindividual differences, supporting systemic rather than regional variation. Despite these differences in heparin sensitivity, baseline proliferation was consistent among SMCs derived from the various individuals. The question arises whether such conflicting results may be explained by different sites of vessel origin or different patient populations.

Such work characterizing the diversity of SMCs with respect to heparin sensitivity has provided an impetus to determine whether this phenomenon might correlate with the clinical problem of vascular graft stenosis. A retrospective study was thus performed on the effect of heparin on proliferation of cultured human SMCs derived from stenotic lesions (at the time of reoperation) and from apparently normal vessels of the same patient as well as on SMCs grown from vessels of patients undergoing primary bypass surgery. Although the vascular tissues in that study were derived from a range of different vessels, including internal mammary artery, common femoral artery, popliteal artery, iliac artery, and saphenous veins, this previous investigation yielded the provocative initial result that SMCs derived from patients with early stenosis showed much lower sensitivity to growth inhibition by heparin than the controls. This effect was apparent whether SMCs were grown from stenotic lesions or undiseased veins. In light of the prognostic potential, the group proceeded with the current prospective study, in which...
SMCs were cultured by outgrowth from redundant sections of veins at the time of initial infrainguinal bypass surgery and the resulting SMC cultures incubated with heparin. A diminished growth inhibition by heparin (median of 20.9%, compared with 54% in control cells) was found with SMCs derived from vessels of patients who subsequently developed graft stenosis over a follow-up period of at least 1 year. In an attempt to explain this phenomenon, the group investigated a subset of the SMC cultures for heparin-binding parameters, which appeared to be correlated with growth inhibition as well as the clinical outcome.

Future Implications
The study by Refson et al provides a basis for expanded investigation of the laboratory assessment of vascular patients by evaluation of heparin effects on SMC growth as a prognostic approach for the development of graft stenosis. In the design and execution of such future studies, certain methodological cautions warrant consideration. The authors point out that more information is needed about the relation of environmental and hereditary risk factors to in vitro properties of SMCs cultured from patients. This is particularly true given the substantial overlap in heparin sensitivities found between the groups of patients with and without stenoses. Such overlap will confound attempts to use this approach for individuals (rather than populations), and it will thus be important to control for other variables and optimize the culture conditions to provide maximally predictive separation between patients. As part of an approach to understanding effects that might be reflected by early pathological changes in the vascular site, it would be helpful to collect tissue samples for histological or physiological evaluation as well as culture. Another area for specific consideration in trial planning would be the choice of consistent heparin formulations, given the well-known heterogeneity of heparin activities, depending on source, processing, etc.

Experiments designed to determine whether the heparin-resistant phenotype is inherited or based on somatic diversity will be important. The finding that the heparin-resistant phenotype persists despite passing in vitro does indeed support a genetic difference but does not necessarily verify a germ-line genotype, because it is possible that particular environmental factors favor either mutation or selection for particular smooth muscle populations from among a group with preexisting functional heterogeneity based on somatic diversity in gene expression. Regional vascular genetic heterogeneity reflected by X-chromosome inactivation patterns has indeed been demonstrated within atherosclerotic plaques as well as normal coronary vessels. It may be speculated that distinct types or intensities of chronic vascular injury result in cell-cycle reentry of specific types of SMCs, or SMCs that develop distinct phenotypes and growth properties once cell proliferation and migration has started. The distinction between systemic and regional genetic variability is significant for future clinical application, because an inherited genetic basis would provide for the possibility of molecular phenotyping for heparin-resistance status based on blood or other peripheral sampling. Local clonal expansion of particular vascular cell types based on selection pressures might also support molecular assignment of the phenotype, but this approach would presumably require screening of vascular tissue(s) in which the selection and clonal expansion was thought to have occurred. Each of these issues will require consideration as the development of a new test for the clinical management of vascular disease is contemplated.

Another intriguing clinical implication of the identification of heparin insensitivity in cultured cells is the suggestion of a state of in vivo functional heparin insensitivity. This implies that an increase in heparin sensitivity of SMCs might be a novel therapeutic target. The discovery of pharmacological interventions capable of inducing such an increase could lead to enhanced in vivo activity of heparin to prevent graft stenosis or might conceivably be manifested as an alteration in SMC proliferative activity without the need for modified heparin administration. Research exploring these as well as the other intriguing questions raised by the study of Refson and colleagues may be expected to yield interesting results in both basic and clinical arenas of vascular medicine in the near future.

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