From Bench to Bedside

Cell Cycle in Vasculoproliferative Diseases
Potential Interventions and Routes of Delivery

Vissa Sriram, MD; Cam Patterson, MD

Abstract—Atherosclerosis and restenosis of epicardial vessels are among the greatest challenges facing the clinical cardiologist, and phenotypic modulation and proliferation of smooth muscle cells are major components of the vasculoproliferative response. Proliferation is regulated by the interplay of regulatory proteins at checkpoints in the cell cycle that alter cellular growth. Activation of the cell cycle and the genetic control of its progression are final common pathways in this process. Investigators have postulated that cell-cycle inhibition using drugs and genetic or physical methods has the potential to reverse or prevent the vasculoproliferative process. The current challenge is to translate in vitro data demonstrating the efficacy of cell-cycle inhibition to clinical trials. At present, the steps that must be taken to meet this goal are (1) to design methods of delivery of these agents to specific sites, (2) to identify appropriate cellular targets to elicit cell-cycle arrest, and (3) to improve the therapeutic ratio by minimizing potential side effects. This review discusses current concepts of the cell cycle, target-regulating mechanisms, and possible interventions in vasculoproliferative diseases. We also discuss ongoing clinical trials that use antiproliferative agents in the hope of limiting the course of these diseases, as well as the promise that antiproliferative therapy holds in the coming decade. (Circulation. 2001;103:2414-2419.)

Key Words: muscle, smooth ■ restenosis ■ gene therapy ■ pharmacology

Results from the World Health Organization’s MONItoring Trends and Determinants in Cardiovascular Disease (MONICA) Project confirm that coronary artery disease is the major determinant of mortality in the Western population. Atherosclerosis is the primary pathological event leading to the decrease in lumen size of coronary vessels in patients with coronary artery disease. For many symptomatic lesions, mechanical interventions such as balloon angioplasty and stent placement are the “standard of care.” However, 30% to 50% of patients undergoing percutaneous coronary interventions will experience restenosis, and 20% of these will require additional interventions, including coronary artery bypass surgery, a procedure that is itself limited by graft failure due to luminal obstruction. Atherosclerosis, postprocedure restenosis, and vein-graft failure comprise the spectrum of vasculoproliferative disorders. This review explores the rationale for antiproliferative therapies targeting the cell cycle in treatment of these diseases. Although local radiotherapy for vascular disease operates in large part by inhibition of cellular proliferation, we focus here on pharmacological and genetic therapeutics aimed at inhibiting specific regulatory events in the cell cycle to arrest proliferation.

Vasculoproliferative Disorders and the Cell Cycle

Vascular injury is common to all vasculoproliferative disorders and may occur in response to factors such as mechanical interventions, hypertension, and hyperlipidemia. Vascular injury triggers a vasculoproliferative cascade that can be divided into three phases: (1) an early phase of platelet activation and thrombus formation, (2) an intermediate phase of smooth muscle cell (SMC) recruitment, and (3) a late proliferative phase. Cytokines and growth factors are the messengers that perpetuate the vasculoproliferative response. Damaged endothelial cells, platelets, SMCs, and macrophages secrete factors that serve as chemoattractants and regulators of growth and gene expression in vascular cells. A robust vasculoproliferative response may itself lead to occlusive coronary lesions that produce myocardial ischemia. In addition, SMC-rich lesions provide the “soil” for alterations in the composition of the extracellular matrix and accumulation of macrophages.

SMCs progress through DNA replication and mitosis in a regulated series of cell-cycle events that comprise the final common pathway downstream of vascular injury during the vasculoproliferative response. Resting SMCs are maintained in a nonproliferative phase (G0). Activated SMCs enter a gap period (G1), during which the cell assembles the factors necessary for DNA replication in the subsequent synthetic (S) phase. After DNA replication is completed, the cells again enter a gap period (G2), when proteins are synthesized in preparation for mitosis (M). Restriction points occurring at the G1-to-S and G2-to-M interphases ensure orderly cell-cycle progression.
progression. Although upstream mitotic signals vary, the major molecular events of the cell cycle are common among all cell types, and it seems unlikely that the molecular events of the cell cycle in SMCs can themselves be targeted with precision. Thus, the clinical specificity of cell cycle–targeting agents is likely to be determined by the ability to deliver these therapies in a spatially restricted fashion.

**Cyclins and Cell-Cycle Control**

The cell cycle is regulated by the synthesis, activation, and degradation of molecules that modulate the proliferative phenotype.\(^5\) The cyclins and their cognate enzymes, the cyclin-dependent kinases (CDKs), exist in stable complexes in their active states and are positive regulators of these events. The CDK inhibitors (CDKIs) are also critical negative regulators of the cell cycle (Table 1). The activities of cyclin–CDK complexes depend on the phosphorylation status of CDKs and the steady-state levels of cyclins. Phase-specific cyclin–CDK complexes regulate progression through the cell cycle to confer specificity and order to this process. Initially, extracellular signals activate CDK4 and CDK2 in series while simultaneously increasing expression of cyclins D, E, and A; these events coordinate DNA replication by regulating the transition through the G\(_1\) and S phases. Subsequently, the G\(_2\)-to-M transition is regulated by cyclin B–CDK1 complexes. Under physiological conditions, CDKs inhibit their respective CDKs to regulate the events in the cell cycle. Failure of antimitogenic events mediated by CDKIs may be an important component of the vasculoproliferative response, and for this reason, timely overexpression of CDKIs is being explored to arrest SMC proliferation.

In addition to the regulatory cyclin-CDK complexes, cyclin–cyclin progression is also modulated by transcription factors that activate CDK and CDK1 expression. Antimitogenic signals activate p53, which induces transcription of p21 and indirectly inhibits activity of the G\(_1\) cyclin–CDK complex. Conversely, the E2F family controls expression of genes required in S phase. Under quiescent conditions, E2F members exist in inactive complexes with retinoblastoma protein. After mitogenic stimulation, the cyclin D–CDK4 complex hyperphosphorylates retinoblastoma protein, leading to dissociation of E2F, which in turn activates the expression of genes such as cyclins E and A and CDK1. In addition to p53 and E2F, GAX and GATA-6 are also relevant cell cycle–associated transcription factors in vascular SMCs.\(^5,6\) Both GAX and GATA-6 inhibit SMC proliferation by inducing expression of p21, and both are downregulated as cells enter the cell cycle. Finally, transcription factors are not the only means by which the cell cycle is regulated in SMCs. Nitric oxide potently downregulates CDK2 and cyclin A and up-regulates p21, resulting in cell-cycle arrest.\(^7\) SMC-specific effects of GAX, GATA-6, and nitric oxide make them attractive therapeutic targets.

Regulation of the cell-cycle machinery is a final pathway for proliferative responses, so it is not surprising that correlations exist between markers of cell-cycle progression and factors associated with the vasculoproliferative response. Studies in animal models invariably demonstrate highly coordinated expression of cyclins, CDKs, and CDKIs after vascular injury.\(^8,9\) These cell-cycle regulators provide a logical site for intervention. As our knowledge of cell-cycle regulation advances, two questions face those intending to target the cell cycle in vascular diseases: (1) Is inhibition of the vasculoproliferative response sufficient to prevent clinical events? (2) Can cell-cycle inhibition be pursued as a therapy with enough specificity to minimize inherent side effects?

**Pharmacological and Genetic Tools to Control the Cell Cycle**

The first applications of antiproliferative therapies for vascular diseases were designed to inhibit specific signaling pathways. Although initial studies testing inhibitors of thrombin and platelet-derived growth factor were promising, such strategies have been slow to translate to humans. This can be interpreted to indicate that the role of proliferative events in vascular lesion formation is overstated. This concern does, in fact, have merit in situations such as late-stage atherosclerotic lesions, in which plaque instability is the primary determinant of clinical events. However, both animal and clinical data indicate that SMC proliferation is a major determinant of postintervention restenosis and vein-graft failure. Many investigators now feel that preventing these outcomes by targeting events far upstream of the cell cycle itself, as has been done previously, is an inefficient way to inhibit proliferation. As the molecules that directly regulate the cell cycle have been identified, the focus in antiproliferative therapies has now shifted to targeting the cell cycle using specific anti–cell cycle drugs and genetic tools (Table 2).

Low-molecular-weight compounds that specifically interfere with cell-cycle proteins provide one means with which the cell cycle can be targeted. Blockade of the ATP-binding site of CDKs is an attractive pharmacological target because the ATP-binding site in CDKs is structurally dissimilar from

### TABLE 1. Molecular Regulators of the Cell Cycle

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Types</th>
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<tbody>
<tr>
<td>Cyclins</td>
<td>A, B(<em>{1,2,3}), C, D(</em>{2,3}), E, F, G, and H</td>
</tr>
<tr>
<td>CDKs</td>
<td>CDK1, CDK2, CDK3, CDK4, CDK5, CDK6, CDK7, and CDK8</td>
</tr>
<tr>
<td>CDKIs</td>
<td>INK4 family p14, p15, p16, p18, and p19, KIP/CIP family p21, p27, and p57</td>
</tr>
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### TABLE 2. Methods of Controlling the Cell Cycle in Vasculoproliferative Disorders

<table>
<thead>
<tr>
<th>Methods</th>
<th>Types</th>
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</thead>
<tbody>
<tr>
<td>Direct—Pharmacological</td>
<td>ATP site–directed competitive inhibitors Flavones (flavopiridol), Olomoucine derivatives (CVT-313)</td>
</tr>
<tr>
<td>Macrolide antibiotic Rapamycin</td>
<td></td>
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<tr>
<td>Microtubule assembly Paclitaxel</td>
<td></td>
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<tr>
<td>Indirect—Genetic</td>
<td>Oligonucleotide-based methods Antisense technology (CDKs and cyclins)</td>
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<td></td>
<td>Transcription factor decoys (E2F) Ribozymes</td>
</tr>
<tr>
<td>Protein overexpression</td>
<td>Viral (CDKIs, Gax, GATA-6), Plasmid-based (p53)</td>
</tr>
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that of other kinases. The most promising agent in this group is flavopiridol. This low-molecular-weight compound is available orally and efficiently blocks the activity of CDKs 1, 2, 4, and 7, but it has negligible effects on other kinases. In vitro observations from our laboratory indicate that flavopiridol can inhibit SMC proliferation in vitro and that the vasculoproliferative response is inhibited by systemically administered flavopiridol after balloon injury to the rat carotid artery. CVT-313, a derivative of the purine analog olomoucine, is another member of the pharmacological CDKI family with potential cardiovascular applications. CVT-313 inhibits CDK2 activity, although it is less potent than flavopiridol, and can inhibit lesion formation when locally administered after carotid injury in the rat model.

Agents that inhibit cell-cycle progression indirectly also have been tested as inhibitors of vascular proliferation. Rapamycin (Rapamune, Sirolimus), a macrolide antibiotic with oral availability, and paclitaxel (Taxol), a chemotherapeutic agent, both induce G1 cell-cycle arrest in SMCs and inhibit neointimal formation in animal models when administered systemically or, in the case of paclitaxel, when deposited into the pericardial space. Paclitaxel stabilizes polymerized microtubules and elicits long-lasting cell-cycle arrest of vascular SMCs. Rapamycin binds to a cytosolic immunophilin, FKBP12, and, through mechanisms that are yet to be explained, potently suppresses SMC proliferation. Preliminary human studies using rapamycin-coated stents are discussed below, and pharmacological inhibitors of the cell cycle clearly hold enormous potential. Indeed, we may be using drugs that modulate cell-cycle activity without realizing it; both nitric oxide and high-dose salicylates inhibit the cell cycle and SMC proliferation in experimental studies and may behave similarly in vivo.

Gene therapy provides a second means to modulate the cell cycle in vasculoproliferative diseases. Gene therapy approaches require choices regarding (1) a method to modulate a gene’s expression or activity and (2) the gene to be modulated. Endogenous gene expression can be downregulated using antisense techniques, which rely on the ability of antisense DNA and RNA molecules to bind to an mRNA molecule in a sequence-specific fashion to increase degradation of the RNA. Gene activity can also be regulated negatively using transcription factor decoy techniques, which utilize oligonucleotides bearing consensus binding sequences for transcription factors in order to competitively prevent their function. When delivered in polyacationic liposome complexes, antisense oligonucleotides and RNAs directed against CDKs 1 and 2 and cyclins B1 and G1 have been used successfully in animal models of neointimal formation. Likewise, a transcription factor decoy strategy targeting E2F has proved effective in the rat carotid injury model. Ribozymes, or catalytic RNAs that target the RNA of a specific gene for degradation, are also promising means for preventing the vasculoproliferative response. Adenoviral delivery of ribozymes targeting c-myb inhibit neointimal formation after balloon injury in a rat model, and local delivery of a proliferating cell nuclear antigen ribozyme inhibits stenosis in a porcine stent model.

Overexpression of cell cycle–inhibitory molecules using viral- or plasmid-based methods is a second approach, and it stands in contrast to the genetic methods designed to downregulate positive cell-cycle effectors. In particular, overexpression of the CDKIs p21 and p27 using adenoviral technology is effective in preventing neointimal formation, as is overexpression of the cell cycle–inhibitory transcription factor p53 using a naked plasmid vector. Adenoviral delivery of the SMC-specific transcription factors has also shown promise as a means to inhibit the vasculoproliferative response. In particular, overexpression of GAX is effective in at least three different vasculoproliferative models. The tests of GAX overexpression are among the most rigorous in the field of gene therapy for vascular disease. Lastly, overexpression of nitric oxide synthase isoforms using gene therapy methods has reduced neointimal formation in animal models, an effect that is likely to be mediated in part via interactions between nitric oxide and the cell-cycle machinery.

It is worth emphasizing that adenovirus- and plasmid-based gene delivery is extrachromosomal and therefore transient, and adenoviruses stimulate an immune response that can preclude repeated administration, thereby limiting the usefulness of these technologies. However, delivery systems using adeno-associated virus and lentiviral vectors may assist in the translation of these techniques to human therapy. In any event, animal studies using genetic methods for the prevention of vasculoproliferative disorders indicate that these methods hold clinical potential. At the same time, it is worth recognizing the limitations of these animal studies. In addition to the obvious species-related differences that exist, the delivery of genetic materials in animal studies is usually by way of direct instillation to a surgically isolated vessel, a method that is only remotely similar to the current human catheter-based technologies described below. We must also await improvements in methodologies related to viral-based gene delivery because none of the options currently available, when contemplated as cardiovascular therapeutics, is ideal with respect to safety, immunogenicity, or stability of gene expression.

**Responses of Vascular Cells to Cell-Cycle Inhibition**

Although it may seem counterintuitive, the response of SMCs to inhibition of the cell cycle is not uniform. For example, p16INK4a is less potent than p21 or p27 in its ability to block CDK2 activity and is a weak inhibitor of the vasculoproliferative response. It should not be assumed that cell-cycle inhibition of SMCs results only in maintenance of the quiescent state. Apoptosis is one possible outcome of cell-cycle inhibition; for example, overexpression of GAX in rat carotid arteries elicits apoptosis at the same time that it inhibits proliferation. Although arguments exist over the utility of inducing SMC apoptosis in vasculoproliferative diseases, it is logical to speculate that reducing the burden of SMCs in vascular lesions will have favorable effects on luminal obstruction.

Hypertrophy is a second potential outcome of cell-cycle inhibition in SMCs. On the basis of its differential induction...
by angiotensin II—a potent stimulus for SMC hypertrophy—the CDKI p27 is hypothesized to serve as a molecular switch that determines whether SMCs hypertrophy or proliferate after growth-factor stimulation. Induction of p27 expression after angiotensin II stimulation favors hypertrophy, whereas serum stimulation, which fails to increase p27 levels, has a potent proliferative action. Although inhibition of proliferation may be desired in vasculoproliferative diseases, hypertrophy can enhance neointimal formation after vascular injury and will reduce vascular compliance, neither of which is a favorable outcome. Unresolved issues such as these underscore the need to understand the effects of cell-cycle inhibitors at the cellular and molecular level before we can expect to predict the clinical effects of these agents.

**Routes of Delivery**

The ultimate success of pharmacological and genetic approaches to vasculoproliferative therapy depends on the precision and efficiency of their delivery to areas of interest in the cardiovascular system. The routes of delivery discussed below are summarized in Table 3. Systemic administration is limited by the dilution effect and by uptake by nonvascular tissues, which narrow the therapeutic window for this approach. A second option is to approach the local site in the course of an interventional procedure. Drugs or genes can be delivered via intrapericardial, intracoronary, interventricular, or epicardial injection; while the subject is undergoing balloon dilatation or stent placement; or in the case of patients undergoing bypass surgery, by treating grafts ex vivo.

Catheter- or stent-based delivery is a logical means of adapting existing interventional techniques to pharmacological or genetic vascular approaches. Catheter-based systems can deliver an agent of choice intravascularly by either pressure- or diffusion-mediated transfer. The pressure-mediated approaches are limited because the efficiency of transduction is relatively low, and their high-flow profile causes damage likely to attenuate the efficacy of these devices. A recent study comparing delivery catheters in pig coronary arteries indicated that adenoviral transduction was similar for each, and although coronary gene expression could be achieved, the efficiency of viral transfer to coronary arteries was rather low. On the basis of these results, it seems that improvements in technique and catheter design will be required to optimize these catheters for human use.

Given the increased frequency with which stents are being deployed in the setting of vascular disease, it is not surprising that interest has turned away from catheter-based drug or gene delivery and toward stent-based approaches. Stents provide an excellent scaffold for delivery at high concentrations over relatively long time periods. In particular, bioreabsorbable, microporous stents are a promising new approach for stent-based drug delivery. Although initial attempts to create biodegradable stents used materials that elicited an inflammatory response, recent data indicate that polymers of poly-L-lactic acid are biocompatible. Stents made from poly-L-lactic acid are biodegradable and have impressive radial strength. The first use of these stents in humans was recently reported. Technology related to poly-L-lactic acid stents is improving rapidly, and success has been demonstrated in delivery of viruses and low-molecular-weight compounds in animal models by this method.

Importantly, coated stents can deliver paclitaxel efficiently to injured rabbit iliac arteries. Lastly, it should be mentioned that the pericardial cavity is a promising space for agent delivery because its relative impermeability maintains high concentrations of a drug or vector for an extended period of time. Safe techniques for pericardial delivery of adenoviruses and pharmacological agents have been developed in animal models, and promising results have been reported in a porcine coronary injury model using the antiproliferative agent paclitaxel, but further refinements are needed before they can be incorporated into clinical trials.

**Antiproliferative Therapy: Prospects for the Future**

Progress in the translation of research in the field of antiproliferative therapies to human applications is occurring rapidly. Given the momentum in this field, one might have expected the first reported human studies to examine the efficacy of adenoviruses or antiproliferative drugs for the inhibition of restenosis after percutaneous interventions. It is somewhat ironic, then, that the first published human trial of antiproliferative therapy for vasculoproliferative diseases is actually the PROject of Ex-vivo Vein graft ENgineering via Transfection (PREVENT) study. This ongoing trial examines the delivery of E2F transcription factor decoy oligonucleotides as a means to inhibit vein-graft stenosis after peripheral bypass surgery.

Direct instillation of vectors has been a means of gene transfer in numerous animal studies. Because of the invasiveness of this method, practical barriers prohibit its application in most human vasculoproliferative disorders. The investigators of the PREVENT trial cleverly chose an exception to these barriers by treating vein grafts to prevent stenosis.
because ex vivo instillation of oligonucleotides only increases the invasiveness of a bypass procedure incrementally. Preliminary, but provocative, data from a Phase I study demonstrate impressive cellular access by this method, with minimal complications and a measurable decrease in vein-graft stenoses after peripheral bypass procedures. The preliminary nature of these data must be emphasized, and the question must be raised whether these methods will be applicable to coronary artery bypass grafting. Nonetheless, these experiments provide the first evidence that antiproliferative gene therapy for vasculoproliferative diseases in humans is feasible and, potentially, efficacious.

Several additional trials to examine the efficacy of pharmacological inhibitors of proliferation and gene therapy methods to treat restenosis after coronary interventions are in early stages or are about to be initiated. In particular, the results of a Phase I study using rapamycin-coated BX Velocity stents (Cordis) in 30 patients have recently been reported. Intimal hyperplasia was assessed at follow-up by intravascular ultrasound and quantitative angiography in these patients. Although the ability to identify beneficial effects of rapamycin in this small, nonrandomized study is limited, the lack of significant neointimal hyperplasia in any of the 30 patients at 4-month follow-up is encouraging. Larger, randomized studies are warranted to determine the extent to which such coated stents will be beneficial in patients with coronary artery disease.

In addition to the rather limited and as yet inconclusive human data, it should be kept in mind that an impressive body of data supports the utility of antiproliferative drugs and gene therapy techniques for prevention of the vascular proliferative response in animal models. Given the history of pharmacological agents’ failing to provide benefit in randomized human clinical trials after having been shown effective in animal models, one has to wonder whether the same fate will be met by antiproliferative agents. We must be mindful that most of the innovations that have led to the introduction of antiproliferative therapies for vascular disease have been technological in nature and have not derived from any major advances in our understanding of the pathophysiology of proliferative processes. Some would argue that this is a recipe for failure, pointing to recent setbacks in the applications of angiogenic gene therapy for vascular disease that have been reported in the lay press. Those with cautious voices seem to be in the minority, however, and it is clear that the introduction of these methods in human clinical trials will accelerate. The efficacy of antiproliferative therapies, using different targets and administered in a variety of ways, in virtually every animal model of vascular disease, provides a compelling argument that these treatments should be beneficial, particularly in human diseases—such as in-stent restenosis—that are primarily proliferative events occurring over relatively short periods of time. The challenges in translating antiproliferative agents to human therapy will be to identify the vasculoproliferative diseases most amenable to this intervention, to determine the most appropriate cell-cycle event to target, and to establish the best means to intervene in this event.

References
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