Local Delivery of Ceramide for Restenosis
Is There a Future for Lipid Therapy?

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Despite the clinical advantage of percutaneous transluminal coronary angioplasty (PTCA) in treating severely narrowed blood vessels, long-term success is often compromised by restenosis. It is unclear what mechanisms cause vessels to renarrow, despite numerous studies involving patients and experimental animal models of arterial injury. Although neointimal thickening was initially considered the major cause, recent evidence suggests that arterial constriction, adventitial thickening, or both may be critical in the restenosis process. The geometric changes of vessel expansion and contraction constitute the definition of remodeling. The present emphasis on remodeling and restenosis has been driven by studies failing to show a direct correlation between neointimal thickening and lumen size, suggesting that intimal mass alone is insufficient to explain narrowing. Analyses of human coronary angioplasty sites at autopsy by our laboratory support the thesis that constrictive remodeling and the initial plaque burden, rather than neointimal formation, are responsible for the failure of angioplasty. Other potential contributors to the pathophysiology of restenosis include arterial spasm, vessel recoil, platelet aggregation, and thrombus formation.

Arterial injury evokes a sequence of events, consisting of medial smooth muscle cell activation, migration and proliferation, and matrix secretion culminating in a thickened neointima. This series of events is linked to complex interactions between cells within the vessel wall as well as circulating blood cells and cytokines. At least in theory, restenosis should be a treatable process with adjunctive pharmacotherapy. Several agents, such as vascular endothelial growth factor, heparin, paclitaxel, urokinase, recombinant hirudin, colchicine, rapamycin sirolimus, cytochalasin B, IIb and IIIa inhibitors, and antisense oligomers, have been tested in animal models or patients. Most of these agents have failed to solve the restenosis problem in humans, but two recent promising agents, paclitaxel and rapamycin, are in clinical trials in Europe.

Several factors may contribute to the failure of studies to be translated into successful human trials. Difficulties in achieving the appropriate dose may offer one explanation. This can be remedied by localized delivery of a drug rather than systemic administration, which should help attain high concentrations while limiting the side effects of potentially toxic agents. Alternatively, most of these agents have generally targeted the reduction of tissue growth as a means of suppressing the intimal hyperplasia, with little emphasis on vascular remodeling, a factor perhaps more critical to the restenotic process, at least in PTCA. Moreover, the importance of cell proliferation in human restenosis is not well supported; coronary atherectomy specimens at various intervals after PTCA fail to show clinical evidence of increased cell proliferation. This observation, however, does not mean that proliferation does not occur. Obviously, smooth muscle cells are the dominant cellular element of restenotic lesions; hence, it is conceivable that replication occurs for transient periods, which may be overlooked because of insufficient sampling. The extent to which smooth muscle cell migration contributes to the restenotic lesion must also be weighed.

Another point to consider is the course of the disease. Animal models of restenosis generally consist of nonatherosclerotic vessels studied within weeks of injury; in humans, this process typically develops over 6 months. Finally, in reference to the underlying disease, there is little information as to the influence of plaque substrate on restenosis. In other words, animal models without underlying atherosclerotic disease may be overly simplistic. For example, it is conceivable that plaques with a large lipid core and increased inflammation provide a more reactive environment than relatively acellular fibrous plaques with small or absent lipid cores. Preliminary evidence in support of this hypothesis comes from an autopsy study of human stents by Farb et al, demonstrating that lipid core size and inflammation correlate with neointima formation and greater restenosis. Today, in-stent restenosis has become an emerging clinical problem, because ≈80% of coronary patients receive stents rather than PTCA alone.

In a study published in this issue of Circulation Research, Charles et al show that local delivery of C₇-ceramide, a cell-permeable bioactive analog of ceramide, reduces neointimal hyperplasia by 50% 2 weeks after balloon stretch injury of the rabbit carotid artery. When neointimal stenosis was quantified as a ratio of neointimal and medial cross-sectional areas, ceramide treatment resulted in a 92% reduction in stenosis. Although the growth-arresting potential of ceramide has been shown previously in culture, this is the first study demonstrating efficacy in an in vitro animal model of restenosis. Administration of ceramide resulted in an early...
inhibition of extracellular signal–related kinase (ERK) and phosphorylation of protein kinase B (PKB/Akt) as a mechanism of decreased neointimal formation. The downregulation of these activities highlights their importance in the mitogenic and cell-survival pathways critical to neointimal hyperplasia in animal models of restenosis. Activation of PKB/Akt and ERK may represent common signaling pathways of vascular smooth muscle cell migration and growth as shared by several agonists, as has been shown recently with angiotensin II. Pathological analysis demonstrated that ceramide treatment did not provoke inflammation, a desirable property for an antirestenotic drug. As an important practical application, this study should prompt trials in larger animal models of restenosis, including those that involve stents. In addition, the development of ceramide-like analogs with greater potency or long-term retention may also prove beneficial.

**Ceramide: Metabolism and Catabolism**

Ceramide forms the central molecule of all sphingolipids. Sphingolipids consist of a long-chain amino-dialcohol base (sphingoid), an amide-linked fatty acyl group, and a polar or glycosidic head group. Although these molecules were initially characterized as serving a structural role in membranes, various derivatives are recognized as second messengers that possess diverse cellular responses. Endogenous ceramide is generated through the activation of distinct sphingomyelinases residing in separate subcellular compartments in response to specific stimuli. Ceramidases are enzymes that hydrolyze ceramide to liberate the fatty acid from the sphingoid base to form sphingosine. The sphingosine released by ceramidases can be phosphorylated by sphingosine kinase to sphingosine-1-phosphate, which is also biologically active in vascular cells. In addition to the parent ceramide, there are cell-permeable analogs, such as C1-Ceramide (N-erythro-sphingosine, N-acetyl), C2-ceramide (N-erythro-sphingosine, N-hexanoyl), or C4-ceramide (N-erythro-sphingosine, N-octanoyl). These exogenous cell-permeant ceramide analogs mimic many of the biological effects of natural ceramide.

It is apparent from the literature that the specificity of cellular responses to ceramide depends on many factors, which include the nature of the stimulus, costimulatory signals, and the cell type involved. This diversity can result in the varied effects of ceramide on cell function, some of which may be proatherogenic. Therefore, differential effects of ceramide may be observed, depending on the dosing and extent of hypercholesterolemia.

**Healing and Restenosis**

Arterial injury produces a wound-healing response initiated by platelet deposition followed by inflammation, angiogenesis, granulation tissue, migration and proliferation of smooth muscle cells, and synthesis of matrix molecules. In the study by Charles et al, neointimal formation was studied at 2 weeks after arterial injury, a time at which healing may be incomplete, especially with drug treatment. It is unknown whether the beneficial effects of ceramide on restenosis represent a true reduction in neointima or delayed healing. Pathological examination was restricted to hematoxylin and eosin–stained slides; special stains were not done for fibrin, for example, which in some instances can be mistaken for collagen.

Other antiproliferative drugs, such as paclitaxel, a novel antimicrotubular agent with antiproliferative as well as anti-inflammatory properties, decreases neointimal formation 28 days after stenting of rabbit iliac arteries. However, this effect is accompanied by incomplete healing up to 6 months after stenting. In the study by Charles et al, inflammation was assessed at only up to 60 minutes after injury; the inflammatory response in this model peaks between 3 to 5 days. It has been shown that ceramides mimic the effects of cytokines, which can induce cellular inflammation via activation of stress-activated protein kinase cascades. Therefore, more detailed pathological studies should be carried out to determine the long-term effects of ceramide treatment on inflammation and healing in vivo.

The dosing of ceramide also deserves comment. In the report by Charles et al, the signaling effects of ceramide were apparent as early as 15 minutes after dosing. This was accompanied by a 50% loss of the parent ceramide analog by 60 minutes after localized delivery. Curiously, the decrease in ceramide mass was associated with an increase in gangliosides and cerebrosides but not sphingosines; phosphorylated sphingosine is a biologically active metabolite associated with prothrombogenic effects.

Whether this short half-life of ceramide is enough to sustain the wave of cellular events in the presence of an injured artery with atherosclerotic disease is debatable. Moreover, the ceramide content of atherosclerotic lesions is markedly elevated relative to normal arteries, in part from the LDL content. The ceramide concentration of lesional LDL is increased 10- to 50-fold compared with plasma LDL. From these data, one would assume that the ceramide levels would be greater in a lipid-rich plaque relative to a more fibrous lesion. This poses the question of whether localized delivery of exogenous ceramide to a plaque abundant in natural ceramide would have any additional effects. Finally, localized delivery of radiolabeled ceramide was observed throughout all layers of the arterial media. It remains to be determined how well exogenous ceramide can penetrate a well-collagenized atherosclerotic plaque.

The model of rabbit balloon injury of normal vessels used by Charles et al produces a neointima consisting of a relatively homogeneous population of smooth muscle cells. The effects of ceramide in a restenosis model with underlying atherosclerotic disease need to be explored. The ceramide-induced decrease in cell proliferation was measured using an antibody directed against proliferating cell nuclear antigen (PCNA). It is recognized that PCNA is a protein that is required for both DNA replication and DNA repair; positive PCNA staining may have represented cellular injury. PCNA is also upregulated during apoptosis, because DNA strand breaks induce an ultimately futile DNA repair process. This effect is probably of little importance in the carotid stretch-injury model, because an increase in apoptosis was not associated with either control or ceramide-treated animals.

**Ceramide and Intracellular Signaling**

The diversity of ceramide as a messenger of cell signaling is well established. Not only can ceramide alone serve as a second messenger in response to a variety of stimuli, but it
may also be converted to other structural or effector molecules. Obviously, ceramide signaling is a nascent area of vascular research, and many of its actions need to be identified. In the article by Charles et al,\textsuperscript{1} ceramide is a potent inhibitor of PKB/Akt and ERK signaling in vascular smooth muscle. Inhibition of PKB/Akt by ceramide has been recently shown to be mediated by dephosphorylation of serine 473.\textsuperscript{34} Moreover, an apparent crosstalk between both PKB/Ark and ERK pathways has also been recognized.\textsuperscript{35} Other pathways of ceramide signaling may involve inhibition of Ca\textsuperscript{2+}-activated K\textsuperscript+ channels or protein kinase C, both promoters of vasodilation. Ceramide can also mimic the effects of certain cytokines, such as tumor necrosis factor-\alpha, causing activation of other regulatory mediators, including nuclear factor-kB, activator protein-1, and c-Jun N-terminal kinase.\textsuperscript{36} Finally, the cytotoxicity associated with ceramide has been linked to its effects on mitochondrial membrane permeability and downstream activation of caspases.\textsuperscript{37}

Summary
How a structurally simple molecule like ceramide is able to mediate so many different, and sometimes paradoxical, physiological responses ranging from cell proliferation and differentiation to inhibition of cell growth and apoptosis is unknown. Moreover, crosstalk between ceramide-induced signal transduction cascades and other signaling pathways will add to the inherent difficulty in distinguishing the specific effects of ceramide in vascular biology. Although the study by Charles et al\textsuperscript{1} shows promise, this is the first step toward our understanding of the actions of ceramide on restenosis in vivo. Clearly, some of the vascular targets of ceramide could be clarified through studies using animals harboring disrupted genes of sphingolipid metabolism. More extensive research on the interaction of ceramide with specific cell types, especially in more complex models of restenosis using stents, is needed. More importantly, a clearer understanding of how vessels renarrow, particularly with stents, may help decipher the signaling pathways that promote restenosis; only then can the development of new therapeutic strategies be clinically effective.

References


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