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Molecular Atherectomy for Restenosis

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Abstract

Receptors for basic (b) and acidic (a) fibroblast growth factor (FGF) are upregulated in activated smooth muscle cells. These cells, which proliferate in response to bFGF, can thus be killed by a conjugate of bFGF and the ribosome-inactivating enzyme, saporin (which, by itself, does not enter the cells). Quiescent smooth muscle cells and other cells that have few FGF receptors are not killed. In vivo, bFGF–saporin transiently inhibits smooth muscle cell proliferation and neointimal accumulation after balloon injury to the rat carotid artery. Delivery of saporin, diagnostic imaging agents, or antisense oligodeoxynucleotides might be made even more selective by linking these substances to antibodies against the extracellular domains of the putative FGF receptor isoform specific for activated smooth muscle cells.

Restenosis after percutaneous transluminal coronary angioplasty (PTCA) has been minimally inhibited by calcium antagonists, nitrates, angiotensin-converting enzyme inhibitors, fish oils, aspirin, ciprostone, thromboxane and serotonin antagonists, heparin, dipyridamole, lovastatin, and warfarin, in single and multidrug regimens, though some trials have lacked statistical power or adequate dosing regimens (Popma et al. 1991, Hermans et al. 1991, Califf and Willerson 1992). The virtual failure of so many logical therapies and the difficulty in predicting which patients will develop restenosis (Weintraub et al. 1993, Kuntz et al. 1993, Bobbio et al. 1991) suggest that the mechanisms of restenosis are not well understood.

Insights into the pathogenesis of restenosis can be sought from its histology. Samples obtained by atherectomy performed 2–6 months after PTCA reveal maturing scar, with foci of activated and quiescent smooth muscle cells (SMCs), and little thrombus or lipid (Waller et al. 1991, Gordon et al. 1990, Potkin and Roberts 1988, Correa et al. 1991, Nobuyoshi et al. 1991). Unfortunately, there is almost no available histology from the first hours to days after angioplasty, and even this is more relevant to the pathogenesis of early fatality than of restenosis. Yet it is precisely during this time that many of the crucial events are thought to occur. As inferred from animal models, the sequence of SMC division, migration, and matrix synthesis is initiated by (1) removal of much of the endothelium and associated basal

lamina, which function as semipermeable selective barriers to plasma mitogens and which, furthermore, contain heparin, transforming growth factor β (TGF β), and other growth inhibitors; (2) rupture of the internal elastic laminae, which exposes SMCs to serum factors and monocytes; (3) exposure of thrombogenic factors such as subendothelial collagens, lipids, tissue factor, and macrophages; (4) stretching of SMCs, thereby directly activating ion channels and proto-oncogenes; (5) separation of adjacent SMCs, thereby disrupting contact inhibition, as well as bathing SMCs in serum mitogens; (6) release of mitogens from ruptured endothelial cells and SMCs; (7) release of chemoattractants from monocytes and expression of intercellular adhesion molecules for monocytes; and (8) stimulation of SMCs (and regenerating endothelial cells) to synthesize and release their own growth factors (Schwartz et al. 1990b, Casscells 1992).

It has not yet been possible to corroborate all of these findings in human tissue, but the descriptions of a few specimens obtained within the first few days after PTCA are broadly consistent with features common to the various animal models (Waller et al. 1991, Farb et al. 1990, Correa et al. 1991, Nobuyoshi et al. 1991). In brief, there are enough similarities with the animal models to justify their continued use, despite the fact that several experimental therapies effective in rat or rabbit models have not shown benefit in pig and baboon models, or in randomized clinical trials.

Clearly, vascular injury is a multifaceted stimulus, but we do not yet know the final common pathways that trigger cell proliferation or cell migration. In vitro, more than a dozen factors in plasma, platelets, endothelial cells, SMCs, and macrophages can each stimulate the proliferation of SMCs (Willerson et al. 1991, Ross 1993, Dzau et al. 1993, Casscells 1991a), and new factors are reported every few months (Gressens et al. 1993, Gadeau et al. 1993, Grove et al. 1993, Shing et al. 1993). In many cases, these factors also upregulate other growth factors and their receptors (Hu et al. 1992, Nicholson and Hajjar 1992, Flaumenhaft et al. 1992, Hajjar and Pomerantz 1992, Schollmann et al. 1992, Stiko-Rahm et al. 1992, Itoh et al. 1993). Thus, it is reasonable to expect some redundancy in this system. Indeed, some “growth” factors (which also act to influence cell differentiation and survival during embryogenesis) (Nathan and Sporn 1991) have been knocked out by homologous recombination with little or no effect on development (Erickson 1993). Our own observations in cultured SMCs suggest that antibodies capable of neutralizing basic fibroblast growth factor (bFGF), a known mitogen for SMCs, have very little effect on SMC proliferation (Casscells et al. 1993). In other words, neutralizing extracellular bFGF (from SMCs) inhibited cell growth only in low serum conditions. Antibodies to bFGF transiently inhibit DNA synthesis in medial SMCs after balloon injury of the rat carotid artery (Lindner and Reidy 1991), and they diminish the stimulation of bFGF expression and migration of cultured SMCs by platelet-derived growth factor (PDGF) (Sato et al. 1991). Antibodies to PDGF have little effect on DNA synthesis after balloon injury, yet inhibit neointimal accumulation, perhaps by inhibiting migration of SMCs (Ferns et al. 1991). Antibodies to insulin-like growth factor I, which is upregulated by balloon injury (Khorsandi et al. 1992), inhibit DNA synthesis by cultured SMCs (Clemmons 1984).

Since there are so many growth factors, including such “honorary” growth factors as epinephrine, high-density lipoprotein, fibronectin, serotonin, thromboxane A₂, angiotensin

II, endothelin, and lipoprotein (a) [for a review, see Casscells (1991a)], it is reasonable to focus on the smaller number of signal pathways. For example, inhibition of tyrosine phosphorylation inhibits proliferation of cultured SMCs (Bilder et al. 1991). Activation of *ras* appears to be critical for cell migration in some cells (Boyer and Thiery 1993, Sosnowski et al. 1993). Another likely growth-regulatory step is phosphorylation of mitogen-activated protein (MAP) kinase (Lange-Carter et al. 1993).

Since growth factors can activate more than one signal pathway (and in some cases, one path has been interrupted without a major inhibition of proliferation), some have sought to inhibit the nuclear events triggered by growth factor stimulation: expression of “immediate-early” and cell-cycle genes, and phosphorylation of their gene products. For example, antisense oligonucleotides to *c-myc*, *c-myb*, *c-fos*, or cyclin A all inhibit SMC proliferation in vitro (Biro et al. 1993, Speir and Epstein 1992). Inhibition of neointimal formation after balloon injury to the rat carotid artery has been achieved with antisense oligonucleotides to *c-myb* (Simons et al. 1992) and to cyclin A (V.J. Dzau personal communication 1993). Thus, the redundancy in the system is not complete. However, these treatments inhibit SMCs only partially and, in some cases, transiently. An alternative approach is to use natural inhibitors such as heparin, TGF β , tissue necrosis factor α , interferon γ (IF γ), atrial natriuretic peptides, and nitric oxide, but these efforts likewise have yielded only partial growth inhibition [for reviews, see Casscells (1991a), Appel (1992), and Scott-Burden and Vanhoutte (1993)]. Other possibilities, such as stimulation of p53 or Rb, have yet to be tried, but there is no evidence that deficiency of these recessive antioncogenes is implicated in restenosis.

While a great deal of research has focused on discovering the Achilles heel in SMC proliferation, other laboratories have assumed that it will take some time to identify the ideal target and to develop agents that can be delivered in a practical way for periods long enough to prevent restenosis. An alternative approach is to kill the activated SMCs, ideally without injuring or otherwise activating the quiescent cells. For example, several groups have used hematoporphyrin or psoralen derivatives, which are taken up by proliferating cells and which sensitize these cells to the lethal effects of light (March et al. 1993). Our laboratory and several others (Pickering et al. 1993, Epstein et al. 1991, Biro et al. 1992) have been pursuing another approach: targeting receptors upregulated by balloon injury, as described below.

bFGF is one of at least eight members of a multigene family of heparin-binding polypeptides (Baird and Böhlen 1990, Burgess and Maciag 1989). In vitro, bFGF enhances the proliferation and survival of dozens of cell types, including endothelial cells and SMCs (Gospodarowicz 1989). It also influences cell migration, matrix production [decreasing collagen synthesis (Tan et al. 1993) and increasing tPA (Moscatelli et al. 1986)], and hormone production and differentiation (Baird and Böhlen 1990). When administered in vivo, it can trigger angiogenesis in some models (Folkman and Klagsbrun 1987), prevent death of some injured neurons and, following vascular injury, enhance the proliferation of both endothelial cells and SMCs (Lindner et al. 1990). Which of these functions are regulated by bFGF in vivo is not yet clear. Gene knockout studies with bFGF have yet to be reported, and there are few descriptive studies of the expression of bFGF in development and in pathologic situations (Fu et al. 1991, Gonzalez et al. 1992, Olson et al. 1992). bFGF

mRNA and peptide are stored in the ovum, and then reexpressed during induction of the mesoderm and subsequently during specific morphogenetic stages (Moon and Christian 1993, Fu et al. 1991). The bFGF mRNA is then found at low levels in adult tissues with the exception of certain loci such as the hippocampus (Baird and Böhlen 1990).

The study of bFGF is made more interesting and complex by some unusual features, including the fact that at least three peptides of 18–23 kD are synthesized as primary translation products from one mRNA transcript (Florkiewicz and Sommer 1989). The largest of these products are translocated into the nucleus, where they are associated with the chromatin and nucleoli (Renko et al. 1990, Yu et al. 1993). Smaller FGF peptides are exported from the cell by some mechanism yet unknown (since they lack a signal peptide) (Abraham et al. 1986) and apparently stored in the extracellular matrix, from which they can be released—at least in tissue culture—by heparin, heparinase, plasmin, or an extracellular phospholipase C (Flaumenhaft and Rifkin 1992). FGF can also be released by physical injury (McNeil et al. 1989). Another interesting feature is that bFGF appears to be phosphorylated in vivo by an ectokinase (Vilgrain and Baird 1991). Thus, it is likely that there is significant posttranscriptional and posttranslational regulation of bFGF in vivo.

Most laboratories have found bFGF to be mitogenic for cultured vascular SMCs, although there are some exceptions, which may reflect differences between SMC lines (as SMCs display heterogeneity in many properties) (Schwartz et al. 1990a, Casscells 1991a) or in culture conditions. For example, we generally find that most isolates grow at maximal rates in 10% or 15% high-quality serum, so that the addition of bFGF—which is not found in serum—nevertheless exerts no additive effect on growth. In contrast, in 1%–5% serum, the additive effect of bFGF on growth can be striking. We (Speir et al. 1991) and others (Gospodarowicz et al. 1988, Weich et al. 1990, Winkles et al. 1987, Mansson et al. 1990) have found that bFGF is expressed by bovine, rat, and human vascular SMCs. In vivo, the mRNA levels are low, as judged by in situ hybridization, but the peptide is relatively abundant. When these cells are cultured, the mRNA transcripts are easily detected by in situ hybridization, and the amounts of the peptide increase sevenfold, suggesting an upregulation at some level (Speir et al. 1991).

With balloon injury, we find some initial loss in bFGF immunoreactivity, but, by 24 h after injury, there is an increase in bFGF immunoreactivity in proliferating SMCs, accompanied by an increase in 18- to 24-kD bFGF detectable by heparin affinity chromatography and immunoblotting (Casscells 1991b). This result contrasts with that of Reidy and colleagues (Olson et al. 1992), but the reasons for the discrepancy are not yet apparent. Miano et al. (1993) have found an increase in bFGF mRNA and FGF-receptor 1 (FGF-R1) mRNA 2–12 h after balloon injury of the rat carotid. Lindner et al. (1990 and 1991) found that infusions of bFGF could enhance both endothelial cell and SMC proliferation after balloon injury, a finding that we have replicated [Casscells et al. (1993); see also Bjornisson et al. (1991)].

Thus, it was logical to ask whether antibodies to bFGF can inhibit SMC proliferation after balloon injury. As noted above, we found that antibodies that could block the mitogenic effect of exogenous bFGF in the presence of 1%–5% serum had almost no effect in the presence of 10% serum, which contains dozens of mitogens. Lindner and Reidy (1991) had

similar results in 3T3 fibroblasts, but they, unlike us, found that antibodies to bFGF could inhibit the DNA synthesis normally observed in medial SMCs after balloon injury in the rat model. Interestingly, the eventual degree of neointimal formation was not altered.

Because of these concerns about the degree of redundancy in the systems controlling SMC proliferation, we have been looking for different approaches. One such approach was suggested by our finding—recently extended by Miano et al. (1993)—that balloon injury helps regulate some type of FGF receptor on SMCs.

Recent studies had yielded conflicting data about the presence of FGF receptor mRNA transcripts in adult tissues. There are at least four FGF receptor genes characterized by extracellular Ig-like loops and an intracellular tyrosine kinase (Johnson et al. 1991, Partanen et al. 1992). Moreover, there are many alternatively spliced transcripts, some of which encode secreted receptor sequences (Xu et al. 1992). Another complicating element is the recent identification of a non-tyrosine-kinase FGF receptor (Burrus et al. 1992) as well as membrane-bound heparan-sulfate proteoglycans (Bernfield et al. 1992) that have recently been shown to be required for high-affinity bFGF binding (Yayon et al. 1991). Finally, FGF binding appears to be subject to posttranslational regulation (Savona and Feige 1991). For these reasons, mRNA analyses at present may not yield physiologically understandable data. Consequently we have focused on bFGF receptor protein and binding assays (Casscells et al. 1992). In vivo autoradiographic experiments showed only low-affinity, heparin-displaceable binding of ^{125}I -labeled bFGF in normal vessels (Figure 1c). We were not able to identify high-affinity FGF receptors in normal vessel homogenates by Scatchard analysis, crosslinking, or western blotting (Figure 1a). Moreover, we have functional evidence of the absence or near absence of bFGF receptors from normal vessels: when bFGF was injected in large doses over 1–3 days, no increase in DNA synthesis rates of any cardiovascular tissues was seen with ^3H thymidine autoradiography or by liquid scintillation counting of tissue digests, confirming the recent results of Whalen et al. (1989). In contrast, when we gave repeated subcutaneous injections of bFGF, to injure the tissue and upregulate FGF receptors, the incorporation of thymidine increased over that in sites repeatedly injected with saline (Figure 1b). Similarly, when we gave bFGF intravenously for 3 days to rats subjected 5 days earlier to left carotid balloon injury, neointimal growth was accelerated only in the injured left carotid and not in the uninjured right carotid. No stimulation was seen if the animals were injected with heat-denatured bFGF or saline (Casscells et al. unpublished).

These data may reflect (a) balloon-induced loss of endothelium, permitting direct access of bFGF to medial SMC; (b) downregulation by injury of some growth inhibitory mechanism(s); or (c) expression of bFGF receptors in response to injury. In fact, upregulation of FGF receptors is supported by several types of data. First, radioligand-binding studies in vessel homogenates revealed that balloon injury stimulated the appearance of high-affinity bFGF binding sites (Figure 1a). Moreover, autoradiograms of histologic sections revealed high-affinity binding of bFGF (that is, not displaced by heparin) to neointimal and medial SMCs of injured vessels but not to quiescent smooth muscle of normal vessels (Figure 1c). Similarly, immunoblotting with an antibody raised against a sequence deduced from the mouse *flg* (FGF-R1) gene demonstrated characteristic bands in injured vessels but none in normal vessels (Figure 1a, insert). Likewise, using these

antibodies in immunohistology (Figure 1d), we found few cells in normal vessels to be immunoreactive, but most proliferating mesenchymal cells were immunoreactive for FGF-R1 (and for smooth muscle α actin). Thus, injuring, like culturing, induces expression of FGF receptors (Casscells et al. 1992).

While a great deal more needs to be learned about which FGF receptors are upregulated (and when and how they are upregulated) following balloon injury, we are already trying to take advantage of these observations for therapeutic purposes. We reasoned that bFGF might serve as a Trojan horse to carry a toxin into the proliferating SMCs. We developed a cytotoxic conjugate of bFGF with saporin, a plant enzyme that inactivates ribosomes by cleaving adenine from ribose in the 28 S RNA of the 60 S subunit (Lappi et al. 1991). It seemed likely that bFGF would be good not only at facilitating entry of saporin into cells, but also at giving saporin access to ribosomes: the unusually slow degradation rate of bFGF and its transit to the nucleus suggest that much of it escapes lysosomal degradation.

In 10% serum, 10 nM bFGF-saporin inhibited protein synthesis by 14 h and DNA synthesis by 24 h (Figure 2a). Cell death began by 24–48 h. This process was irreversibly initiated by 1 h of exposure to bFGF-saporin. These effects were not seen in the presence of 400-fold excess bFGF, and saporin alone inhibited cell growth only at concentrations 500-fold greater than those required for bFGF-saporin (Figure 2b).

By 96 h after a single exposure to 10 nM bFGF-saporin, only 10% of cells were viable (Figure 2c). The survival of 10% of the cells may be due to failure of bFGF-saporin to bind or enter the cells or to resistance to saporin at an intracellular site. Some SMC clones may have few or no FGF receptors. Interestingly, in postconfluent cultures, bFGF-saporin has almost no effect, consistent with their low density of FGF receptors at confluence (Figure 3).

We also found that the initial response to low (0.1–1.0 nM) concentrations of bFGF-saporin was an increase in protein and DNA synthesis; at doses of 1 nM, the increase eventually declined at 48–72 h. This initial stimulation was best seen in the presence of 1% serum, which revealed the mitogenic capacity of bFGF (Figure 2b). Presumably bFGF exerts its effect sooner and at a lower concentration than saporin. Little initial stimulation was detected in 10% serum with 1 or 10 nM bFGF-saporin. In 0.1 nM bFGF-saporin, [3 H]leucine incorporation was reduced by 33% with no change in cell number, suggesting that bFGF-saporin can exert a cytostatic effect at subcytotoxic concentrations.

Another intriguing finding is that endothelial cells are relatively resistant to the effects of bFGF-saporin. We have repeatedly found that aortic endothelial cells are stimulated by concentrations of bFGF-saporin that kill aortic SMCs from the same animals; the endothelial cells were killed only at 50 times higher concentrations (Biro et al. 1991). Recent data suggest some possible mechanisms for these findings. It is well known that 10% serum, which does not contain FGFs (Zimering et al. 1990, Casscells et al. 1990), is not an optimal growth medium for most endothelial cells, which require bFGF or another endothelial growth factor to achieve optimal growth rates. A growth medium of 10% or 15% serum is usually optimal, however, for SMCs. Serum does contain a number of components that usually inhibit endothelial cell proliferation, but enhance SMC proliferation, including

TGF β , IF γ , serotonin, angiotensin II, and others (Casscells 1991a). Hence, there is stimulation of endothelial cells by FGF or by low concentrations of bFGF-saporin in the presence of 10% serum. Stimulation of SMCs by bFGF-saporin is only apparent in growth media that have low percentages of serum.

The resistance of the endothelial cells to the lethal effects of bFGF-saporin may be explained on the basis of their four times lower (than SMCs) density of cell surface FGF receptors (Biro et al. 1991). Not only must toxins be internalized, they also must escape the lysosomes (and lysosomal degradation) if they are to reach the ribosomes in the cytoplasm. Endothelial cells, with only a few thousand receptors per cell, may not take up enough bFGF-saporin to saturate the degradative processes; therefore no toxin molecules ever reach the ribosomes. Endothelial cells may also have better detoxifying mechanisms. At any rate, the different responses suggest that bFGF-saporin could be used in vivo at doses that simultaneously enhance the proliferation of endothelial cells and kill SMCs. Indeed, in the rat carotid artery model, FGF-saporin given intravenously kills activated SMCs but not quiescent SMCs (Figure 4) or endothelial cells (Biro et al. 1991). The optimal dose and the dosing regimen of bFGF-saporin have not yet been determined. These exciting results did not persist: animals studied at 28 days after injury and treatment did not show the reduction in neointima seen when tissues were harvested at 7–10 days. It remains to be determined whether such catch-up proliferation can be avoided by using dosing regimens that completely eliminate all activated cells (so that no remaining cells are responsive to the mitogens released by dying cells).

Recently, we have been trying to determine which components of balloon injury are responsible for the upregulation of FGF receptors. Among the possibilities are stretch, inflammatory cytokines, platelet products, and loss of endothelial growth inhibitors. When we made aortic SMCs quiescent by depriving them of serum and then added fresh serum, by 6 h there was a dramatic increase in the abundance of mRNA transcripts for bFGF and FGF-R1. This result suggests a novel way in which thrombosis may interact with oncogenes in the vessel wall.

We have also been trying to determine which type of FGF receptor is upregulated by balloon injury to SMCs. This is a complex task, in light of the varied types of FGF receptors described above. Moreover, FGF-R1 (*flg*) can be alternatively spliced, thereby giving rise to multiple isoforms, for example, as many as 100 isoforms in hepatoma cells (Hou et al. 1991). Splices in the third immunoglobulin domain confer some ligand specificity for bFGF compared with acidic FGF, and vice versa. However, most of the known FGF receptors bind many or most of the ligands of the eight-member FGF gene family. Thus, we remain unsure of the purpose for the many isoforms of the FGF receptors.

One possibility is suggested by recent findings by our laboratory and others, namely, that the individual isoforms of the FGF receptors are expressed by particular cell types at specific times (Patstone et al. 1993, Xu et al. 1992, Peters et al. 1993). It is thus possible that coronary SMCs, proliferating in response to balloon injury, express a unique isoform of FGF receptor. If such an isoform exists in the extracellular domain, a monoclonal antibody could presumably be raised to target these cells in vivo. For example, such an antibody could be

used to target a radionuclide for diagnostic imaging to learn whether FGF receptor expression after balloon injury predicts restenosis.

These antibodies are currently being raised in our laboratory. They are being screened for specificity for proliferating human SMCs, and then screened to determine whether they are internalized by the cells, in which case they could be used for intracellular delivery of anti-sense oligonucleotides and toxins. Meanwhile, the potential of the current recombinant bFGF-saporin is being pursued in animal models. Even if a narrow therapeutic-toxic ratio requires that therapy be local, the conjugate or the recombinant fusion protein should have the attractive feature of stimulating endothelial cells while killing SMCs.

The prospects for such a therapy are in fact more promising than those for immunotoxin and mitotoxin therapy for most solid tumors (Fitzgerald and Pastan 1989), for the following reasons: solid tumors are poorly vascularized in the center (Folkman and Klagsbrun 1987), and the drugs simply do not reach the center of the tumor, particularly because the drugs must leave the vessels to get to the tumor cells (Dvorak et al. 1991). In contrast, after balloon injury, the endothelium is largely gone, which leaves no barrier between the blood (where the bFGF-saporin is at highest concentration) and the SMCs, whose rate of proliferation is highest in the layer contacting the bloodstream. The final reason is that SMCs themselves are a more realistic target because they are not malignant. Their growth does stop after about three cycles, and they do not have the genetic instability or the cell surface diversity of tumor cells. Thus, there is less chance of a receptor-negative clone taking over. In immunotoxin therapy for cancers, it is necessary to kill 99.99% of cells to have a clinically important effect (Vitetta and Thorpe 1992). In contrast, in restenosis, it may only be necessary to kill ~90% of the activated SMCs to show clinical benefit.

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Figure 1.

FGF receptors are increased by balloon injury. **(a)** Detectable binding of basic fibroblast growth factor (bFGF) by membranes from balloon-injured adult arteries: *open bars*, total counts per minute (cpm) bound; *hatched*, nonspecific cpm; and *filled*, specific cpm. Membranes were prepared as described by Burrus et al. (1992). (*Inset*) Immunoblotting indicates an FGF receptor in injured (*lane 2*) but not in normal (*lane 1*) arteries. **(b)** Repeated injections of bFGF increase thymidine incorporation in injured tissue (A) but not in uninjured tissue (B). **(c)** In vivo autoradiographic evidence that FGF receptors are expressed after vascular injury. Adult rats were subjected to balloon dilation of the carotid artery and injected i.v. 2 days later with 60 ng of either mitogenically active or heat-denatured ¹²⁵I-labeled bFGF with or without 10 μg unlabeled bFGF, followed 1 h later by i.v. heparin (165 U/kg). (c1) Uninjured vessel causes almost no precipitation of silver grains, indicating little or no heparin-resistant (high affinity) binding. (c2) Binding of bFGF 48 h after balloon injury. (c3) At day 4, bFGF binds preferentially to cells in neointima (N). (c4) Competition by excess unlabeled bFGF (no grains over neointima). **(d)** Immunocytochemical localization of an FGF receptor after vascular injury. (d1) Rat aorta, 14 days after balloon injury, stained with a polyclonal antiserum (R129) raised against a synthetic peptide replicate (CSSGEDSVFSHEPLPEEP) of an intracellular domain deduced from FGF receptors 1 and 2 (Johnson et al. 1990). Many of the proliferating smooth muscle cells exhibit the brown immunoperoxidase reaction product. L, lumen. Methyl green counterstain, ×130. (d2) Injured segment of same rat stained with the polyclonal antibody

R131 (raised to the peptide RITGEEVEVRDR in loop of FGF-R1). (*d3 and d4*) *d1* and *d2* at higher magnification ($\times 790$). Brown (immunoperoxidase positive) smooth muscle cells are indicated by *arrowheads*. Monocyte (M) is immunoreactive but most erythrocytes (E) show only background staining. Normal segment shows little immunoreactivity of the medial layer with R131. The near absence of stain for the *flg* gene product is shown in *d5*. (*d6*) Normal rabbit serum is unreactive with injured vessels (or normal vessels, not shown). Adapted from Casscells et al. (1992), with permission.

Figure 2.

Effects of basic fibroblast growth factor (bFGF)–saporin on protein and DNA synthesis and cell number. **(a)** Time course of effect of various concentrations of bFGF–saporin on protein synthesis by cultured rat aortic smooth muscle cells (SMCs), eighth passage, plated at 30,000 cells/cm² in 10% fetal bovine serum (FBS) in leucine-free M199. At 20 h, cells received 0.01–10 nM bFGF–saporin. At 14, 18, or 24 h, cells were pulsed with [³H]leucine and counted by liquid scintillation. For clarity, means are shown without SD, which were 10% of means. Also omitted are the data with 10 and 100 nM saporin, which did not differ from controls. **(b)** Inhibition of DNA synthesis by bFGF–saporin, but not saporin or bFGF–saporin competed by bFGF, in rat aortic SMCs. Cells were plated in M199–1% FBS at 10,000/cm² and exposed to additives and [³H]thymidine at 24 h, for 24 h more. In 10% FBS (*top panel*), no initial enhancement of DNA synthesis was noted even at low doses of bFGF–saporin. In 1% FBS (*bottom panel*), however, there is an initial increase in DNA synthesis with low (but not high) concentrations of bFGF–saporin. DNA synthesis decreases

further by 48 and 72 h (not shown). (e) Killing of SMCs by bFGF-saporin. Cells were plated at 10,000/cm² in M199–1% FBS for 24 h and then exposed to the indicated concentrations of bFGF-saporin, saporin, or bFGF for 72 h, at which time cells excluding trypan blue were counted. Adapted from Casscells et al. (1992), with permission.

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Figure 3.

Quiescent smooth muscle cells (SMCs) are not killed by basic fibroblast growth factor (bFGF)–saporin. **(Left)** Subconfluent ($2 \times 10^4/\text{cm}^2$) P₁₂ rat aortic SMCs in 10% fetal bovine serum (FBS) were exposed to indicated concentrations of saporin or bFGF–saporin for 1 h and then washed for 10 min with 10 $\mu\text{g}/\text{mL}$ heparin to remove proteoglycan-bound bFGF–saporin, refed 10% FBS–M199, and trypsinized and counted 96 h later. **(Right)** Confluent ($2 \times 10^5/\text{cm}^2$) cells were made more quiescent by 72 h in serum-free M199 and then exposed to bFGF–saporin (*open circles*) or saporin (*solid circles*), washed, refed, and counted as above.

Figure 4.

After arterial injury, basic fibroblast growth factor (bFGF)–saporin kills proliferating smooth muscle cells (SMCs) and inhibits neointimal accumulation. **(a)** Twenty-four hours after balloon injury, the carotid artery was occluded by proximal and distal ties and 10 μ g of bFGF–saporin in 20 μ l saline was instilled for 15 min followed by release of the ligatures, and the animals were killed 11 days later. In **a**, the medial (M) SMCs are lysed, and the layers of elastica are collapsed. The lumen is largely occluded by organizing thrombus (T). Some hemorrhage and inflammatory cells are noted in the adventitia (A). H&E, $\times 186$. Saporin alone was nontoxic (not shown) and bFGF–saporin was not toxic to the uninjured right carotid of a rat subjected to left carotid balloon injury and bFGF–saporin 75 μ g/kg i.v. **(b)** The intimal endothelium (I) and medial (M) SMCs appear normal and no cells are synthesizing DNA, as illustrated by the absence of silver grains (*small arrowhead*) over the cell nuclei. The inset (**i**) illustrates an S-phase SMC from a ballooned artery treated with saline H&E, $\times 744$. **(c)** The dramatic inhibition by bFGF–saporin (75 μ g/kg i.v. at 24, 48, 72 h after balloon injury) of neointimal SMC accumulation (*small arrowheads*): 83% less than in animals injected with 40 μ g/kg (equimolar) saporin **(d)** or saline, as determined by Zeiss Videoplan 2 planimetry of neointimal areas normalized to medial areas. All samples were coded for “blinded” morphometry. By two-tailed unpaired *t*-test, $p = 0.004$. However, toxicity was indicated by an 8% weight loss and by a few areas of medial SMC lysis (*large arrowheads* in **c**). Mortality (including perioperative) was 5% in both the saporin and the FGF–saporin groups. For this reason, lower doses were tested, which produced the results

graphed in **e**: effects of lower doses of bFGF–saporin on injured arteries. (A) The numbers of intimal and medial cells from the central (nonendothelialized) segments of carotid artery from rats treated 24 h after balloon injury with a 15-min local application of 1 µg bFGF–saporin (bF-S) or saline (C), followed by killing of the animals 10 days later. The samples were coded for “blind” counting by two observers with intra- and interrater reproducibilities of 92%. (B) Rats were given a single dose of 75 µg/kg bFGF–saporin or 40 µg/kg i.v. saporin (an equimolar dose) 24 h after carotid balloon injury and were killed 12 days later. Data are given as ratio of neointimal to medial surface areas. The *t*-test for unpaired samples was used. Adapted from Casscells et al. (1992), with permission.