Increased Expression of Macrophage Colony–Stimulating Factor After Coronary Artery Balloon Injury Is Inhibited by Intracoronary Brachytherapy

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Background—The mechanisms underlying the reduced neointimal proliferation (NP) by intracoronary brachytherapy (ICBT) are unknown. We hypothesized that ICBT inhibits NP by reducing expression of macrophage colony–stimulating factor (M-CSF).

Methods and Results—Thirty coronary arteries from 10 pigs were divided into 3 groups of 10 each: control (C), balloon injury (BI), and BI followed by ICBT (16 Gy at 0.5-mm tissue depth with a 32P balloon system). Pigs were killed at 24 hours (n = 3) and at 7 (n = 4) and 14 (n = 3) days. Expression of M-CSF was assessed by Western blot, ELISA, and quantitative immunostaining. Persistently increased levels of M-CSF after BI (to 1.4 ± 0.2 nmol/L [ELISA] and 29.4 ± 4.9% of cross-sectional area stained [immunocytochemistry]; P < 0.001 versus control for both) were observed in the injured arteries. Treatment of BI arteries with ICBT reduced M-CSF expression compared with BI alone (to 0.7 ± 0.1 nmol/L [ELISA] and 13.5 ± 2.9% of cross-sectional area stained [immunocytochemistry]; P < 0.001 versus BI and P = NS versus control for both) and remained similar to control M-CSF expression for the 14-day study period. Neointimal thickness increased after BI (to 4.8 ± 2.9 mm²; P < 0.001 versus control), but this was reduced by ICBT (1.4 ± 0.4 mm²; P < 0.001 versus BI).

Conclusions—In porcine coronary arteries, BI is associated with increased expression of M-CSF and NP, but neither occurs after ICBT. The beneficial effects of ICBT on NP involve inhibition of M-CSF expression. (Circulation. 2002; 105:2411-2415.)

Key Words: muscle, smooth ▪ radioisotopes ▪ cells ▪ restenosis ▪ proliferation

Even technically successful angioplasty and stenting invariably cause mechanical injury to the vessel wall. This trauma initiates a complex inflammatory healing response that involves the expression of growth factors and cytokines that promote cellular proliferation. The subsequent neointimal hyperplasia may be fairly localized and of little clinical significance but also may be exaggerated and result in compromised arterial flow, necessitating further intervention. Stenting reduces the risk of restenosis somewhat but nevertheless results in greater neointimal proliferation. Radiation therapy has been used successfully to inhibit proliferation in other pathological conditions, and intracoronary brachytherapy (ICBT) has therefore been developed in an attempt to limit neointimal proliferation after intracoronary intervention. ICBT delivers radiation locally to the arterial wall at the time of angioplasty or stenting by means of catheter-based delivery systems using γ or β irradiation sources, with demonstrated efficacy in reducing the incidence of restenosis. The mechanism by which ICBT inhibits restenosis is not fully understood, but it appears to involve reduced smooth muscle cell (SMC) proliferation and delayed healing responses to vascular injury. Macrophage colony–stimulating factor (M-CSF) is a multifunctional proinflammatory protein that regulates the differentiation, proliferation, and survival of mononuclear phagocytic lineage cells such as macrophages and SMCs. Increasing evidence has suggested an important role for M-CSF in de novo human atherosclerotic lesions and experimental animal models. In this study, we tested the hypotheses that coronary artery balloon injury (BI) results in augmented levels of M-CSF and that the mechanism by which ICBT reduces restenosis and neointimal proliferation involves reduction in the levels of M-CSF.
Methods

Experimental Model, Injury, and Irradiation Procedure

The porcine coronary injury model was used in the study. The Institutional Animal Care and Use Committee approved all procedures. Ten adult swine, 30 to 40 kg in weight and 4 months old (S & S Farms, Ranchita, Calif), received antiplatelet therapy with 325 mg aspirin q.d. and 75 mg clopidogrel q.d. commencing 3 days before the procedure. Animals were fasted for 12 hours before surgery and were immobilized with intramuscular acepromazine 0.5 mg/kg, ketamine 20 mg/kg, and atropine 0.05 mg/kg during the procedure to prevent intracoronary thrombosis, which is often caused by angioplasty in the porcine model. Anesthesia was induced with 5 to 8 mg/kg thiopental IV, followed by intubation and ventilation. Anesthesia was maintained with 1% to 2% isoflurane. Antiarrhythmic therapy was administered with bretylum as necessary. Coronary angiography was performed via a carotid artery approach, and heparin was administered (100 to 125 U/kg IV). Each of the 3 coronary arteries (total of 30 arteries) was randomly assigned to one of the following interventions: control, in which no intervention was performed (n = 10 arteries); oversized BI followed by sham ICBT (n = 10 arteries); and BI followed by ICBT (n = 10 arteries). For BI, a 3.5 × 18- or 4.0 × 18-mm balloon was inflated to 12 atm twice for 30 seconds each time, resulting in a 35% overstretch injury. For ICBT, a Radiance 32P balloon system was used to deliver a dose of 16 Gy at 0.5-mm depth (RDX Coronary Radiation Delivery System, a generous gift from Radiance Medical System Co, Irvine, Calif). The balloon material of this system has integrated radioactive solid-phase 32P, which is a pure β-emitter, with an Eβ of 1.71 MeV and a half-life of 14.28 days. The radiation balloon was deployed at the treatment site with low-pressure inflation. Pilot studies using this system have demonstrated that the isotope is not removable from the balloon under usual clinical conditions. Three pigs were killed at 24 hours, 4 pigs at 7 days, and 3 pigs at 14 days. Immediately after death, pig hearts were excised and coronary arteries isolated and flushed with ice-cold PBS. Treated sections were identified and cut perpendicular to their long axis into 2 halves. The first half was snap-frozen and stored at −70°C. The second half was fixed in 4% formalin/50 mmol/L BHT/0.2% EDTA overnight at room temperature and embedded in paraffin for immunohistochemistry. Tissue sections were deparaffinized before the immunostaining by subsequent washes (xylene, then ethanol 100%, 95%, and 80% diluted in distilled H2O).

Protein Extraction

Coronary arterial segments were cleaned of surrounding fat, snap-frozen in liquid nitrogen, and ground to powder. A lysis buffer was used for protein extraction. Protein concentration was determined by use of the Coomassie blue protein assay (Pierce).

Western Blot Analysis

A total of 50 μg of tissue homogenate from the coronary arteries was electrophoretically separated on 12% SDS-PAGE gels and incubated first with a polyclonal goat anti-human M-CSF antibody (Santa Cruz Biotechnology) and then with horseradish peroxidase conjugated anti-sheep antibody. Detection was performed with an enhanced chemiluminescence protocol (Amersham). Results were quantified by densitometry.

ELISA

This assay used a quantitative sandwich enzyme immunoassay kit (R&D Systems) to detect M-CSF antigen. A monoclonal antibody specific for M-CSF was precoated onto a microplate. M-CSF present in porcine arterial homogenates was allowed to bind to the immobilized antibody. After any unbound substances had been washed away, an enzyme-linked polyclonal antibody specific for M-CSF was added to the wells. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to develop color in proportion to the amount of M-CSF bound in the initial step.

Immunohistochemistry

Polyclonal goat anti-human M-CSF antibodies (Santa Cruz Biotechnology) were used for immunostaining of M-CSF. Mouse monoclonal antibodies were used for immunostaining of macrophages (CD18 68 KP-1, DAKO, 1:200) and SMCs (HHF-35, DAKO, 1:800). From each artery, 5 sections 10 μm thick at 50-μm intervals were stained by standard immunohistochemistry methods. For each of the above stains, slides were incubated with 5% normal goat serum for 30 minutes, and then primary antibody was applied overnight at +4°C. Control slides were incubated with a goat nonimmune IgG or PBS. The sections were incubated with the biotinylated secondary antibody for 30 minutes and then with avidin-biotin for 30 minutes. Sections were exposed to 3-amino-9-ethylcarbazole for color development and counterstained with hematoxylin. An observer blinded to treatment assignment assessed histological sections with computer-assisted morphometric software (Image Pro; MediaCybernetics), a Nikon E600 microscope, and a Nikon digital camera. For M-CSF, the percentage of positively stained area as a function of the total vessel wall area was determined by computer-assisted morphometry of the vessel wall. The same software was used to measure the neointimal area in the vessel wall.

Statistical Analysis

Data are presented as mean ± SD. Differences between groups were evaluated by ANOVA followed by paired group comparisons using the modified Bonferroni test. A value of P < 0.05 was considered to indicate a statistically significant difference.

Results

Western Blot Analysis

Western blots demonstrated constitutive production of M-CSF in control uninjured arteries (Figure 1). M-CSF protein levels were markedly increased in balloon-injured arteries at 7 days after BI. Treatment of balloon-injured arteries with ICBT sharply attenuated this increased M-CSF expression to levels that appeared qualitatively similar to those seen in uninjured control arteries. Significant results were noted at 24 hours and 14 days after injury (data not shown).

ELISA

ELISA results are summarized in the Table and in Figure 2. M-CSF expression in control arteries averaged 0.72 ± 0.10 nmol/L. At 24 hours after BI, M-CSF expression increased significantly to 1.55 ± 0.24 nmol/L (P < 0.01). Significantly elevated M-CSF levels persisted for the 14-day study period (1.29 ± 0.14 nmol/L at 7 days and 1.23 ± 0.16 nmol/L at 14

Figure 1. Western blot showing M-CSF protein expression in control arteries (C; n = 4), increased expression after BI without ICBT (BI; n = 4), and inhibition of this increase after treatment with ICBT (ICBT; n = 4). Bottom, α-Actin control. Results are derived from 12 arteries from 4 animals, with 4 arteries per condition.
M-CSF Expression by ELISA and Immunocytochemistry and Neointimal Thickness Measured by Computer-Assisted Histomorphometry

<table>
<thead>
<tr>
<th>Method of Measurement</th>
<th>Control</th>
<th>Balloon Injury</th>
<th>ICBT</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF ELISA, nmol/L</td>
<td>0.7±0.1</td>
<td>1.4±0.2*</td>
<td>0.7±0.1†‡</td>
</tr>
<tr>
<td>Immunocytochemistry, % stained area</td>
<td>10.3±3.4</td>
<td>29.4±4.9*</td>
<td>13.5±2.9†‡</td>
</tr>
<tr>
<td>Neointimal thickness, mm²</td>
<td>0.10±0.02</td>
<td>0.48±0.29*</td>
<td>0.14±0.04§</td>
</tr>
</tbody>
</table>

Data are pooled from all time points and expressed as mean±SD.

*All P values <0.001 vs control.
†All P values <0.001 vs balloon injury.
‡P=NS vs balloon control.
§P<0.02 vs control.

days; P<0.02 and P<0.01, respectively, compared with control uninjured arteries). In contrast, treatment of balloon-injured arteries with ICBT abolished the increase in M-CSF levels observed after BI alone. At 24 hours, M-CSF levels in balloon-injured arteries treated with ICBT averaged 0.79±0.05 nmol/L (P<0.01 compared with BI alone), which was not significantly different from control arteries. The inhibitory effects of ICBT on M-CSF levels persisted for the duration of the study (0.67±0.12 nmol/L at 7 days after injury and after ICBT; 0.77±0.15 nmol/L at 14 days; P<0.03 and P<0.01, respectively, compared with BI alone; P=NS compared with control uninjured arteries for both time points). Thus, by 24 hours after arterial BI, M-CSF levels increased significantly and remained elevated, but ICBT completely prevented this increase and maintained M-CSF expression at levels comparable to those observed in control uninjured arteries.

Immunohistochemistry

Immunohistochemistry corroborated the findings from ELISA (Figure 3, A and B, and the Table). The M-CSF immunoreactive area in control uninjured arteries was 10.3±3.4%. By 24 hours after BI alone, the M-CSF immunoreactive area in arteries increased significantly to 31.6±5.4% (P<0.0001 compared with control arteries). As was the case with arteries assessed with ELISA, the M-CSF immunoreactive area remained persistently and significantly elevated compared with control arteries for the entire duration of the study. The M-CSF immunoreactive area was 28.5±4.4% at 7 days and 28.4±6.4% at 14 days (P<0.001 and P<0.02, respectively, compared with control uninjured arteries).

ICBT inhibited the increase in M-CSF immunoreactivity after BI. At 24 hours after injury, the M-CSF immunoreactive area in ICBT-treated arteries was 14.5±3.1% (P<0.02 compared with balloon-injured arteries; P=NS compared with control uninjured arteries). The inhibitory effect of ICBT on M-CSF immunoreactivity persisted for the duration of the study (13.4±3.4% at 7 days and 12.9±2.9% at 14 days; P<0.003 and P<0.04, respectively, compared with balloon-injured arteries). M-CSF immunoreactive area in balloon-injured arteries treated with ICBT did not differ significantly from control uninjured arteries at all time points measured and also did not vary significantly from one another. Thus, ICBT treatment of balloon-injured arteries both stably and persistently inhibited M-CSF immunoreactivity to levels similar to control.

Neointimal Area

As shown in Figure 4, A and B, and the Table, neointimal area in control arteries averaged 0.104±0.022 mm². After BI, neointimal area increased 5- to 8-fold by 14 days (0.157±0.012 mm² at 24 hours, 0.469±0.075 mm² at 7 days, and 0.826±0.075 mm² at 14 days; P=NS, P<0.02, and P<0.007, respectively). Neointimal area in balloon-injured arteries treated with ICBT was significantly smaller than in control arteries (0.108±0.028 mm² at 7 days and 0.18±0.02 mm² at 14 days; P<0.02 and P<0.02, respectively, compared with BI alone). Thus, ICBT resulted in little increase of neointimal area after BI compared with control.

Discussion

The present study shows that uninjured porcine coronary arteries constitutively produce immunoreactive M-CSF protein, which is significantly increased after BI. In contrast, ICBT after BI drastically attenuated this response concomitantly with reduced neointimal proliferation. Our findings support the hypothesis that reduced neointimal thickening after ICBT may result, in part, from a reduction of M-CSF levels.

Several lines of evidence support a central role for M-CSF expression in atherosclerosis and perhaps also in intimal proliferation after arterial injury. First, M-CSF functions as a growth factor for SMCs and cells of the mononuclear phagocytic lineage and is expressed by all major cell types in atherosclerotic lesions. Second, M-CSF expression is increased by oxidized lipoproteins and apoipoprotein E–null mutant mice form extensive atherosclerotic plaques, but this does not occur in the absence of M-CSF. Collectively, these findings, together with the results reported here, further suggest a central and early role for M-CSF in proliferative intimal hyperplasia after mechanical trauma to the arterial wall and suggest that therapeutic interventions that limit M-CSF expression might be useful in optimizing outcomes after percutaneous coronary intervention.
Our results seem most consistent with the concept that increased expression of M-CSF after injury is part of a general response to injury orchestrated by early growth-response genes such as Egr-1. The arterial biological response to the trauma caused by such arterial interventions as angioplasty is a complex interplay of cytokines, SMC migration and proliferation, extracellular matrix synthesis, and vascular remodeling. Recent studies have shown that recognition elements for Egr-1 appear in the promoters of all these genes and are present in the promoter.
of the M-CSF gene as well.23 It is therefore possible that Egr-1 commonly regulates the gene expression of a number of growth-promoting activities after injury, including M-CSF. It is possible that the suppression of increased M-CSF after ICBT may be the result of radiation-induced inhibition of Egr-1 expression after injury, but this hypothesis has not been directly tested.

Alternatively, it is possible that M-CSF deficiency exerts antiproliferative effects by decreasing postinjury inflammation. M-CSF is a proinflammatory cytokine necessary for the survival, differentiation, and proliferation of mononuclear phagocytes, such as monocytes and macrophages.32–34 Diminished expression of M-CSF could result in enhanced apoptosis of monocytes and macrophages, which would in turn be associated with attenuated local inflammation. It is also possible that M-CSF might not be directly affected by ICBT, and one or more of several possible indirect mechanisms could explain our findings. These include enhanced stability of the M-CSF cognate receptor (c-fms) mRNA and/or protein, or facilitation of downstream signaling after ligand binding to c-fms. The possibility that effects on upstream promoters and/or repressors of M-CSF gene transcription mediated the effects of ICBT on the M-CSF levels observed in our study also cannot be excluded. Further studies are necessary to discern which of these possibilities may be operative.

Acknowledgments

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References

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