Interferon-\(\gamma\) Inhibits Arterial Stenosis After Injury

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Background. Arterial injury initiates a proliferative response among the smooth muscle cells of the artery. This leads to the formation of a thickened intima that may reduce the diameter of the arterial lumen. Such intimal lesions often develop after vascular surgery and angioplasty procedures. Previous cell culture studies have shown that the lymphokine, interferon-\(\gamma\) (gIFN), inhibits smooth muscle cell proliferation.

Methods and Results. We therefore tested whether administration of exogenous gIFN could inhibit the development of intimal lesions. Rat carotid arteries were denuded with a balloon catheter, resulting in the formation of a standardized intimal lesion. The animals were then treated with recombinant rat gIFN at 200,000 units (approximately 400,000 units or 100 \(\mu\)g/kg body wt) administered parenterally once daily for 7 days. Autoradiographic analysis of \(^3\)H-thymidine incorporation revealed that gIFN reduced the early smooth muscle replication by approximately 75%. gIFN treatment for 1 week resulted in a 50% reduction of intimal cross-section area at 2 weeks after injury when compared to control rats injected with buffer alone. The difference in lesion development persisted in rats analyzed 10 weeks after injury, suggesting that proliferative events during the first week determine the long-term development of the intima. Inhibition of lesion development was accompanied by expression of the class II histocompatibility (Ia) gene, RT1B, suggesting that both were directly related to the administration of gIFN.

Conclusions. These results show that gIFN is a potent inhibitor of the formation of arterial proliferative lesions in vivo. It is possible that gIFN could be useful in preventing arterial stenosis after surgery and angioplasty in man. (Circulation 1991;84:1266-1272)

The development of arterial stenosis after mechanical injury is a major clinical problem in vascular surgery and cardiology. Injury to arterial tissue during angioplasty and reconstructive vascular surgery leads to the formation of intimal thickenings composed of proliferating vascular smooth muscle cells (SMC). In approximately one third of cases, such intimal thickenings will encroach upon the lumen sufficiently to cause new ischemic symptoms of the end-organ.1-3

The proliferation of SMC is controlled by specific growth factors and cytokines acting in paracrine networks.4-8 It was previously thought that the major growth regulating factors were released by platelets adhering to deendothelialized arterial surfaces, but inflammatory cells may be equally important as growth regulators for SMC.4-8

Macrophage products such as the platelet-derived growth factor and interleukin-1 promote SMC proliferation in cell culture4,5,9,10 and are expressed in the arterial wall during atherosclerosis and repair processes after experimental injury.11-13 T lymphocytes are also present in vascular tissue during atherosclerosis and vascular repair,6,8,14-17 but their role in vascular growth control in vivo has been unclear. The T lymphocyte product, interferon-\(\gamma\) (gIFN), inhibits SMC proliferation in culture18,19 and indirect evidence suggests that gIFN is secreted locally in the arterial intima during the vascular response to injury.18 Cells expressing interferon-induced genes were found to be quiescent, whereas nonresponding cells proliferated markedly.18 This suggests that gIFN is an endogenous growth inhibitor during the vascular response to injury.

We have now tested the effect of exogenous parenterally administered gIFN on the formation of an intimal thickening after balloon catheter injury in an experimental rat model. Our results indicate that gIFN treatment inhibits the formation of such intimal lesions.

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Methods

Animals

Five-month-old male Sprague-Dawley rats weighing 400–500 g were obtained from ALAB, Södertälje, Sweden. They were fed standard rat pellets and kept in the animal house for 2 weeks before use. No signs of infection or other diseases were observed. Animal care complied with the recommendations of the Swedish National Agricultural Board.

gIFN Treatment

Recombinant rat gIFN\(^ {20}\) was purchased from Holland Biotechnology, Leiden, The Netherlands. Lyophilized recombinant protein was dissolved in phosphate buffered saline (PBS), pH 8.2, with 0.5% heat-inactivated rat serum according to the manufacturer’s recommendations. Aliquots were stored at −70°C and thawed immediately before use. Because freezing and thawing were found to reduce gIFN activity, all aliquots used for injection were kept frozen and thawed only once before use. Rats were injected intraperitoneally with these preparations in volumes of 250–1,000 μl and controls were injected with equal volumes of PBS. The bioactivity of the recombinant protein was assessed by its capacity to induce RT1B expression in cultured rat aortic smooth muscle cells.\(^ {18}\)

Antibodies

OX19,\(^ {21}\) W3/13,\(^ {22}\) OX6,\(^ {23}\) and OX17\(^ {24}\) monoclonal mouse anti-rat antibodies (MAb) were obtained from Seralab, Crawley-Down, UK. Biotinylated F(ab')\(^ {2}\) fragments of goat-anti-mouse IgG was purchased from Amersham, Bucksh., UK.

Catheter Surgery

Arterial injury was inflicted in the common carotid artery with a Fogarty 2F balloon catheter as previously described.\(^ {16,18,25}\) Rats were anesthetized with a combination of fluanisone and fentanyl (Hypnorm\(^ {R}\), Janssen Pharma, Belgium) i.m. before surgery.

Morphology

Anesthetized rats were killed by perfusion with 4% paraformaldehyde in PBS, pH 7.4. The common carotid artery was removed, snap-frozen in liquid nitrogen, and sectioned in a cryostat. Three sections at 500-μm intervals were analyzed in each rat. The point-sampling method was used for morphometric quantitation of lesions in hematoxylin-eosin-stained sections.\(^ {26}\)

Cell Replication Analysis

\(^ {3}\)H-Thymidine (500 μCi/100 g body wt i.p.; Amersham) was administered over 24 hours as described previously.\(^ {18}\) Rats were killed as described above and cryostat sections of the carotid arteries dipped in autoradiographic emulsion. The autoradiograms were developed after 2 weeks of exposure and counterstained with hematoxylin. Two hundred cells were counted per section and corresponding areas were analyzed in sections from different rats. Cell replication rate was determined as \((^{3}\text{H}-\text{thymidine positive cells/all hematoxylin positive cells})\).

Immunohistochemistry

Frozen sections were fixed in methanol with 1% hydrogen peroxide and preincubated with 2% normal goat serum in PBS with 5% fat-free dry milk. They were incubated with the specific mouse-anti-rat monoclonal antibodies described above, at optimal dilutions in PBS/dry milk, rinsed in PBS, incubated with biotinylated goat anti-mouse-IgG, rinsed, incubated with peroxidase-conjugated streptavidin (Amersham), rinsed, incubated with 1 mM 3-amino-9-ethyl carbazole in 20 mM acetate buffer, pH 5.2,\(^ {27}\) rinsed, and counterstained with hematoxylin. Optimal concentrations of antibodies were determined by checkerboard titrations on rat spleen sections.

Statistical Analysis

Differences between means were analyzed using Student’s \(t\) test. Each experimental group was compared with a concomitant control group. Regression functions were estimated by the maximum likelihood method under the assumption that the intimal cross-section area was normally distributed. It was further assumed that the regression function was of the form, \(y=a+b/\log(x+1)\), where \(y=\text{intimal area}; x=\text{time after injury} \); and \(a\) and \(b\) are constants. Confidence bands at the 95% level were determined for the regression functions as well as for the difference between the functions. The difference represents the reduction of intimal cross-section area (\(y\)) due to the treatment with gIFN. Two-sided tests were used.

Results

The effect of gIFN on the vascular response to injury was studied in a standardized rat model. A balloon catheter was used to denude the intimal surface of the carotid artery; this results in the formation of an intimal thickening of relatively uniform size and cellular kinetics.\(^ {28–30}\) The intimal lesions thus induced were amenable to quantitation 2 weeks after injury.\(^ {28–31}\)

Rats were treated with daily injections of recombinant rat gIFN in the postoperative period. The design of the experiment is shown in Figure 1. In the first series of experiments, 200,000 units (50 μg, or approximately 100 μg/kg body wt i.p.) of gIFN was injected once daily for 7 days. Rats were killed 14 days after surgery and the arteries analyzed morphologically and immunohistochemically.

Since vascular smooth muscle cells respond to gIFN by de novo expression of the class II major histocompatibility protein, RT1B (also called I-A, Reference 32), the effect of gIFN could be monitored by expression of RT1B in the intima. Figure 2 shows a sixfold increase in the frequency of RT1B expressing cells in the intima of gIFN treated rats. This indicates that vascular cells respond in situ to parenteral gIFN administration.
The size of the intimal lesions 2 weeks after injury was determined by morphometry. As shown in Figures 3 and 4, gIFN treatment significantly reduced the size of lesions, which were only approximately 50% of those in control rats injected with PBS. Parenteral gIFN treatment therefore not only induces RT1B expression but also inhibits the formation of a proliferative intimal lesion. There was no significant correlation between the frequency of RT1B expressing cells and the degree of inhibition of intimal lesions, suggesting that variability in lesion size among gIFN treated rats was determined by other factors.

We analyzed arterial SMC replication after injury by \(^{3}H\)-thymidine labelling in order to identify any antiproliferative effect of the drug treatment. It has been shown that SMC undergo a first wave of replication in the media during the first week of injury, migrate into the intima, and then start proliferating in the growing intima.

The uptake of \(^{3}H\)-thymidine by medial SMC at day 7 was significantly reduced in gIFN treated rats, indicating that gIFN inhibits SMC replication in vivo (Table 1). In contrast, SMC replication at day 14 was not affected by gIFN (Table 1), suggesting that gIFN did not exert any further growth inhibitory effect at this time point.

In another series of experiments, rats were treated in a similar manner with balloon catheterization followed by gIFN treatment (200,000 units) for 7 days. The animals were then kept for either 7, 14, or 70 days before sacrifice. The growth of intimal lesions could then be plotted as a function of time (Figure 5). These growth curves were analyzed by nonlinear regression. Ninety-five percent confidence bands of the regression functions differed significantly between gIFN and PBS treated groups from day 14 after surgery and onward throughout the study period. This indicates that gIFN treatment results in a reduction of the development of intimal thickenings which persists for (at least) 10 weeks.

The dose–response relationship was studied in rats treated with gIFN at either 20,000, 100,000, or 200,000 units for 7 days. Lesions were analyzed 14 days after injury (Table 2). No significant effects were observed in rats given 20,000 or 100,000 units.

Finally, the effect of different treatment intervals was studied in rats given 200,000 units of gIFN for either 2, 4, or 7 days. As shown in Table 2, the intimal lesion size was significantly reduced already after 4 days of gIFN treatment. In rats treated for only 2 days with gIFN the mean lesion size was lower but not significantly reduced compared to PBS treated controls.

**Discussion**

Our previous work has led to the identification of gIFN as an endogenous growth inhibitor for vascular SMC. We now demonstrate that exogenously administered recombinant gIFN inhibits the develop-
ment of vascular proliferative lesions after injury. This supports the idea that gIFN is an important inhibitor of SMC proliferation in vivo and makes it attractive to test whether gIFN can be used to inhibit postsurgical arterial stenosis in man.

Daily, intraperitoneal administration of recombinant rat gIFN for 7 days led to a significant inhibition of arterial SMC replication at day 7 and to a significant reduction in intimal lesion size at day 14. Previous cell culture studies have demonstrated that gIFN is a potent inhibitor of SMC replication and acts by blocking the progression of the cells through the G1 phase of the cell cycle.18 Taken together, the in vivo and in vitro studies suggest that gIFN inhibits arterial SMC proliferation after vascular injury and that this results in a reduction of the size of the intimal lesion.

Surprisingly, there was no difference in intimal or medial SMC replication between gIFN treated and control groups at day 14 after injury. This implies that a difference in growth rate during the first week results in a long lasting difference in lesion size. The time frame for growth regulation was further assessed in rats treated with gIFN for only 2 or 4 days. It was found that a 4-day regimen was sufficient to significantly reduce the size of lesions at 14 days post injury.

![Bar graph illustrating effect of interferon-γ (gIFN) on intimal lesion size after balloon catheter injury. Rats (n=5 per group) were treated for 7 days, either with gIFN at 200,000 units daily or with phosphate buffered saline (PBS). Intimal cross-section areas were calculated by point-sampling morphometry of tissue 2 weeks after injury (mean±SE). **Indicates significant difference (p<0.01).](http://circ.ahajournals.org/)

**Figure 3.** Photomicrographs showing intimal lesions 14 days after balloon catheter injury in a PBS treated rat (a) and a rat that had received interferon-γ (gIFN) at 200,000 units daily for 7 days (b). Arrows indicate the internal elastic lamina between intima and media. Hematoxylin and eosin was used for staining. Magnification, ×180.

**Figure 4.** Bar graph illustrating effect of interferon-γ (gIFN) on intimal lesion size after balloon catheter injury. Rats (n=5 per group) were treated for 7 days, either with gIFN at 200,000 units daily or with phosphate buffered saline (PBS). Intimal cross-section areas were calculated by point-sampling morphometry of tissue 2 weeks after injury (mean±SE). **Indicates significant difference (p<0.01).

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**Table 1. Smooth Muscle Replication After Interferon-γ Treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of analysis (days)</th>
<th>Vessel compartment</th>
<th>Cell replication (% 3H-Thy-pos. cells) (mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>7</td>
<td>Intima</td>
<td>n.d.</td>
</tr>
<tr>
<td>gIFN</td>
<td>7</td>
<td>Intima</td>
<td>n.d.</td>
</tr>
<tr>
<td>PBS</td>
<td>7</td>
<td>Media</td>
<td>8.3±1.4**</td>
</tr>
<tr>
<td>gIFN</td>
<td>7</td>
<td>Media</td>
<td>2.1±0.94**</td>
</tr>
<tr>
<td>PBS</td>
<td>14</td>
<td>Intima</td>
<td>31±0.98†</td>
</tr>
<tr>
<td>gIFN</td>
<td>14</td>
<td>Intima</td>
<td>31±6.6†</td>
</tr>
<tr>
<td>PBS</td>
<td>14</td>
<td>Media</td>
<td>1.1±0.29‡</td>
</tr>
<tr>
<td>gIFN</td>
<td>14</td>
<td>Media</td>
<td>1.9±0.66‡</td>
</tr>
</tbody>
</table>

Intimal lesions were induced with a balloon catheter. The rats (n=5 per group) were treated either with interferon-γ at 200,000 units daily for 7 days or with an equal volume of phosphate buffered saline. 3H-thymidine was administered over 24 hours either 7 or 14 days after surgery, and the rats were sacrificed immediately afterwards. The frequency of replicating cells was determined by autoradiography. n.d., not determined.

**Significant difference between gIFN and PBS groups, p<0.01.**

†No significant difference between gIFN and PBS groups.

‡No significant difference between gIFN and PBS groups.
The development of an intimal thickening depends not only on SMC proliferation but also on their capability to migrate from the media into the intima. In a similar experimental model, it was recently shown that platelet depletion reduces intimal thickenings following injury without affecting cell replication. This is probably due to an effect on SMC migration. We cannot rule out that gIFN might affect SMC migration and that this could contribute to the reduced lesion size in gIFN treated rats. There is, however, no experimental evidence for such a mechanism. Furthermore, the potent antiproliferative effect of gIFN on SMC both in vivo and in vitro should be sufficient to explain the inhibition in lesion size observed in the present study.

The SMC response to gIFN was manifested not only by a reduced intimal lesion development, but also by expression of the class II major histocompatibility protein, RT1B, in the arterial intima of gIFN treated rats. RT1B is expressed not only by SMC but also by several other cell types including monocytes, macrophages, and activated T lymphocytes. We cannot exclude that some RT1B+ intimal cells may belong to these categories. However, the spindle-shaped morphology of most RT1B+ cells as well as the low frequency of cells positive for the macrophage- and T cell-specific monoclonal antibodies, ED1, and OX19 (data not shown) suggested that the overwhelming majority of RT1B+ intimal cells were SMC.

The choice of doses of gIFN was based on work by van der Meide et al. who found that infusion of approximately $2 \times 10^7$ units per 24 hours led to a biological response manifested by RT1B expression. Furthermore, injections of $10^7$–$10^8$ units led to significant plasma levels within an hour and gIFN was eliminated relatively slowly with detectable plasma levels several hours after injection. No dose-dependent toxicity was observed when gIFN was given in doses up to $9 \times 10^6$ units per 24 hours.

Our own experiments showed that gIFN treatment at $2 \times 10^7$ units daily resulted in a significant reduction of lesion size combined with an increased expression of RT1B on the target cells. No significant reduction of lesion size was accomplished in rats treated with smaller doses of gIFN. It is, however, possible that an effect may be detected in a larger series of experiments, because mean values of lesion areas in these rats were lower than those of control rats.

The present data shed light on the role of gIFN in the vascular response to injury. Indirect evidence was first obtained for gIFN as a regulator of SMC gene expression in the intima. It was also shown that type I IFN, which has partly overlapping activities with gIFN, inhibits the development of experimental atherosclerosis in fat-fed rabbits. An inverse correlation was then observed between expression of the gIFN induced class II MHC genes and SMC replication in vitro and in vivo. This suggested that gIFN may serve as an endogenous growth inhibitor in the intima. We now show that gIFN when administered exogenously significantly inhibits lesion formation after arterial denudation. This supports the idea that gIFN is an important regulator of tissue responses after injury.

gIFN is, to our knowledge, the fourth pharmacological substance that has been shown to inhibit the arterial proliferative response to injury. Clowes and Kanno discovered already in 1977 that heparin can inhibit the development of intimal thickenings, and clinical studies are underway to determine whether heparin is useful as an inhibitor of post-surgical arterial restenosis. In 1988 we showed that cyclosporin A inhibits intimal lesions after balloon catheter injury. This was initially thought to be related to the immunosuppressive effect of this drug, but further work has shown that cyclosporin A exerts a direct inhibitory effect on growth factor-induced SMC replication.

Finally, two antihypertensive drugs have recently been found to inhibit intimal lesion formation after mechanical injury. The calcium antagonist, nifedipine, reduces lesion size after injury in cholesterol-
fed rabbits and the angiotensin convertase inhibitor, cilazapril, could reduce intimal thickenings after balloon catheter injury. It is, however, yet unclear to what extent the antiproliferative effect is related to the antihypertensive effect and it is also not known whether a brief treatment period is sufficient to persistently inhibit lesion development.

In conclusion, several compounds have been identified that may be useful for inhibiting the development of vascular stenosis after angioplasty and vascular surgery. Further experimental studies and clinical trials will be necessary to determine to what extent these drugs will be useful in clinical medicine.

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References


KEY WORDS • angioplasty • atherosclerosis • cell proliferation • smooth muscle, vascular • interferon type II
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