Suppression of Aortic Atherosclerosis in Cholesterol-fed Rabbits by Purified Rabbit Interferon

Alan C. Wilson, Robert G. Schaub, Randee C. Goldstein, and Peter T. Kuo

The effectiveness of rabbit Interferon in suppressing atherosclerosis was evaluated in rabbits fed a diet containing 1% cholesterol. Ten male New Zealand White rabbits received Intramuscular injections of 1 million units of Interferon twice a week, while a control group of 10 rabbits received injections of buffer. Both groups had average serum cholesterol levels of over 2000 mg/dl during the 8-week experimental period. Interferon treatment resulted in no significant hypolipidemic effect or changes in lipoprotein composition. Atherosclerotic lesions in aortas were quantified both macroscopically and microscopically. Interferon treatment decreased the grossly visible lesion area significantly from 25±4% to 8±1% (mean±SEM, p<0.005) compared to the untreated group. Microscopic analysis of serial cross-sections of aortic segments revealed significant (p<0.01) reductions in both lesion size and frequency in the Interferon-treated group. Electron microscopy also showed that Interferon treatment reduced the pathological effects of cholesterol feeding. Tissue analysis showed that total aortic cholesterol was reduced by 28% by Interferon treatment, while the aortic phospholipid concentration was increased by 25%. The possibility exists that the Interferon preparation used contained other biological response modifiers and that the observed effects may be totally unrelated to Interferon. These results suggest that the mechanism of atherosclerosis suppression in these cholesterol-fed rabbits is not related to the lowering of serum cholesterol but may be associated with inhibition of lesion initiation.

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Atherosclerotic lesions develop as the result of the interplay between the multiple risk factors associated with increased incidence of atherosclerosis, the cells of the arterial wall, and the circulating blood cells.1 Increased understanding of these interactions is critical to developing new approaches to prevention and therapy.

Since the discovery of Interferon as an antiviral agent in 1957, interest has grown in its nonantiviral activities,2 particularly in its use in cancer therapy.3 Interferons exhibit a wide range of effects, each depending on the type of interferon and the nature and state of differentiation of the target cell. Interferons are involved in the inhibition of cell growth and proliferation, regulation of the expression of specific genes, modulation of cell differentiation, and activation of certain cell types in the immune system, for example, macrophages and natural killer cells.4

The Interferon inducers, polynosinic-polycytidylic acid and 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidinone (U-54,461), have been shown to suppress atherosclerosis in rabbits.5 It is important to establish whether the anti-atherogenic effect is a direct effect of these drugs or is mediated by interferon. The purpose of the present study was to evaluate the anti-atherogenic effectiveness of the administration of purified rabbit interferon in the cholesterol-fed rabbit model. The results presented in this report support the findings of the inducer studies and may suggest novel approaches to the pharmacology of atherosclerosis control.

Methods

Interferon

Rabbit interferon (lot 83016, titer 1.5×10^6 IRU/ml, specific activity 1.3×10^7 IRU/mg) purified from RK13 rabbit kidney cultures stimulated with para-influenza-1 virus was obtained from LEE BioMolecular Research Laboratories, San Diego, CA. The titer was assayed by dye uptake assay and normalized to international reference units (IRU) against a National Institutes of Health rabbit interferon standard.6 The interferon titer was confirmed by independent assay at The Upjohn Company. Because of the cell culture source, there is a possibility of the presence of other biological response modifiers in the material.

Interferon Nomenclature

Unlike the mouse and human interferons, which are designated alpha or beta based on various physical properties, no information is yet available concerning the subdivision of rabbit interferon into alpha or beta components.

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Animals and Diet

After being assigned into two hypercholesterolemic-response matched groups, 20 New Zealand White male rabbits (weight, 3 to 4 kg) were fed an atherogenic diet1 (1% cholesterol, 4% peanut oil supplied by ICN Nutritional Biochemicals, Cleveland, OH) for 8 weeks. Each rabbit in the interferon-treated group received injections of 1 x 10^9 IRU of rabbit interferon twice a week intramuscularly in alternate rear legs. This route of administration was chosen because interferon is rapidly cleared after intravenous injection (t½ = 13 minutes), while fairly stable levels of serum interferon are maintained for at least 12 hours after intramuscular injection.8 The rabbits in the untreated cholesterol-fed group were handled similarly except that the injections consisted of sterile 0.01 M phosphate buffer, pH 7.2. A third group of 10 rabbits was maintained on regular chow as normal controls. The studies involving experimental animals were in accordance with institutional guidelines.

Experimental Procedure

Body weight and food consumption were recorded twice weekly. At 4 and 8 weeks, the animals were fasted for 16 hours, and blood samples for blood chemistry, interferon, and lipoprotein analysis were withdrawn from the marginal ear veins into plastic tubes on ice and were allowed to clot; the serum was collected. The blood for platelet studies was collected into plastic tubes containing citrate (final concentration, 3.8%). Lipoproteins were separated from serum by sequential preparative ultracentrifugation after adjustment of solvent densities to 1.006, 1.019, and 1.063 g/ml with KBr,8 and the cholesterol and triglyceride contents of serum and lipoprotein fractions were determined by the methods described in the Lipid Research Clinics Program Manual.10

Postmortem Studies

After sacrifice, samples of liver, spleen, kidney, and adrenal tissue were taken for pathological examination. The whole aorta from the aortic valve to the iliac bifurcation was removed intact from each animal and was opened longitudinally along the anterior side. The adventitia and adhering adipose tissue were removed, and the wet weight of the aorta was determined. Samples (3 mm diameter) for light and electron microscopic examination were punched out from identical sites in the aortic arch (distal to the origin of the coeliac trunk) and the thoracic aorta (distal to the fifth intercostal arteries) and were fixed in 2% glyceraldehyde in Tyrode’s buffer. The remainder of the aorta was fixed in 10% buffered formaldehyde, and the lipid-rich lesions on the surface of the aorta were stained with 1% Sudan IV.11 After tracing on an overlaid clear plastic sheet, the aortic surface area and its lipid-staining lesions were quantitated by an electronic graphic calculator (Model 1224, Numonics, Lansdale, PA). The punch samples were freeze-sectioned and stained for neutral lipid with oil red O. A Zeiss Videoplan image analysis unit was used for computer-assisted morphometric planimetry of lesions in 137 of these serial freeze-sections. The stained lesion area in each section

Table 1. Effect of Interferon Treatment on Serum Lipid and Lipoprotein Concentrations in Rabbits Fed a Cholesterol Diet

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Buffer (mg/dl)</th>
<th>Interferon (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cholesterol (at start of study)</td>
<td>67±9</td>
<td>63±4</td>
</tr>
<tr>
<td>Serum cholesterol (after 8 weeks)</td>
<td>2259±232</td>
<td>2164±309</td>
</tr>
<tr>
<td>Triglyceride (after 8 weeks)</td>
<td>295±158</td>
<td>286±99</td>
</tr>
<tr>
<td>VLDL cholesterol (d&lt;1.006)</td>
<td>1450±168</td>
<td>1328±222</td>
</tr>
<tr>
<td>IDL cholesterol (d=1.006-1.019)</td>
<td>461±94</td>
<td>442±43</td>
</tr>
<tr>
<td>LDL cholesterol (d=1.019-1.063)</td>
<td>222±61</td>
<td>196±55</td>
</tr>
<tr>
<td>HDL cholesterol (d&gt;1.063)</td>
<td>46±6</td>
<td>41±11</td>
</tr>
</tbody>
</table>

The values are means±SE. n=10 for total cholesterol and triglyceride, n=7 for ultracentrifugal separations.

HDL=high density lipoprotein, IDL=intermediate density lipoprotein, LDL=low density lipoprotein, VLDL=very low density lipoprotein.

Purified rabbit interferon (1 x 10^9 IRU) was injected intramuscularly twice a week for 8 weeks.

was expressed as a percent of the total vessel section area (intima + media). Tissue sampling and macroscopic and microscopic area analyses were performed without knowledge of prior treatment to avoid subjective bias.

The aortas were dried by lyophilization and were stored at -20°C until analysis. Total lipid extracts12 were prepared by cutting each aorta into small pieces and homogenizing three times in methanol/chloroform/water (2:1:0.8, vol/vol/vol) in a tapered glass-glass homogenizer. Determinations of total, esterified, and free cholesterol and phospholipids were performed in duplicate after thin-layer chromatography of the total lipid extracts. Analysis of DNA content was performed in duplicate on the defatted, dry tissue by the method of Burton.13

Statistical Analysis

The statistical significance of between-group differences was tested by one-way analysis of variance followed by Duncan’s multiple range test by using the Statistical Analysis Systems computer program.14

Results

Serum Lipids

The serum of both groups of rabbits became milky within 4 weeks, and total serum cholesterol increased from baseline levels of about 65 mg/dl to over 2000 mg/dl and remained at this level throughout the experiment (Table 1). Serum triglyceride concentrations after 8 weeks were in the range of 300 mg/dl in both the buffer-treated and the interferon-treated groups.

Ultracentrifugal fractionation revealed that the hypercholesterolemic response to the atherogenic diet was predominantly in the density d<1.006 g/ml lipoprotein.
Table 2. Effect of Interferon Treatment on Visible Lipid Staining Lesions and Aorta Composition In Cholesterol-fed Rabbits

<table>
<thead>
<tr>
<th>Aorta parameter</th>
<th>Buffer</th>
<th>Interferon</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grossly visible (aortic lesion area %)</td>
<td>25.3±4.1</td>
<td>7.6±1.3*</td>
<td>—</td>
</tr>
<tr>
<td>Total C (mg/g, dry wt)</td>
<td>10.40±1.73</td>
<td>7.44±0.97</td>
<td>1.77±0.92</td>
</tr>
<tr>
<td>C ester (mg/g, dry wt)</td>
<td>3.66±1.65</td>
<td>2.84±0.96</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td>PL (mg/g, dry wt)</td>
<td>1.80±0.18</td>
<td>2.26±0.19</td>
<td>—</td>
</tr>
<tr>
<td>DNA (mg/g, dry wt)</td>
<td>0.19±0.03</td>
<td>0.18±0.02</td>
<td>—</td>
</tr>
<tr>
<td>Dry weight (g)</td>
<td>0.21±0.05</td>
<td>0.27±0.09</td>
<td>0.14±0.03</td>
</tr>
</tbody>
</table>

The results are means±SEM, n=10 in each group. Whole aortas from root of arch to iliac bifurcation were analyzed individually in duplicate as described in the Methods section.

* p<0.005, analysis of variance compared to buffer group.

Macroscopic and Chemical Findings

In rabbits fed the atherogenic diet without interferon treatment, grossly visible lipid-staining lesions covered a mean 25.3% (range 12% to 43%) of the total aortic surface (Table 2), mostly affecting the aortic arch. Interferon treatment significantly decreased the lipid staining surface affected by lesions to a mean of 7.6% (range 3% to 25%, p<0.005) compared to the untreated atherosclerotic group. Aortic dry weight and DNA content were not significantly different in the untreated or interferon-treated groups. Treatment with interferon resulted in a 28% decrease in the contents of esterified and total cholesterol and a 25% increase in phospholipid content.

Microscopic Analysis

Examination of serial freeze-sections of thoracic aorta and aortic arch from untreated cholesterol-fed rabbits showed markedly raised intima with large proliferations of lipid-laden "foam" cells and some pericellular lipid in the intimal-medial layers. In rabbits treated with interferon, the lesions consisted of fewer layers of intimal foam cells overlying an otherwise normal-looking arterial wall.

Statistical analysis indicated that interferon treatment significantly reduced the amount of visible lipid accumulation (Table 3). In the aortic arch, a significant reduction in lesion area in the aortic wall from 24.5±4.2% to 12.9±1.6% was found (p<0.01). In the thoracic aorta (which was less severely atherosclerotic than the arch), a smaller decrease in the lipid accumulation in the wall was observed from 6.9±1.5% to 5.1±1.4%. Pooling the data from both areas, the overall 46% reduction in lipid accumulation from 15.7±2.5% to 8.7±1.2% was found to be significant (p<0.01) compared to the untreated group.

Table 3. Morphometric Analysis of Microscopic Lipid Accumulation in Serial Cross-sections of Aortic Arch and Thoracic Aorta Wall from Cholesterol-fed Rabbits Treated with Interferon

<table>
<thead>
<tr>
<th>Aortic section</th>
<th>Buffer</th>
<th>Interferon</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arch (n=66)</td>
<td>24.5±4.2</td>
<td>12.9±1.6*</td>
<td>—</td>
</tr>
<tr>
<td>Thoracic (n=71)</td>
<td>6.9±1.5</td>
<td>5.1±1.4</td>
<td>—</td>
</tr>
<tr>
<td>Arch + thoracic (n=137)</td>
<td>15.7±2.5</td>
<td>8.7±1.2*</td>
<td>—</td>
</tr>
</tbody>
</table>

The values are means±SE, n=number of serial freeze sections of aorta examined. Lesion area is expressed as the sum of areas of the individual lipid-staining lesions in each microscopic section×100/total section area.

*p<0.01, analysis of variance compared to buffer group.

A frequency plot (Figure 1) of the pooled data shows that in interferon-treated rabbits there were significantly fewer large lesions (those covering more than 20% of the total section area) than in untreated rabbits. The largest lesions...
Figure 2. Transmission electron micrographs of lesion areas from interferon-treated (A and B) and untreated cholesterol-fed rabbits (C and D). Many of the foam cells in the interferon-treated group appear to be in the early stage of development, with small lipid-containing vacuoles (arrows). Many of the cells are of smooth muscle cell origin (S). The foam cells in the untreated group are more complex, containing larger numbers of electron-translucent vacuoles (arrows), which distend the cells and increase their size. More foam cells of macrophage origin (M) are found in addition to those of smooth muscle cell origin (S). The origin of some foam cells in the untreated group could not be identified. Bars=2μm.

Transmission Electron Microscopy

Examination of electron micrographs from treated and untreated rabbits confirmed and extended the observations made by light microscopy.

In the interferon-treated rabbits (Figures 2A and 2B) less lipid accumulation was apparent within the cells of the lesions, with smaller lipid-containing vacuoles (arrows), and in some areas there was almost no lipid accumulation. Many of the cells were of smooth muscle origin (S) and appeared to be more "normal" in both their morphology and size compared to the nontreated rabbits.

In the untreated animals, generally, the smooth muscle cells and macrophages of the lesion had taken up large quantities of lipid (Figures 2C and 2D). Because of the amount of lipid that was deposited in some of the cells, it was difficult to determine many of the important characteristic criteria of the cells, and morphologic identification of the cell precursor was difficult. These cells were also larger than normal intimal cells. A typical foam cell (Figure 2D) in an intimal lesion from an untreated, cholesterol-fed rabbit had the features of a macrophage (M). There was no basal lamina; it had a regularly shaped nucleus with peripherally clumped chromatin prominent Golgi apparatus and many lipid droplets.

Pathology of Other Organs and Serum Chemistry

Postmortem histologic studies of liver, kidney, spleen, and adrenals of each rabbit were performed to compare the effect of interferon administration. The livers of both treated and untreated groups of rabbits showed a range of mild to marked fatty changes, with little or no bile stasis, and with early signs of fibrosis and necrosis. There appeared to be no correlation between interferon-treatment and the severity of the hyperlipidemic changes. All the sections of kidney were normal. The spleens exhibited a few foamy histiocytes, again with no correlation with the interferon treatment. The adrenals of the untreated cholesterol-fed rabbits showed necrosis and many cholesterol clefts, while the interferon-treated group appeared to be normally lipid-laden, with no cholesterol clefts and only mild necrosis. No significant effect
of interferon treatment was detected by serum chemistry determinations of liver enzymes serum aminotransferases (SGOT and SGPT), alkaline phosphatase, and gamma-glutamyltransferase.

**Platelet Activity**

Platelets from the interferon-treated and untreated cholesterol-fed rabbits were tested for sensitivity to aggregation in response to both arachidonic acid and collagen. No effect of the interferon treatment could be detected. Rabbit interferon added in vitro directly to the measuring cuvettes had no effect on platelet aggregation. In addition, washed platelets from hypercholesterolemic rabbits showed no differences in aggregability compared to platelets from normolipidemic rabbits.

**Discussion**

A previous report described the inhibition of atherogenesis by interferon inducers in cholesterol-fed rabbits, but the mechanism of action was unclear since no serum cholesterol-lowering effect was found. The interferon inducers might have acted by inducing interferon production at the lesion site or at a distant site. Alternatively, the effect might have been a direct one with no involvement of interferon at all. To clarify the mode of action, the aim of this study was to determine whether the suppression could be reproduced by treatment with purified rabbit interferon.

Interferon treatment was found to decrease the development of grossly visible atherosclerotic plaques by an average of 70% in cholesterol-fed rabbits. This finding was supported by results of morphometric analysis of serial sections of aortic wall, which showed a 47% decrease in lipid staining lesion area and a significant shift in the lesion size-frequency distribution. These results indicate that intramuscular administration of purified rabbit interferon was effective in achieving substantial and significant suppression of atheromatous lesion formation in the aortas of cholesterol-fed rabbits. The protection did not appear to arise from a hypolipidemic action, and the precise mechanism of action remains unclear. There is a possibility that the interferon preparation used contained other biological response modifiers and that the observed effects may be totally unrelated with interferon. It is of interest that other classes of antiatherogenic agents, the calcium and calmodulin antagonists, also accomplish their effect without lowering serum cholesterol levels. The following mechanisms may explain the observed results.

**Mechanism of Lipoproteins**

The relationship between interferon treatment and lipoprotein metabolism has been examined in healthy men by Cantell et al. and Enholm et al. Daily subcutaneous injections of leukocyte interferon \((3 \times 10^6 \text{ RU})\) for 1 week decreased the level of high density lipoprotein cholesterol, very low density plus low density lipoprotein cholesterol, and apolipoprotein A-I by about 15%. Simultaneously the activity of both postheparin plasma lipoprotein lipase and hepatic lipase decreased by 40%. In patients being treated for various carcinomas, Dixon et al. and Massaro et al. found similar effects with alpha-interferon. With beta-interferon, the same authors reported a dose-related decrease in low density lipoprotein but not high density lipoprotein, and an increase in very low density lipoprotein.

In the present study, interferon treatment for 8 weeks produced no significant changes in the beta-lipoprotein fractions. A transient significant decrease in high density lipoprotein cholesterol was detected in serum samples drawn at 4 weeks (data not shown). The relevance of this decrease to the present antiatherogenic effect is unclear, since decreased levels of high density lipoprotein have been associated with an increased risk of coronary heart disease. While small differences were seen, it is unlikely that cholesterol-lowering is responsible for the suppression of atherosclerosis because serum cholesterol levels in excess of 2000 mg/dl were found in both groups of rabbits.

**Mechanism of Arterial Wall Cells**

Current models of atherogenesis implicate many factors that modify arterial wall cells and circulating blood cells. In these various models, smooth muscle cells, endothelial cells, platelets, monocytes and their derived macrophages all interact with one another and with serum lipoproteins. Interferon may affect one or more of these interactions directly so as to modify cholesterol accumulation. It has been suggested that gamma-interferon is responsible for smooth muscle expression of la antigens during the response to arterial injury in rats. Studies in vitro with HeLa-S3 cells have shown that beta-interferon stimulates cholesterol and phospholipid synthesis but inhibits cholesterol ester synthesis, possibly by inhibition of low density lipoprotein endocytosis. The same workers have described the inhibition of concanavalin A-stimulated calcium flux by beta-interferon. Alternatively, the influence of interferon may be indirect, perhaps being mediated through the action of prostaglandins, since a distinct interrelationship apparently exists between interferon and prostaglandin metabolism.

**Mechanism of Monocyte Macrophages**

In the cholesterol-fed rabbit, hypercholesterolemia is accompanied by rapid development of arterial fatty streaks with foam cells of smooth muscle cell or monocyte-macrophage origin. In the early stages of the formation of atheromatous lesions, macrophages derived from circulating blood monocytes appear to act as scavenger cells, taking up \(\beta\)-VLDL from the areas of lesion formation during hyperlipidemia, while their failure to do so results in the accumulation of lipid at the lesion. As the concentration of blood lipids and lipoproteins was not changed, the infiltration of lipoprotein cholesterol ester must remain the same. The possibility, then, is that interferon increases the removal processes and/or modifies the composition of aortic cell membranes to allow the accommodation of part of the excess cholesterol ester. The capacity for cholesterol efflux from monocyte-derived macrophages from cholesterol-fed rabbits has been shown to be significantly greater than that found in macrophages from normocholesterolemic rabbits. In addition, it has been reported that interferon and polyanionic interferon inducers activate macrophages to in-
increased phagocytosis and cholesterol ester accumulation. The data presented here indicate that intramuscular injection of rabbit interferon was effective in significantly reducing the visible aortic atherosclerosis induced by feeding rabbits a diet containing cholesterol and peanut oil. In this short-term study in rabbits response-matched to cholesterol feeding, atherosclerosis suppression was judged objectively by "blinded" observers from the reduction in macroscopic lesion area on the surface and the decrease in area and size of microscopically analyzed lesions in the artery wall.

These reductions in atherosclerosis were achieved without any significant lowering of serum cholesterol or changes in lipoprotein cholesterol distribution, adding support to the conclusion that other approaches as well as lowering serum cholesterol may be protective against atherogenesis. This study further suggests that it may be possible to pharmacologically influence vascular cells to reduce lipid accumulation in the face of substantial hyperlipidemia. It should be noted that the severe side effects of interferon therapy rule against its probable utility in prophylaxis of atherosclerosis. Substantiation of the observed results and further studies in long-term models of atherosclerosis, such as the Watanabe heritable hyperlipidemic rabbit, perhaps utilizing interferon inducers or other agents, will be necessary before clinical applications can be envisioned.

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