Etoposide Treatment Suppresses Atherosclerotic Plaque Development in Cholesterol-Fed Rabbits

Margarita de la Llera-Moya, George H. Rothblat, Jane M. Glick, and James M. England

To study the mechanisms by which monocytes/macrophages and smooth muscle cells contribute to atherosclerotic lesions, we studied atherosclerotic plaque formation in cholesterol-fed rabbits treated with etoposide, a drug that has been shown to have several effects that could interfere with the proposed interactions between these two cell types (M.W. Aarnoudes et al, *Virchows Arch B 1984;47:211–216* and M. Rozencweig et al, *Cancer 1977;40:334–342*). Our results show that long-term etoposide treatment of New Zealand White rabbits maintained on a high-cholesterol diet decreases the extent of fatty streak formation in the aortic intima. Moreover, the plaques formed in the presence of etoposide are thinner and at least focally have less fibrous tissue and fewer smooth muscle cell–derived foam cells than do plaques in control rabbits. These effects are independent of the extent of the diet-induced hyperlipemia or an effect of etoposide on blood cell count and may be related to the inhibition of intimal cell proliferation by etoposide. (*Arteriosclerosis and Thrombosis 1992;12:1363–1370*)

**Key Words** • atherosclerosis • plaque progression • foam cells • macrophages • smooth muscle cells • hypercholesterolemia

Macrophages and smooth muscle cells are important constituents of the atherosclerotic plaque in naturally occurring lesions and in lesions obtained from cholesterol-fed experimental animals. Recently, monoclonal antibodies specific for macrophage or muscle cell antigens have been used to identify the phenotype of lipid-laden foam cells within atherosclerotic plaques. Based on antibody reactivity, the majority of cells in atherosclerotic lesions have been classified as either macrophage-derived (MFC) or smooth muscle cell–derived (SMFC) foam cells. However, much remains to be learned regarding the role of these two cell types in the development of atherosclerosis. Current models of atherosclerotic plaque development propose that the macrophage plays a crucial role in the initiation and progression of lesions. It is thought that blood monocytes adhere to and penetrate through the vascular endothelium at sites prone to lesion development, which are also areas where modified lipoproteins can accumulate. Macrophages can then avidly internalize these lipoproteins via unregulated scavenger receptors to accumulate large intracellular stores of cholesteryl esters and become foam cells in fatty streaks. By mechanisms that are not understood but that may involve the release of chemotactic and growth factors, these lipid-loaded macrophages can in turn trigger the migration and proliferation of smooth muscle cells into the intima, a hallmark of a progressing atherosclerotic plaque. The mechanism by which smooth muscle cells become foam cells in vivo has not been established, but it has recently been reported that smooth muscle cells in vitro can express unregulated cell-surface receptors for modified lipoproteins. It has also been proposed that lipid-rich macrophages can stimulate the formation of SMFCs by releasing cholesteryl ester–rich droplets that can then be internalized by smooth muscle cells. Thus, macrophages may promote atherosclerosis by various effects on smooth muscle cells.

To investigate the mechanisms by which smooth muscle cells and monocytes/macrophages contribute to the formation of atherosclerotic lesions, we studied the effects of the cytostatic drug etoposide on atherosclerotic plaque development in cholesterol-fed rabbits. We chose to study the effects of etoposide on aortic lesions because this drug has been reported to produce cytopenia in rabbits, and as discussed above, circulating monocytes have been implicated in the early events of fatty streak formation in several animal models of atherosclerosis. Our results show that long-term treatment of cholesterol-fed New Zealand White (NZW) rabbits with etoposide decreases the extent of plaque formation in the aortic intima. The plaques formed in etoposide-treated rabbits are thinner, have decreased intimal accumulation of fibrous tissue as visualized with histological stains, and have fewer SMFCs as measured immunocytochemically with the monoclonal antibody HHF-35. Although we observed dramatic differences in lesion development in the drug-treated animals, these effects were found to be independent of the extent of diet-induced hyperlipemia or changes in blood cell populations. We propose that etoposide impairs plaque development by inhibiting intimal cell proliferation.

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Methods

Rabbits

Adult male NZW rabbits (average weight, 2.5 kg) were used in all experiments. The rabbits were individually caged in stainless steel cages and were housed in an animal care facility approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with National Institutes of Health guidelines. Conventional NZW rabbits were obtained from Ace Animals (Boyertown, Pa.). Specific pathogen-free (SPF) NZW rabbits were from Hazleton Research Products (Denver, Pa.). NZW-SPF rabbits were used in some experiments because they are more inbred than the conventional rabbits and less prone to subclinical infections. NZW-SPF rabbits were isolated from all other rabbits and were shown to be Pasteurella free on receipt and at the end of our studies. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Etoposide Treatment and Monitoring

Rabbits were treated with the cytostatic drug etoposide (VP16-213, VePesid, Bristol-Myers Oncology Division, Evansville, Ind.), a potent inhibitor of monocytic murine leukemia23 that has been shown to others to produce monocytopenia in Chinchilla rabbits.24 We obtained etoposide as a gift from Dr. William Bradner (Bristol-Myers) and used a total weekly dose of 40 mg/kg body wt injected subcutaneously as two 20 mg/kg doses. Etoposide was diluted with sterile phosphate-buffered saline to the appropriate dose just before injection. Since etoposide is formulated in a complex vehicle that maintains the drug in solution, we injected etoposide 1 week before the start of treatment. Since etoposide is formulated in a complex vehicle that maintains the drug in solution, we injected etoposide 1 week before the start of treatment.

Tissue Preparation

Rabbits were given a lethal dose of sodium pentobarbital (Butler Co., Columbus, Ohio). Immediately after death, blood was collected by heart puncture; serum was prepared and stored at 4°C until use. The aorta was dissected from the arch to the bifurcation, gently rinsed with normal saline, and opened along the posterior wall. After removing as much of the periaortic tissue as possible, the opened aortas were flattened on strips of paper, intima side up. The vessels adhered to the paper strips and were fixed face down with 10% buffered formalin (Fisher Scientific, Pittsburgh, Pa.) overnight at room temperature.

 collects glutamine pyruvate transaminase and bilirubin were elevated in etoposide-treated rabbits on the high-cholesterol diet, but similar elevations were also seen with the diet alone, and the average values for these parameters were not significantly different between these two groups (p=0.69 for glutamine pyruvate transaminase and p=0.18 for bilirubin). Histological evaluation of liver and spleen samples from rabbits that had been treated with etoposide for several weeks showed no abnormalities.

Diet

Atherosclerotic lesions were induced by feeding the rabbits a 2% cholesterol–6% peanut oil diet for 8 weeks.24 This atherogenic diet was prepared by dissolving the cholesterol (USP grade, anhydrous, Sigma Chemical Co., St Louis, Mo.) in the peanut oil (Planters) and then using this mixture to coat pellets of the standard chow (Agway Pro Lab Diet, Ithaca, N.Y.). The diet was prepared every 2 weeks and was stored in sealed containers at 4°C. All rabbits on the cholesterol diet were initially fed 100–120 g/day, and the amount eaten was monitored. If necessary, the amount of diet given each animal was adjusted to ensure that both etoposide-treated and untreated rabbits consumed the same amount of cholesterol. Total cholesterol was measured on postprandial plasma samples, obtained once a week from the ear vein, using a standard enzymatic assay (Sigma procedure No. 352, Sigma), and these weekly measurements were plotted against time. The area under each curve was integrated by weighing the paper containing the traces of each curve to calculate the average daily plasma cholesterol level for each rabbit. In the experiments with NZW-SPF rabbits, after 4 weeks the 2% cholesterol diet was cut with equal amounts of standard chow to lower the cholesterol content to 1% and to maintain the serum cholesterol levels below 2,000 mg/dl. Total triglyceride was also measured enzymatically (triglyceride-GPO, procedure No. 339, Sigma) in some samples. In addition, agarose electrophoresis (Paragon Lipo Electrophoresis System, Beckman Instruments Inc., Brea, Calif.) was done on plasma samples obtained from all rabbits at various intervals during cholesterol feeding and at the time the animals were killed. Electrophoresis was done to ensure that both etoposide-treated and untreated rabbits developed the beta-migrating very low density lipoprotein (β-VLDL) that has been described in the literature.25

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Analysis of Lesions

Fixed aortas were stained with Sudan IV to visualize areas of atherosclerotic plaque. Staining was done by floating the vessels face down for 25 minutes at room temperature in a solution of 0.5% Sudan IV (Fisher Scientific Co., Fair Lawn, N.J.) in freshly prepared 70% isopropanol.26 The stain was differentiated with several rinses in 70% isopropanol. Templates of the stained vessels were drawn on clear acrylic sheets, and the extent of gross lesion was quantified with a dot-counting method by superimposing each template over a dot grid with a 2-mm2 grid size (Letratone LT-914, Letraset, England) and counting the total number of dots covered by each vessel and the number covered only by lesions.27 Tissue for histological analysis (3 mm thick) was taken from the macroscopically most apparent plaques. At least two plaques were sampled from each aorta; one plaque was always from the aortic arch, and the other sample of plaque was usually taken from the thoracic aorta, although in some animals prominent plaques in the abdominal aorta were sampled. Adjacent histological sections were stained with hematoxylin-eosin, Verhoeff-van Gieson’s elastin, and Masson’s trichrome stains. To analyze the cellular origin of foam cells found in the aortic lesions, immunocytochemical staining was performed using the well-characterized monoclonal antibodies HHF-35 and RAM-11.28 HHF-35 was obtained from Enzo Biochemicals Inc. (New York), and RAM-11 was a kind gift from Dr. Allen M. Gown. Staining with these antibodies was done with standard procedures using an avidin-biotin complex/peroxidase technique for visualization (Vectastain ABC kit, Vector Corp., Burlingame, Calif.). To determine if there were significant quantitative histological differences in plaques obtained from either etoposide or control rabbits, histological sections of plaques from the aortic arch were stained with Masson’s trichrome stain and HHF-35, coded, and randomized; these sections were then reviewed by four independent observers who assigned each section a score from 1 (low reactivity) to 4 (high reactivity). Scores from all observers were averaged for each experiment, and the statistical significance of the results was analyzed by using Student’s t test. Measurement of lesion thickness was done by using a microscope eyepiece micrometer. The mean intimal thickness was calculated from measurements of the distance from the luminal surface to the internal elastic lamina, determined at 22.5-μm intervals throughout the entire length of the plaque section, and Student’s t test was also used to determine statistical significance. Although histological assessment was done in both experiments, quantitative histological analyses were done only on slides prepared from tissue obtained in experiment 2.

Chemical Analysis of Tissues

Formalin-fixed vessels from all rabbits in experiment 2 were rinsed with 70% isopropanol followed by normal saline. The vessels were blotted gently, and wet weights were determined. Entire individual aortas were minced, except for two 3-mm-long sections taken from each vessel for histological analysis. The minced tissues were resuspended in 2.5 ml water for each 0.5 g wet weight and were homogenized with a Polytron. Aliquots (0.5 ml) of homogenates were transferred to tared test tubes to determine wet weights. Total lipids were extracted from equivalent aliquots according to the method of Folch et al.28 Cholesterol was measured by gas-liquid chromatography with cholesterol methyl ether (Sigma) as an internal standard.29 Total cholesterol was measured after hydrolysis by following the method of Ishikawa et al.,30 and the cholesterol ester content was calculated by difference. Although all extracts contained a residual amount of Sudan IV, it was determined that it did not interfere with the quantification of either cholesterol or its esters.

Experiments With Cells in Culture

J774 macrophages were seeded in 35-mm wells (Cell Wells, Costar, Corning, N.Y.) and grown to confluency in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum. Test media for cell incubations contained 10% of each serum to be tested in RPMI-1640 supplemented with gentamicin (50 μg/ml, Tri Bio Laboratories, State College, Pa.). Incubations with test media were started by replacing growth media with the test media after gently rinsing the cells with RPMI-1640. Cells were incubated with test media for 24 hours at 37°C. Cell incubations were done in triplicate. At the end of the incubations test media were removed, and the cell monolayers were rinsed with warm phosphate-buffered saline. Cell lipids were extracted by incubating the monolayers with isopropanol overnight as previously described.29 Cell cholesterol and cholesteryl ester content was quantified by gas-liquid chromatography as described above. Cell protein was quantified by the method of Markwell et al,31 after the monolayers were extracted with isopropanol.29

Statistical Analysis

Statistical comparisons between groups were made by using analysis of covariance, t test, Mann-Whitney non-parametric tests, and linear correlation. Differences were considered significant at a probability value of 0.05 or less. The quantitative data are reported as mean±SD. The analysis of covariance was done by our statistician, Dr. Edward Graceley. Other statistical tests were done by using a computerized program (Graph PAD In Stat, Graph PAD Software).

Results

Plasma Cholesterol and Lipoproteins

Plasma cholesterol in both etoposide-treated and control rabbits increased progressively from a normal level of 40–60 mg/dl (NZW, 35±12.5 mg/dl; NZW-SPF, 50±8.5 mg/dl) to over 1,500 mg/dl after 4 weeks on the high-fat diet. The average daily plasma cholesterol level was calculated for each rabbit by integrating the areas under the cholesterol-versus-time curves obtained from the weekly plasma cholesterol measurements. There were equivalent increases in the average daily plasma cholesterol levels and body weights for both etoposide-treated and control rabbits (Table 1), and there were no significant differences in the average amount of diet eaten by each group. However, the amount of diet eaten by each rabbit was not significantly correlated to either the daily plasma cholesterol level or the plasma cholesterol level measured at the time the animals were killed.
TABLE 1. Average Weights and Plasma Cholesterol Levels for Control and Etoposide-Treated Rabbits

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Weight (kg)*</th>
<th>Plasma cholesterol (mg/dl)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZW rabbits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=12)</td>
<td>3.1±0.3</td>
<td>1,906±513</td>
</tr>
<tr>
<td>Etoposide (n=11)</td>
<td>3.0±0.2</td>
<td>1,470±498</td>
</tr>
<tr>
<td>NZW-SPF rabbits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=9)</td>
<td>2.9±0.2</td>
<td>1,614±479</td>
</tr>
<tr>
<td>Etoposide (n=11)</td>
<td>2.8±0.1</td>
<td>1,474±391</td>
</tr>
</tbody>
</table>

NZW, New Zealand White; SPF, specific pathogen free.
*Mean±SD of all weekly weight determinations for each group of animals.
†Mean±SD of average daily plasma cholesterol values for each group of animals.

Plasma triglyceride levels increased slightly from a basal level of 50–90 mg/dl (NZW, 58±18 mg/dl; NZW-SPF, 82±31 mg/dl) with the high-fat diet and seemed to be accentuated when plasma cholesterol levels were over 1,000 mg/dl (NZW, 181±93 mg/dl; NZW-SPF, 97±57 mg/dl). Agarose gel electrophoresis of serum samples obtained at various intervals during cholesterol feeding demonstrated that the hypercholesterolemia seen in all of the cholesterol-fed rabbits was accompanied by the appearance of a characteristic β-VLDL particle, which is considered to be atherogenic.25

To determine if lipoproteins from the etoposide-treated rabbits could be internalized by macrophage-like cells to produce foam cells, J774 macrophages were incubated with normolipemic rabbit serum and with hyperlipemic serum obtained from untreated and etoposide-treated rabbits that had been on the atherogenic diet for 4 weeks. As shown in Table 2, when added at equivalent cholesterol concentrations, hyperlipemic sera from either drug-treated or control rabbits caused a similar increase in cellular cholesterol. As expected, the excess cholesterol was stored as cholesteryl ester in droplets that were evident in the lipid-loaded cells when these were examined by phase microscopy.29

Effects of Etoposide on Aortic Atherosclerosis

Since a high-cholesterol diet induces lipid-rich atherosclerotic lesions in the rabbit aorta, Sudan IV was used to quantify the extent of intimal area covered by plaque.26,27 Pooled data obtained from both conventional and NZW-SPF rabbits demonstrated a significant difference in the percentage of the intimal surface that was stained with Sudan IV in the etoposide-treated group compared with the control group (control, 37±25% versus etoposide, 13±15%, p<0.001). Figure 1 shows the extent of Sudan IV staining in each vessel as

TABLE 2. Cholesterol Accumulation in J774 Cells Exposed to Serum From Control and Etoposide-Treated Rabbits

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total</th>
<th>Unesterified</th>
<th>Esterified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10% FBS (0.034 mg/ml cholesterol)</td>
<td>29±1</td>
<td>29±1</td>
<td>0</td>
</tr>
<tr>
<td>2. 10% rabbit serum+etoposide (0.030 mg/ml cholesterol)</td>
<td>21±1</td>
<td>21±1</td>
<td>0</td>
</tr>
<tr>
<td>3. 10% HRS (2.25 mg/ml cholesterol)</td>
<td>554±48</td>
<td>211±21</td>
<td>343±27</td>
</tr>
<tr>
<td>4. 10% HRS+etoposide (2.24 mg/ml cholesterol)</td>
<td>477±30</td>
<td>168±8</td>
<td>309±22</td>
</tr>
</tbody>
</table>

FBS, fetal bovine serum; HRS, hyperlipemic rabbit serum. Confluent monolayers of J774 cells were incubated with the indicated sera for 24 hours, after which the cholesterol content was determined as described in "Methods." Normal rabbit serum plus etoposide was pooled from three rabbits. Hyperlipemic rabbit serum was obtained from single rabbits. Three different hyperlipemic sera from etoposide-treated rabbits were tested. Data shown are from a typical experiment. Values are mean±SD of three determinations.
a function of the average daily plasma cholesterol concentration. The data are presented separately for each group of rabbits. As shown in Figure 1A (conventional NZW rabbits) and Figure 1B (NZW-SPF rabbits), etoposide-treated animals developed fewer Sudan IV-positive lesions at all levels of hyperlipemia, and an analysis of covariance of the extent of lesions compared with the area under the cholesterol-versus-time curve for each rabbit demonstrated that this difference was significant in each experiment (significance of \( F = 0.022 \) for experiment 1 and 0.030 for experiment 2). We measured the cholesterol content of the aortas obtained in the second experiment. A comparison of the extent of intimal area covered by Sudan IV-positive lesions and the cholesterol content of the tissue demonstrated a significant correlation for both etoposide-treated animals (\( r = 0.74 \)) and the untreated control animals (\( r = 0.84 \)), and the average cholesterol content of the aortas in the control group showed a trend toward an elevated value compared with the drug-treated group by the Mann-Whitney two-sample test (\( p = 0.063 \)). Despite quantitative differences in the extent of plaque, as expected the lesions were most frequently found in the aortic arch and at the intercostal ostia in the thoracic aorta of both control and etoposide-treated animals in both experiments.\(^{17}\)

**Effect of Etoposide on the Microanatomy of Atherosclerotic Plaques**

The thickness and cellular composition of cholesterol-fed atherosclerotic plaques in etoposide-treated rabbits differed from those of controls. The mean thickness of plaques in the etoposide-treated group was 88.5 ± 16.9 μm compared with 138.2 ± 16.0 μm in the control group (\( p = 0.0243 \)). The plaques in control animals showed considerable morphological heterogeneity in that the central areas of the plaques were predominantly cellular, with only focal accumulation of fibrous (trichrome- or elastin-stain-positive) tissue (Figures 2A and 2E), whereas peripheral areas of plaques were often less cellular and contained substantial accumulations of fibrous tissue (Figures 2C and 2G). In contrast, atherosclerotic plaques in etoposide-treated animals appeared to be more homogeneous and contained sparser accumulations of fibrous tissue than did plaques in control animals (Figures 2B, 2D, 2F, and 2H). Mononuclear antibodies reactive for either a macrophage-specific antigen (RAM-11) or muscle-specific actin (HHF-35) were used in immunohistochemical assays to analyze the cellular constituents of atherosclerotic plaques. As expected, plaques in control animals contained large, globular foam cells that were RAM-11 positive and smaller, spindle-shaped foam cells that were HHF-35 positive; these are designated macrophage-derived foam cells (MFCs) or smooth muscle cell-derived foam cells (SMFCs), respectively. In control animals, MFCs and SMFCs were not uniformly distributed within plaques. MFCs were relatively more abundant in regions in which extracellular tissue was sparse (Figure 2I). Conversely, SMFCs were relatively more abundant in regions rich in fibrous tissue (Figure 2O). In contrast to the control animals, fewer SMFCs appeared to be present within plaques of etoposide-treated animals (Figures 2J, 2L, 2M, and 2P). To determine if these histological impressions of quantita-tive differences in plaque components between the two groups of rabbits were significant, slides stained with either Masson’s trichrome or HHF-35 monoclonal antibody were scored in a blinded analysis by four independent observers as described in “Methods.” Average scores for trichrome-positive collagen material were significantly different by a one-tailed \( t \) test (\( p = 0.0046 \)). Average scores for the HHF-35 stain for SMFCs were also significant by one-tailed \( t \) test (\( p = 0.0498 \)). Thus, the plaque that formed in the control animals was enriched in fibrous tissue and showed a trend toward a higher content of SMFCs.

**Effects of Etoposide Treatment on Blood Cell Populations**

Since an initial aim of this investigation was to employ etoposide to reduce the number of circulating monocytes and to determine if such a reduction influenced plaque development, careful attention was given to monitoring monocyte levels throughout the entire treatment period. Coulter counts of RBCs and WBCs, as well as differential counts on blood smears, were performed for all animals on a weekly basis. Platelet counts were done every 3–4 weeks. In all rabbits fed cholesterol, there was a significant decrease in the RBC count and hematocrit when compared with rabbits fed a standard diet; however, no decreases in the total WBC count were seen with either the high-cholesterol diet alone or the high-cholesterol diet and etoposide (Table 3). The platelet count was the same in both drug-treated and untreated cholesterol-fed rabbits (data not shown). Etoposide treatment did not change the average lymphocyte count but did cause a slight but significant increase in the number of granulocytes in NZW rabbits (Table 3).

Since both the percentage of the WBC population represented by monocytes as well as the absolute number of WBCs can vary widely in normal rabbits, there was considerable overlap in the absolute monocyte counts of both control and etoposide-treated groups throughout the experiment. Thus, it was difficult to assess whether the etoposide-treated rabbits were consistently different in their monocyte counts throughout their 8 weeks on the high-cholesterol diet, and a stringent statistical analysis of all the data points using analysis of covariance could not demonstrate statistical differences between etoposide-treated and control animals with either conventional or NZW-SPF rabbits (data not presented). Moreover, the average monocyte count during treatment could not be statistically correlated with the extent of plaque measured with Sudan IV staining (\( r = 0.216 \)).

**Discussion**

Our experimental results demonstrate that treatment of cholesterol-fed rabbits with the drug etoposide causes a statistically significant decrease in the extent of plaque formed in the aortic intima. The decrease in plaque formation is independent of the extent of diet-induced hyperlipemia (Figure 1). In both control and treated animals, plaque was most frequently found in the aortic arch and at the intercostal ostia in the thoracic aorta. The lesions formed in the absence of etoposide resembled early fibrous plaques, which have been described in rabbits and nonhuman pri-
FIGURE 2. Photomicrographs showing the effect of etoposide on the microanatomy of atherosclerotic plaques. Panels A–D show sections stained with the Verhoeff–van Gieson technique for elastin in which elastin fibers stain black (arrow indicates focal elastin accumulation in panel A). Panels E–H show sections stained with the Masson's trichrome technique, in which collagen fibers stain blue (arrow indicates focal collagen accumulation in panel E). Panels I–P are immunoperoxidase assays, in which tissue sections were reacted with either monoclonal antibody RAM-II, directed against a rabbit macrophage-specific antigen (panels I–L) or monoclonal antibody HHF-35, directed against muscle cell specific actin (panels M–P; arrows indicate scattered HHF-35–positive foam cells in panel N). Panels I–P have been counterstained with hematoxylin. Bar represents 100 μm.
Table 3. Blood Cell Counts of Control and Etoposide-Treated Rabbits

<table>
<thead>
<tr>
<th>Experiment</th>
<th>RBCs (counts/1x10^12)</th>
<th>WBCs (counts/1x10^9)</th>
<th>Lymphocytes (counts/1x10^9)</th>
<th>Granulocytes (counts/1x10^9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>NZW rabbits</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=12)</td>
<td>5.4±0.6</td>
<td>3.9±0.3*</td>
<td>6.8±1.3</td>
<td>6.7±2.6</td>
</tr>
<tr>
<td>Etoposide (n=11)</td>
<td>5.4±0.6</td>
<td>3.4±0.7*</td>
<td>6.8±1.3</td>
<td>6.4±1.7</td>
</tr>
<tr>
<td>NZW-SFP rabbits</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=9)</td>
<td>5.0±0.5</td>
<td>4.1±0.5*</td>
<td>8.0±1.8</td>
<td>9.8±4.6</td>
</tr>
<tr>
<td>Etoposide (n=11)</td>
<td>5.0±0.5</td>
<td>3.5±0.5*</td>
<td>8.0±1.8</td>
<td>9.6±1.4</td>
</tr>
<tr>
<td>Published values</td>
<td>5.3 (4.5-6.1)</td>
<td>9.7 (5.1-18.4)</td>
<td>4.9 (2.6-9.2)</td>
<td>2.2 (0.6-6.7)</td>
</tr>
</tbody>
</table>

RBCs, red blood cells; WBCs, white blood cells; NZW, New Zealand White; SPF, specific pathogen free. Values represent mean±SD of all measurements (see "Methods") before (average for all rabbits) and after (average for rabbits in each group) the treatment phase of the experiment. Published values are from Reference 32. *p<0.05.

mation, which represents a developmental transition between the fatty streak and more advanced lesions. In contrast, the atherosclerotic lesions in cholesterol-fed rabbits that had been treated with the cytostatic drug etoposide were less extensive and thinner and, at least focally, contained less fibrous tissue and SMFCs when compared with the more fibrous lesions seen in the untreated control animals. Since numerous studies in animal and human arterial tissues suggest that with time some fatty streaks develop into fibrous plaques, we propose that etoposide treatment suppresses this conversion and therefore reduces the progression of early atherosclerotic lesions to more advanced plaques.

The mechanism by which etoposide suppresses plaque progression and development is not established, but several observations indicate that its action in this study is not solely due to a systemic effect of the drug. First, there were no significant differences in total plasma cholesterol concentrations between drug-treated and the control rabbits, and it was shown that all cholesterol-fed rabbits had circulating β-VLDL. Moreover, hyperlipemic serum from drug-treated animals was shown to induce macrophage foam cell formation in vitro. Thus, etoposide treatment did not interfere with the intestinal absorption of cholesterol or the formation of abnormal, remnant-like lipoproteins. Second, although etoposide has been shown to cause monocyteopenia in short-term experiments with Chinchilla rabbits, no consistent, significant difference in the average absolute monocyte number could be demonstrated between etoposide-treated and untreated rabbits during the 8 weeks the rabbits were on a high-cholesterol diet. Likewise, there was no difference in the total RBC, WBC, or platelet count between these two groups. Thus, the reduction in atherosclerotic plaque development seen with etoposide treatment does not appear to be due to a significant drug-induced reduction in the number of circulating monocytes or platelets, cells that have been implicated in atherogenesis. Third, the prominent accumulation of MFCs in the atherosclerotic lesions of the etoposide-treated animals suggests that the drug does not interfere with either the recruitment of monocytes/macrophages into areas of the vessel wall prone to lesion development or the intracellular accumulation of lipid that leads to the appearance of foam cells. However, the observation that both the extent and thickness of lesions are diminished in etoposide-treated animals does suggest that drug treatment results in a quantitative defect in overall plaque formation. Moreover, the fatty streaks that formed in the etoposide-treated animals did not seem to readily mature to more advanced fibrous plaques.

The results presented here raise the possibility that etoposide affects processes that are intrinsic to the plaque. It is thought that the progression of atherosclerotic plaques from fatty streaks to more advanced fibrous lesions is closely related to the cellular components of these lesions and that the appearance and proliferation of SMFCs in the vascular intima heralds this transition. Our results indicate that the accumulation of SMFCs and fibroelastic tissue in plaques is not solely related to the duration of exposure of the vessel wall to elevated levels of plasma cholesterol, since both etoposide-treated and control rabbits became hyperlipemic shortly after the start of the atherogenic diet. However, we did find that in control rabbits there was a spatial correlation between the accumulation of SMFCs in plaques and the presence of fibroelastic tissue. This was particularly evident at the periphery of plaques but could also be seen in other areas of plaques that contained accumulations of SMFCs. Since the population of SMFCs may influence the composition of the extracellular matrix in the atherosclerotic plaque, agents that disrupt the accumulation of SMFCs could suppress fibroelastic plaque development. In addition, since the appearance of SMFCs in plaques is at least partially dependent on the intimal proliferation of smooth muscle cells and etoposide has been shown to be cytostatic for a variety of cell types, we speculate that the etoposide-mediated suppression of fibroelastic plaque development may be at least partially explained by the inhibition of smooth muscle cell proliferation in the intima. The cytostatic effect of etoposide may also explain the decreased extent of fatty streak formation in the treated rabbits, since experimental evidence suggests that areas of active endothelial cell proliferation are more permeable to lipoprotein and other blood components and the accumulation of these constituents is thought to be a precursor of plaque formation. Therefore, it is possible that the frequency of such high-permeability areas is reduced because of the cytostatic effects of etoposide. Finally, there is evidence that macrophage proliferation occurs at sites of lesions and
that this step may be also impaired in etoposide-treated rabbits, thereby diminishing the extent of atherosclerotic lesions.

Our results do not eliminate the possibility that etoposide interferes with atherosclerotic plaque development by mechanisms other than cytostatic effects. For example, there is much evidence to suggest that growth and chemotactic factors may be locally released from various cells to act in a paracrine fashion at sites of plaque development. It is possible that etoposide could interfere with these putative mechanisms by inhibiting the maturation or activation of monocytes or the activation of tissue macrophages, so that the monocyte-derived foam cells found in lesions of drug-treated rabbits lack some as-yet-undefined paracrine function critical to the progression of plaques.

Although future studies are needed to identify the mechanism by which etoposide inhibits atherosclerotic lesions, it is clear that this animal model offers an opportunity to investigate factors that promote the initiation and progression of the macrophage-rich fatty streak and to develop therapeutic interventions that retard plaque development.

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