Dexamethasone-Induced Suppression of Aortic Atherosclerosis in Cholesterol-Fed Rabbits
Possible Mechanisms

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We investigated the mechanisms by which corticosteroids affect atherosclerosis. Male New Zealand White rabbits were injected with 0.125 mg dexamethasone (n = 10) or vehicle (control group, n = 10). Both groups were fed a 1% cholesterol diet for 8 weeks. Although the dexamethasone-treated animals exhibited a greater degree of hyperlipidemia, they exhibited significantly less atherosclerotic plaque of the aortic surface than control animals (7.8% versus 47.2%). Immunofluorescence study of the aortic plaque specimens showed that dexamethasone administration reduced both macrophages and T lymphocytes. In vitro, dexamethasone suppressed the proliferation and differentiation of U937 cells and inhibited uptake and degradation of β-very low density lipoproteins by mouse peritoneal macrophages. These findings suggest that dexamethasone suppresses the development of atherosclerosis in the aorta of rabbits by inhibiting recruitment and proliferation of macrophages and the formation of foam cells in plaques. (Arteriosclerosis and Thrombosis 1993;13:892–899)

Key Words • dexamethasone • atherosclerosis • macrophages • rabbits • pathogenesis

Atherosclerotic plaque is characterized by intimal cell proliferation, lipid accumulation, and connective tissue deposition. Because plaque has features similar to those associated with chronic inflammation, such as the accumulation of macrophages, anti-inflammatory agents have been evaluated in the treatment of atherosclerosis. Anti-inflammatory drugs, including corticosteroids, have been found to suppress the development of atherosclerosis in both cholesterol-fed New Zealand White rabbits and Watanabe heritable hyperlipidemic rabbits. In a different model of experimental atherosclerosis, dexamethasone inhibited both the leukocyte accumulation and intimal thickening induced by cuff sheathing in the rabbit carotid artery; indomethacin, a cyclooxygenase inhibitor, had little effect. However, the mechanisms by which corticosteroids exert an inhibitory effect on atherosclerotic plaque formation remain to be elucidated. In particular, the phenomenon of reduced plaque formation in the presence of enhanced hyperlipidemia in corticosteroid-treated, cholesterol-fed rabbits has not been explained. Since monocyte-derived macrophages are thought to play an important role in atherogenesis, we attempted to clarify the mechanism of action of corticosteroids by focusing on the effect of corticosteroid treatment on macrophages. We investigated the effects of dexamethasone on plasma lipoproteins and on the distribution of macrophages and T lymphocytes in plaques of cholesterol-fed rabbits. We also conducted in vitro experiments using monocyte/macrophage-like cells.

Methods

Animal Experiments

Twenty male New Zealand White rabbits weighing 2.2–2.5 kg were divided into two groups: a dexamethasone-treated group (n = 10) and a control group (n = 10). Both groups were fed a 1% cholesterol diet for 8 weeks. During those 8 weeks, the dexamethasone-treated group received daily injections of 0.125 mg i.m. (0.050–0.057 mg/kg body wt) of dexamethasone (Banyu Pharmaceutical Co., Tokyo) in 0.1 mL of vehicle. The control group received only vehicle, which contained sodium bisulfate (0.5 mg/mL), methyl p-hydroxybenzoate (1.5 mg/mL), propyl p-hydroxybenzoate (0.2 mg/mL), and creatinine (8 mg/mL). Blood was sampled at 4-week intervals for the determination of hemoglobin, serum total cholesterol (TC), total protein, and glucose. Very low density lipoprotein (VLDL, d<1.006 g/mL) was separated from plasma by ultracentrifugation, and the concentrations of protein, TC, and triglyceride (TG) were measured. TC/TG was calculated as an index of the composition of lipids in VLDL. After 8 weeks, all rabbits were killed by injection of sodium pentobarbital (150 mg i.v.); the whole aorta was removed and incised longitudinally. The aortic surface involved by plaque was traced within 12 hours of removal of the aortas, assessed quantitatively by planimetry with a computer, and expressed as the proportion (percent) of the area of...
plaque involvement to that of the entire area of the aorta.

All experiments were conducted in accordance with institutional guidelines for animal studies.

**Immunofluorescence Study of Aortic Specimens**

Small portions were obtained from the aortic base from two representative animals in each group with the highest or the second highest serum cholesterol level, snap-frozen in liquid nitrogen, and stored at −80°C until studied. Frozen tissues were cut into 2-μm sections with a cryostat. Sections were fixed with acetone at room temperature for 10 minutes and air-dried. After being washed with 0.01 mol/L phosphate-buffered saline (PBS), the sections were incubated for 20 minutes with an optimal concentration of the mouse monoclonal anti-macrophage immunoglobulin M (IgM) antibody (HAM-56, Enzo Diagnostics, New York). The sections were washed three times with PBS and then incubated with fluorescein isothiocyanate (FITC)–conjugated rabbit antibody against mouse IgM (HAM-56). Similarly, to stain the T lymphocytes in plaques, the sections were first incubated with goat anti-rabbit T-lymphocyte polyclonal antibody (CL-8800, Cedarlane Laboratories, Hamby, Canada) and then stained with FITC-conjugated rabbit anti-goat IgG. In addition, small portions from the aortic base were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

**Proliferation and Differentiation of U937**

**Proliferation of U937 cells and [3H]thymidine incorporation into cellular DNA.** U937 cells (CRL 1593, American Type Culture Collection, Rockville, Md.) were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal calf serum (FCS, Cell Culture Laboratories, Cleveland, Ohio) in a humidified CO₂ incubator at 37°C. Cell growth was arrested in cultures of U937 cells at a cell concentration of ≈1x10⁴ cells/mL by transfer of cells to RPMI 1640 with 0.5% FCS; cells were incubated in this medium for 48 hours. The incubated cells were seeded in RPMI 1640 with 10% FCS (growth medium) at a density of 1.5−1.6x10⁶/mL and were incubated for 48 hours with dexamethasone in concentrations of 10⁻⁶ to 10⁻⁴ mol/L (Sigma Chemical Co., St. Louis, Mo.) dissolved in ethanol. The final concentration of ethanol in the incubation medium was 0.1% (vol/vol). After 48 hours of treatment, the cells were counted with a hemocytometer (model CC-130, Sysmex, Tokyo).

DNA synthesis during dexamethasone treatment was evaluated by [3H]thymidine (TdR) incorporation. The U937 cultures, which had been growth arrested and incubated for 48 hours in RPMI 1640 with 0.5% FCS, were incubated in a growth medium containing 10⁻⁶ to 10⁻⁴ mol/L dexamethasone and incubated for 24 hours. Aliquots (1 mL) with a density of 4x10⁶ cells/mL were incubated with 3.7 kBq/mL of [3H]TdR (NEN Research Products, Boston) for 2 hours at 37°C. The cells were then washed twice with Dulbecco’s PBS, and the radioactivity in trichloroacetic acid (TCA, Katayama Chemical, Osaka, Japan)–insoluble fractions was measured.

**Differentiation of U937 by phorbol esters.** The U937 cell line is a well-characterized representative of monocytic cells that, on stimulation with differentiation-inducing agents such as phorbol esters, mature along the monocytic pathway. When stimulated by phorbol 12-myristate 13-acetate (PMA, Sigma), these cells adhere...
Binding, Uptake, and Degradation of β-VLDL by
Mouse Peritoneal Macrophages

Unstimulated ddY mice weighing 25–30 g were anesthetized with ethyl ether in air, and peritoneal cells were harvested in Dulbecco's PBS.11 Cells were collected by centrifugation (400g, 10 minutes) and washed once with Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co.). Aliquots (1 mL) were dispensed into 12-well dishes (Corning 25815, Corning, N.Y.) in DMEM containing 20% (vol/vol) FCS and then incubated with the indicated concentration of DMEM containing 0.3% bovine serum albumin (BSA, Sigma) by the iodine monochloride methanol extraction. After the medium was replaced by DMEM containing 0.3% bovine serum albumin (BSA, Sigma) with or without 10⁻⁷ mol/L dexamethasone, the sample was assayed to determine the radioactivity associated with the cells.13-14 In all cases, the cells were incubated with the indicated concentration of ¹²⁵I-β-VLDL in the presence or absence of a 20-fold excess of unlabeled β-VLDL. Specific binding, uptake, or degradation was calculated by subtracting the amount of ¹²⁵I-β-VLDL measured in the presence of unlabeled β-VLDL from the amount measured in the absence of unlabeled β-VLDL.

Each data point represents the average of duplicate incubations. The protein content of the lipoproteins and cell extracts was determined by the method of Lowry et al.15

Other Assays

Serum levels of cholesterol,16 TG,17 total protein, and glucose were determined by established procedures. Data are expressed as mean±SD. Statistical significance was determined by Student's t test, with the probability level set at p<0.05.

Results

Macroscopic and Microscopic Findings

As illustrated in Figures 1A and 1B, dexamethasone suppressed plaque involvement of the aortic surface by 83% compared with control rabbits (p<0.001). Microscopic study of plaques from the aortic base showed a markedly raised intima with proliferation of foam cells in control rabbits. In dexamethasone-treated rabbits, the lesions consisted of fewer layers of foam cells.

Immunofluorescence Study

Immunofluorescence microscopic study using anti-macrophage monoclonal antibody (HAM-56) showed clusters of positively stained cells in atherosclerotic plaques of the control animals (Figure 2A). There was a marked decrease in these cells in the dexamethasone-treated rabbits (Figure 2B). Similarly, aortic specimens
stained with anti-T-lymphocyte polyclonal antibody (CL-8800) showed some T lymphocytes in the plaques of the control rabbits but almost no T lymphocytes in aortic specimens from dexamethasone-treated animals (Figures 2C and 2D).

**Body Weight, Organ Weight, and Laboratory Parameters**

The serum TC level in the dexamethasone-treated group was more than twice that of the control group (Table 1): 1,804±612 mg/dL (control) versus 4,089±1,181 mg/dL (dexamethasone-treated) (p<0.001). Serum total protein levels in the dexamethasone-treated group were significantly higher than in the control group (p<0.001). Hemoglobin and serum glucose did not differ significantly between the two groups. Baseline body weights were similar in both groups: 2,266 ±163 g (control) versus 2,266±111 g (dexamethasone-treated). At autopsy, the body weight of the dexamethasone-treated animals was significantly lower (p<0.001) at 4 weeks but was significantly higher (p<0.001) at 8 weeks.

**Protein, TC, TG, and TC/TG Levels**

Hemoglobin and serum glucose did not differ significantly between the control rabbits but almost no T lymphocytes in aortic specimens from dexamethasone-treated animals (Figures 2C and 2D).

**Analysis of VLDL Fractions**

Analysis of VLDL fractions revealed a significant increase in protein (p<0.01), TC (p<0.01), and TG (p<0.001) in the dexamethasone-treated group compared with the control group at 4 weeks (Table 2). In the dexamethasone-treated group, the TC/TG was significantly lower (p<0.001) at 4 weeks but was significantly higher (p<0.05) at 8 weeks.

**Proliferation and Differentiation of U937 Cells**

The U937 cells used in this study consistently showed a viability of >95%, as determined by trypan blue dye exclusion. As shown in Figure 3, top panel, dexamethasone at concentrations of 10⁻⁸ to 10⁻⁶ mol/L significantly increased the cell viability (p<0.05; 10⁻⁷ to 10⁻⁶ mol/L, p<0.001) suppressed the increase in number of U937 cells at 48 hours of culture. Cell viability was >90% after treatment with 10⁻⁷ to 10⁻⁶ mol/L dexamethasone, similar to cell viability in the control group (Figures 3, top). When DNA synthesis was evaluated by [³H]Tdr incorporation, U937 cells incubated with 10⁻⁶ mol/L dexamethasone for 24 hours showed a significantly increased percentage of cells in S phase and a decreased percentage of cells in G₂/M phase (Figure 3, bottom). Again, cell viability did not differ between the control and dexamethasone-treated cells. These results suggest that dexamethasone suppressed the proliferation of U937 cells without causing significant toxic effects.

**Binding, Uptake, and Degradation of ¹²⁵I–β-VLDL by Mouse Peritoneal Macrophages**

The effects of dexamethasone at 10⁻⁷ mol/L on ¹²⁵I–β-VLDL binding, uptake, and degradation as a function of ligand concentration appear in Figure 5. Treatment with 10⁻⁷ mol/L dexamethasone did not alter the binding of ¹²⁵I–β-VLDL to mouse peritoneal macrophages (Figure 5, top panel). In contrast, dexamethasone treatment reduced ¹²⁵I–β-VLDL uptake and degradation (Figure 5, middle and bottom panels). The saturation curve of ¹²⁵I–β-VLDL degradation showed a pattern in the effect on receptor activity: the apparent Vₘₐₓ was reduced, whereas the Kₘ did not change.

**Discussion**

The mechanism by which corticosteroids inhibit diet-induced atherosclerosis in rabbits has not been established. Our results showed that dexamethasone reduced the development of grossly visible atherosclerotic plaques by an average of 83% in cholesterol-fed rabbits, despite the development of a greater degree of hyperlipidemia in treated animals. These findings are consistent with previous reports.²⁻⁶ Less plaque formation in the presence of more highly elevated serum cholesterol levels contradicts the assumption that the severity of atherosclerosis is proportional to serum cholesterol levels. We performed lipoprotein analysis, an immunofluorescence study of plaques, and in vitro experiments to examine the effects of dexamethasone on U937 cells and mouse peritoneal macrophages in an attempt to explain this paradox. Our analysis of VLDL fractions indicated that VLDL concentration was increased in dexamethasone-treated animals, but there was no con-
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FIGURE 3. Bar graphs showing effects of dexamethasone on proliferation of U937 cells. Upper panel: Cell count after 48 hours of culture. Cultures of U937 cells at a concentration of \( \geq 1 \times 10^6/\text{mL} \) were growth arrested by being transferred to RPMI 1640 with 0.5% fetal calf serum and were then incubated for 48 hours. The incubated cells in a concentration of \( 1.5-1.6 \times 10^5/\text{mL} \) were then incubated in the growth medium with or without (control cells) the indicated concentration of dexamethasone for 48 hours. The number of cells was counted by hemocytometer. Lower panel: [\(^3\)H]Thymidine (Tdr) incorporation into cellular DNA. U937 cells, growth arrested and incubated as in upper panel, were incubated in growth medium containing the indicated concentration of dexamethasone for 24 hours. Aliquots (1 mL, \( 4 \times 10^5 \) cells/mL) were incubated with 3.7 kBq/L [\(^3\)H]TdR for 2 hours. After aliquots were washed with Dulbecco’s phosphate-buffered saline, radioactivity in trichloroacetic acid–insoluble fractions was counted. Each point represents the mean of five incubations. The cell viability was determined by trypan blue dye exclusion. Each point represents the average of triplicate determinations. •, Cell viability without dexamethasone; ○, cell viability with the indicated concentration of dexamethasone. *p<0.05; ***p<0.001 (vs. control).

FIGURE 4. Bar graph showing adherent U937 cells (%) after 72 hours of incubation with phorbol 12-myristate 13-acetate (PMA) and dexamethasone. U937 cells (3.5-3.6x10^5/mL) were incubated with 10^-7 mol/L PMA for 72 hours in the absence or presence of the indicated concentration of dexamethasone. For the dimethyl sulfoxide (DMSO) control, the cells were incubated in growth medium containing only 0.1% DMSO and ethanol. Percent of adherent cells is expressed as number of adherent cells divided by number of nonadherent cells plus adherent cells times 100 (%). Each point represents the mean of six incubations. The viability of residual nonadherent cells is expressed as the average of triplicate determinations. □, Cell viability without dexamethasone; •, cell viability with dexamethasone. ***p<0.001 (vs. 0).

Persistent difference in TC/TG of VLDL fractions between the two groups throughout the experiment.

Macrophages specifically and saturably bind glucocorticoids via their cytosol receptors,18 which, in turn, modulate numerous macrophage functions.19 Our in vitro results using the monocyte/macrophage cell line U937 showed that dexamethasone suppressed not only proliferation of these cells but also their PMA-induced differentiation without significantly affecting cell viability. These observations are consistent with previous reports.10,20 It has been shown that proliferating macrophages exist in atherosclerotic lesions from cholesterol-fed rabbits.21 The systemic administration of corticosteroids has induced a prolonged monocytopenia in blood and a characteristic reduction of mononuclear phagocytes at the site of inflammation during steroid treatment.22 Moreover, corticosteroids have also been found to reduce monocyte chemotaxis in response to various inducers.23 Together with these findings, our immunofluorescence study data suggest that dexamethasone inhibited the recruitment and proliferation of monocyte-derived macrophages in the aortic wall.

Although dexamethasone did not alter the binding of \( ^{125}\text{I}-\beta\)-VLDL to mouse peritoneal macrophages, it reduced \( ^{125}\text{I}-\beta\)-VLDL uptake and degradation. Related studies conducted under somewhat different experimental conditions have shown that exposure of cultured fibroblasts and arterial smooth muscle cells to physiological concentrations of hydrocortisone (4.1 \times 10^{-8} \text{ mol/L}),\) of cultured human monocyte-derived macrophages to dexamethasone,23 and of cultured human fibroblasts to serum from glucocorticoid-treated patients26 reduces their capacity to degrade low density lipoprotein (LDL). Recent reports indicate that uptake of \( ^{125}\text{I}-\beta\)-VLDL is mediated by the LDL receptor,27-29 suggesting that, in our experiments, dexamethasone suppressed the degradation of \( ^{125}\text{I}-\beta\)-VLDL via the LDL pathway. Our results with dexamethasone are similar to those in an earlier report24 showing that hydrocortisone reduced LDL uptake and degradation by cultured human fibroblasts without affecting binding. A decreased uptake of \( ^{125}\text{I}-\beta\)-VLDL to macrophages in association with unchanged binding suggests a dexamethasone-related internalization defect of \( ^{125}\text{I}-\beta\)-VLDL, as Henze et al24 demonstrated.
preincubated with or without 10⁻⁷ mol/L dexamethasone for 36 hours at 37°C. Then cells were incubated at 4°C for 2 hours with the indicated concentration of 125I-B-VLDL with or without 10⁻⁷ mol/L dexamethasone. Cell-associated 125I-B-VLDL was determined. In all studies of binding, uptake, and degradation, incubation with 125I-B-VLDL was carried out in the presence or absence of a 20-fold excess of unlabeled B-VLDL. Each point is the average of duplicate determinations.

FIGURE 5. Top panel: Binding curve of 125I-B-very low density lipoprotein (VLDL) to mouse peritoneal macrophages. Monolayers of mouse peritoneal macrophages were preincubated with or without 10⁻⁷ mol/L dexamethasone for 36 hours at 37°C. Then cells were incubated at 4°C for 2 hours with the indicated concentration of 125I-B-VLDL with or without 10⁻⁷ mol/L dexamethasone. Cell-associated 125I-B-VLDL was determined. Middle panel: Uptake of 125I-B-VLDL. Mouse peritoneal macrophages were incubated with 125I-B-VLDL at 37°C for 5 hours, and cell-associated 125I-B-VLDL was measured. Bottom panel: Degradation of 125I-B-VLDL. After cells were incubated at 37°C for 5 hours with the indicated concentration of 125I-B-VLDL, the amount of 125I-labeled trichloroacetic acid-soluble materials excreted into the medium was determined. In all studies of binding, uptake, and degradation, incubation with 125I-B-VLDL was carried out in the presence or absence of a 20-fold excess of unlabeled B-VLDL. Each point is the average of duplicate determinations.

using LDL. When Henze et al measured the net LDL uptake to cultured fibroblasts and arterial smooth muscle cells by trypsinization, they found that hydrocortisone reduced the internalization of LDL. In addition to an enhanced hepatic production or secretion of VLDL caused by dexamethasone administration, these alterations in β-VLDL metabolism in macrophages may play a role in the dexamethasone-induced enhancement of hyperlipidemia. It is conceivable that, despite the enhanced hyperlipidemia induced by a high-cholesterol diet, dexamethasone suppressed macrophages that would be expected to take up and degrade excess β-VLDL in the aortic wall, leading to a decrease in the formation of foam cells. However, a toxic effect of dexamethasone on macrophages can be excluded, since we observed no difference in the amount of cell protein or the number of cells (data not shown).

These observations suggest that dexamethasone enhances the accumulation of β-VLDL in plasma but suppresses the recruitment of monocyte-derived macrophages to the aortic wall, as evidenced by the presence of fewer macrophages in the plaques of the dexamethasone-treated rabbits. In addition, this agent may have inhibited foam cell formation via a reduction in the uptake of β-VLDL by macrophages.

The T lymphocytes frequently found in association with macrophages in plaques may be involved in atherogenesis. Immunofluorescence study in our experiments showed that dexamethasone greatly diminished T lymphocytes in plaques. Since corticosteroids suppress the production of interleukin-1 by macrophages, they may thus exert an inhibitory effect on T-cell accumulation in plaques.

Corticosteroids have been used to treat a variety of chronic diseases, such as rheumatoid arthritis and systemic lupus erythematosus, over long periods. Nevertheless, there is little information on the effects of corticosteroids on atherosclerosis. In humans, retrospective studies, including pathological data obtained at autopsy, suggested that these agents, through their hyperlipidemic action, adversely affected atherogenesis. The inconsistency between such reports and our experimental findings raises the question of whether corticosteroids may exert a different action on atherogenesis in humans versus cholesterol-fed rabbits. Although we have no data to answer that question, recent studies using cultured human monocyte-derived macrophages have shown that dexamethasone stimulates acetyl-LDL receptor activity. If this were true in vivo, dexamethasone would augment the scavenger receptor activity of the macrophages for the denatured LDL, which is thought to play a central role in atherogenesis, and would thus accelerate atherosclerosis in humans. Therefore, differing atherogenic lipoproteins in humans and cholesterol-fed rabbits may account for the different response of macrophages to corticosteroid treatment.

The dose of dexamethasone (0.050–0.057 mg/kg) used in our animal experiment is comparable to that administered clinically. However, when administered in vivo for long periods as in this experiment, dexamethasone causes a variety of side effects, including growth retardation or atrophy of adrenal glands (Table 1), indicating that the antiatherogenic effect of dexamethasone cannot be practically applied in the clinical setting. Nonetheless, our data show that, irrespective of the presence of hyperlipidemia, atherosclerotic plaque formation can be suppressed by modulating the function of macrophages. Recently, the suppression of aortic atherosclerosis by purified rabbit interferon was observed in cholesterol-fed rabbits in which, interestingly, interferon administration did not change the
serum lipid levels but decreased the number of foam cells of macrophage origin in the atherosclerotic lesions. This result, as well as our findings, suggests that approaches other than the lowering of serum cholesterol that may affect the inflammatory aspects of atherosclerosis may protect against atherogenesis.

In conclusion, our observations suggest that dexamethasone suppresses the recruitment and proliferation of macrophages and the formation of foam cells in atherosclerotic lesions, thereby inhibiting the development of aortic atherosclerosis in cholesterol-fed rabbits. These findings may provide new insights into the pharmacological control of atherosclerosis.

Acknowledgments

We would like to thank S. Aoyama, M. Doi, and K. Adachi for their excellent technical assistance.

References

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Possible mechanisms.
K Asai, C Funaki, T Hayashi, K Yamada, M Naito, M Kuzuya, F Yoshida, N Yoshimine and F 
Kuzuya

doi: 10.1161/01.ATV.13.6.892
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville 
Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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