Glucocorticoid Resistance Caused by Reduced Expression of the Glucocorticoid Receptor in Cells From Human Vascular Lesions

Paula J. Bray, Baoheng Du, Victor M. Mejia, Steven C. Hao, Ezra Deutsch, Chenzhong Fu, Robert C. Wilson, Hartmut Hanauske-Abel, Timothy A. McCaffrey

Abstract—Mechanisms that control the balance between cell proliferation and death are important in the development of vascular lesions. Rat primary smooth muscle cells were 80% inhibited by low microgram doses of hydrocortisone (HC) and 50% inhibited by nanogram concentrations of transforming growth factor-β1 (TGF-β1), although some lines acquired resistance in late passage. However, comparable doses of HC, or TGF-β1, failed to inhibit most human lesion-derived cell (LDC) lines. In sensitive LDC, HC (10 μg/mL) inhibited proliferation by up to 50%, with obvious apoptosis in some lines, and TGF-β1 inhibited proliferation by more than 90%. Collagen production, as measured by [3H]proline incorporation or RIA for type III procollagen, was either unaffected or increased in the LDCs by HC. These divergent responses between LDC lines were partially explained by the absence of the glucocorticoid receptor (GR) and heat shock protein 90 mRNA in 10 of 12 LDC lines, but the presence of the mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase type II. Western blot analysis confirmed the absence of the GR protein in cells lacking GR mRNA. Immunohistochemistry of human carotid lesions showed high levels of GR in the tunica media, but large areas lacking GR in the fibrous lesion. Considering the absence of the GR in most lines, the effects of HC may be elicited through the mineralocorticoid receptor. Functional resistance to the antiproliferative and antifibrotic effects of HC may contribute to excessive wound repair in atherosclerosis and restenosis. (Arterioscler Thromb Vasc Biol. 1999;19:1180-1189.)

Key Words: glucocorticoid receptor ■ transforming growth factor-β1 ■ atherosclerosis ■ cell proliferation ■ collagen production ■ smooth muscle cells ■ restenosis

Restenotic narrowing of arteries after angioplasty or endarterectomy is caused principally by fibroproliferative intimal hyperplasia and contractile remodeling by a cell resembling both the vascular smooth muscle cell (SMC) and myofibroblast.1,2 Serial angiography after angioplasty suggests that almost all patients will exhibit a fibroproliferative narrowing after angioplasty, although in most patients the stenosis will spontaneously regress to exhibit a net luminal gain.3 However, in 40% of patients, this lesion fails to regress, but instead progresses to clinical restenosis (reviewed in Reference 4). Restenosis appears to be caused by the migration of cells into the intima, subsequent proliferation, and extracellular matrix deposition. In animal models, neointimal regression is caused by apoptosis of the lesion cells.5 In humans, growing evidence suggests that restenosis may be caused by a failure in growth inhibitory and apoptotic systems that would normally mediate lesion regression.6,7 Thus, the persistence and slow proliferation of cells in the injured vessel wall may be caused by a failure in endogenous inhibitory systems.

One defective inhibitory system involves the transforming growth factor-β1 (TGF-β1) receptor pathway. Lesion-derived cells (LDCs) from human atherosclerotic plaques are resistant to the inhibitory effects of TGF-β1 because of a decrease in the TGF-β type II signaling receptor.7 In some patients, microsatellite instability in the TGF-β type II receptor is responsible for the resistance to TGF-β1 in LDCs.8 Another major inhibitory system of SMC proliferation are glucocorticoids (GCs). When the hormone is bound to the glucocorticoid receptor (GR) complex, a fibrosuppressive effect is exerted, which is elicited in several ways: inhibition of SMC proliferation in vitro,9,10 inhibition of collagen production,11–13 induction of apoptosis in lymphocytes and fibroblasts (for review see Reference 14), and anti-inflammatory or immunosuppressive actions.15,16 Given these diverse antifibrotic and antiproliferative actions, GCs should be good candidates for preventing restenosis.

Animal studies have generally supported a beneficial effect of GCs on both primary, cholesterol-induced atherosclerosis and postangioplasty restenosis. In cholesterol models of...
atherosclerosis, treatment with hydrocortisone has consistently been shown to lessen the severity of atherosclerosis in various rabbit models. In models of balloon-catheter injury, GCs typically inhibit neointimal proliferation in vivo in both rat and rabbit models, although shorter, 1-week treatments with dexamethasone have failed to inhibit intimal hyperplasia in rabbits. Despite these in vivo effects of steroids, 2 clinical trials have failed to observe a beneficial role of GCs in preventing restenosis in humans. Both studies administered high doses of prednisolone, a synthetic GC, after successful coronary angioplasty, and both concluded that high-dose steroid therapy does not influence the overall rate of restenosis after coronary angioplasty when compared with the untreated control groups.

In vitro studies have observed that corticosteroids inhibit proliferation of SMCs derived from atherosclerotic arteries. However, to see any significant decrease in cell number ($60\%$ to $70\%$), highly concentrated doses of steroids were needed. In the case of hydrocortisone, 10 000-fold higher than physiological levels were required to see a $70\%$ inhibition of SMC proliferation. Some diseases are known to involve acquired resistance to GCs. The results indicate that the majority of cell lines from human endarterectomy lesions are resistant to the anti proliferative effects of hydrocortisone and this may be caused by the absence of the GR or other members of the GR multiprotein complex.

## Methods

### Vascular Specimens

Vascular lesions were acquired as waste surgical material, under Institutional Review Board approved protocols, from patients undergoing surgical endarterectomy at the New York Hospital/Cornell Medical Center. Portions of the vascular lesions were explanted for cell culture, and the remainder was fixed in 4% buffered formaldehyde and paraffin-embedded for immunohistochemical analysis.

### Cell Culture

Vascular lesions from carotid and femoral or iliac endarterectomy were finely diced, and the explants placed in 25-cm$^2$ tissue culture flasks containing medium 199 (M199; Gibco BRL). Vascular lesions from carotid and femoral or iliac endarterectomy at the New York Hospital/Cornell Medical Center. Portions of the vascular lesions were explanted for cell culture, and the remainder was fixed in 4% buffered formaldehyde and paraffin-embedded for immunohistochemical analysis. The present studies compared with the untreated control groups.

### Cell Proliferation

The effect of the steroids on the rate of DNA synthesis was examined by semiautomated methods. Cells were plated at 1 10$^4$ cells/well of 96-well microtiter plates at least 24 hours before the assay. Hydrocortisone ($0.01$ to $10\mu$g/mL) was added to M199 plus 1% FBS for 20 hours before the cells were pulsed with $[^{3}\text{H}]$thymidine (1 $\mu$Ci/mL; NEN) for 4 hours. Cells were collected with trypsin/EDTA and a cell harvester (Wallac, Inc), and the DNA-incorporated label was determined by scintillation counting (Betaplate; Wallac, Inc) ($n=3$ per point). Cell proliferation was also determined over a 5-day period with exposure to hydrocortisone or TGF-$\beta_1$, or both, followed by trypsinization and physical counting of the cells using a particle counter (Coulter ZBI).

### Protein Synthesis

The rate of protein synthesis was measured by $[^{3}\text{H}]$proline incorporation, which is partially incorporated into proline-rich proteins such as collagen. Cells were plated as described for cell proliferation. Hydrocortisone was added to the cells (M199/1%FBS) at least 4 hours before the cells were pulsed with $[^{3}\text{H}]$proline (4 $\mu$Ci/mL; 1-[5- $^{3}\text{H}]$proline; NEN) for 24 hours. The supernatants were precipitated in 10% trichloroacetic acid (TCA). The adherent cell monolayer was washed twice with PBS and then dissolved with 1 mol/L NaOH for 30 minutes at 37°C. The TCA-precipitated supernatant and cell monolayer were counted using a scintillation counter. All concentrations were tested in replicates of 3 wells.

### Collagen Secretion

Pooled triplicate supernatants from the cell proliferation assays were analyzed further by radioimmunoassay techniques for the presence of antigenic material derived from the N-terminal pro-peptide of type III (PIINP; Incstar) collagen. A 1-step equilibrium-type assay using polyclonal rabbit antibodies against purified human pro-peptide antigen was used. All samples were measured in duplicate, interpolated to a standard curve, and expressed in nanograms×10$^5$ cells.

### RNA Extraction

Total RNA was extracted from cell cultures by RNAzol B (Tel Test). Briefly, the supernatant was removed and RNAzol B was added to the cells and rapidly harvested (0°C to 4°C) with a sterile scraper. The cell suspension was collected, chloroform was added, and the mixture was centrifuged at 14 000g for 15 minutes at 4°C. The

### Oligonucleotide Primers Used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward No.</th>
<th>Reverse No.</th>
<th>PCR Product</th>
<th>Accession No.</th>
<th>Reference</th>
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<td>657–676</td>
<td>428 bp</td>
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<td>Rat GR</td>
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<td>1468–1497</td>
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<td>M14053</td>
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<tr>
<td>MR</td>
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<td>3168–3194</td>
<td>426 bp</td>
<td>M16801</td>
<td>36</td>
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<td>11β-HSD2</td>
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<td>676–697</td>
<td>304 bp (RNA)</td>
<td>U27318</td>
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<td>Hsp 70</td>
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<td>Hsp 90</td>
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<td>3617–3638</td>
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<td>54</td>
</tr>
<tr>
<td>hGR$\beta$</td>
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<td>2930–2951</td>
<td>452 bp</td>
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<td>TGF-$\beta$ receptor I</td>
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<td>1491–1514</td>
<td>255 bp</td>
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MR indicates mineralocorticoid receptor; Hsp, heat shock protein; hGR, human GR; and 11β-HSD2, 11β-hydroxysteroid dehydrogenase type II.
aqueous phase was collected and mixed with an equal volume of isopropanol and centrifuged at 14,000g for 15 minutes at 4°C, and the resulting pellet was washed with ethanol.

**Reverse Transcription–Polymerase Chain Reaction (RT-PCR)**

The oligonucleotide primers used for RT-PCR analysis of selected steroid hormone receptors are listed in the Table. Total RNA (500 ng) was reverse transcribed into cDNA with MuLV reverse transcriptase at 42°C with random hexamers (Perkin Elmer) followed by PCR with Taq polymerase in a 100-µL reaction containing 0.2 mMol/L of dNTPs and 0.15 µMol/L of each primer. The PCR profile varied for particular primer pairs but generally consisted of an initial 1-minute denaturation at 95°C, then 35 cycles of 1-minute denaturation at 95°C, 2-minute annealing at 60°C, 1-minute extension at 72°C, and finally a 10-minute extension at 72°C. Fifteen microliters of PCR product were separated in 1.5% (wt/vol) agarose gel and stained with ethidium bromide.

**Western Blot Analysis**

The conditioned media of the LDCs was removed and concentrated (Centricon 10; Amicon), and protein lysates were made from the LDCs. Lysis buffer (40 mMol/L Tris-base, 1% Triton X-100, 2 mMol/L MgCl₂, 0.1 mMol/L PMSF, 5 µg/mL leupeptin; pH 8.8) was added to the LDCs, which were then harvested and sonicated for 5 s. Protein content was determined by the bicinchoninic acid (BCA) assay (Pierce), and 30–50 µg was used for analysis. Samples were boiled for 10 minutes, analyzed by SDS-PAGE, and electroblotted onto polyvinylidene difluoride (PVDF membranes) (NEN Life Science) in 160 mMol/L glycine, 25 mMol/L Tris base, and 20% methanol transfer buffer. The PVDF membrane was then blocked in Tris-buffered saline (TBS)-milk (20 mMol/L Tris-HCl, 150 mMol/L NaCl, and 4% powdered milk; pH 7.4) for 2 hours. The membrane was exposed to the anti-GR antibody (diluted 1:100, Novacastra Laboratories) overnight, then washed in TBS before application of the peroxidase-conjugated anti-mouse IgG (diluted 1:1000), which was detected by chemiluminescence (NEN Life Science).

**Apoptosis Assays**

**Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling**

Fragmented DNA was labeled in situ by use of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method (Boehringer-Mannheim). Briefly, LDCs were plated on 8-well chambered glass slides at least 24 hours before treatment. Hydrocortisone (10 µg/mL) was added to the cells for 24 and 48 hours before the TUNEL assay (M199/1%FBS). The cells were fixed in 4% buffered formaldehyde and permeabilized (0.1% Triton X-100, 0.1% sodium citrate). The fragmented DNA was labeled with fluorescein dUTP at strand breaks identified by terminal deoxynucleotidyl transferase (TdT) for 1 hour in the dark at 37°C. The cells were then analyzed directly under a fluorescence microscope. Negative controls were performed by omitting the TdT. For each treatment group 10 fields (approximately 400 cells) were counted and the percentage of TUNEL-positive cells calculated.

**Colorimetric MTT Assay**

The MTT reduction by mitochondria is a measure of metabolism and therefore only detects living cells. Inhibition of MTT reduction has been used as a quantitative assay for apoptosis. Cells were plated as described for cell proliferation. Hydrocortisone was added to the cells (M199/1%FBS) at least 20 hours before the cells were pulsed with MTT (0.5 mg/mL) for 4 hours. The medium was removed and DMSO was added to dissolve the dark blue crystals; the mixture was thoroughly shaken at room temperature for 30 minutes. The optical density of each well was determined by absorbance at 540 nm.

**Immunocytochemistry**

Portions of endarterectomy specimens were fixed in phosphate-buffered 4% formaldehyde, paraffin-embedded, and immunostained by standard procedures for the avidin-biotin enhanced immunoperoxidase detection (Vector Laboratories). Primary antibodies included a monoclonal antibody to detect the GR (NCL-GR; Novacastra Laboratories), HAM56 to detect macrophages, a mouse IgG control antibody (Cappel Cooper Diagnostics), and HHF35 to detect SMC-specific actin (kindly provided by A. Gown®). Positive immunostaining was detected with DAB, and tissues were counterstained with hematoxylin.

**In Situ Hybridization**

LDC lines were plated onto glass multichamber slides (Labtek) and allowed to adhere for at least 24 hours before fixation with DEPC-treated 4% buffered formaldehyde for 20 minutes. The fixed monolayer was prehybridized with hybridization buffer (2 mg/mL nuclease-free BSA, 20% dextran sulfate, 20 mMol/L EDTA, 1 mg/mL yeast tRNA, 200 µg/mL poly(A), 4 × SSC in Denhardt’s solution), and then the mRNA for the GR was detected in LDC by in situ hybridization (ISH) of a digoxigenin-labeled probe in hybridization buffer at 45°C overnight. Free probe was washed with 2 × SSC at 37°C. Hybrids were detected with anti-digoxigenin/alkaline phosphatase visualized with Fast Red substrate.

**Results**

**Rat SMC Sensitivity to GCs**

Primary SMCs isolated from Fisher 344 rat aortas were significantly growth-inhibited (P<0.05) by treatment with increasing concentrations of hydrocortisone for 20 hours (Figure 1A), as measured by [3H]thymidine incorporation. In both of the rat lines tested (R8 and R13), strong inhibition (approximately 60% to 90%) occurred at low doses of hydrocortisone, 0.125 to 1.25 µg/mL. Higher doses of hydrocortisone further inhibited proliferation, which was maximal at 1250 µg/mL of hydrocortisone with an 80% to 90% decrease in DNA synthesis. Later passages of one of the rat SMC lines (R13-P32) were resistant to the antiproliferative effects of hydrocortisone, suggesting an acquired resistance to hydrocortisone in these SMCs. Essentially identical results were obtained with dexamethasone when corrected for the 30-fold greater potency of this synthetic steroid (not shown). Further, the inhibitory effect of GCs on DNA synthesis was corroborated by marked decreases in cell numbers over a 6-day period. Dexamethasone, at 0.040 µg/mL (equivalent to 1.2 µg/mL of hydrocortisone) caused a 62% decrease in cell proliferation of R8 and 40% decrease in R13.

The inset in Figure 1 demonstrates the presence of the GR by RT-PCR (404 bp) in both early (P4) and late passages (P29 or P32) of these 2 rat SMC lines. Thus, serial subpassage can,
but need not, cause rat SMCs to become resistant to hydrocortisone, and in this case, the functional resistance is apparently not caused by the loss of the GR mRNA.

Both rat SMC lines were also sensitive to the antiproliferative effects of TGF-β1 in early passages, with a 65% decrease in cell proliferation at 10 ng/mL TGF-β1. Later passages of R13, but not R8, became resistant to the antiproliferative effects of TGF-β1, thus paralleling the response to hydrocortisone and dexamethasone. TGF-β resistance with in vitro aging of the R13 line could be attributed to loss of the type II receptor (not shown), in a manner similar to the known loss of the type II receptor in SMCs derived from aged rats.41 TGF-β resistance may also explain the acquired resistance to hydrocortisone in R13 because hydrocortisone has been reported to inhibit cell proliferation via activation of TGF-β.42

Effects of Hydrocortisone on DNA Synthesis in LDCs

LDCs were treated with increasing concentrations of hydrocortisone or TGF-β1 under conditions similar to the rat studies. After 20 hours of low-dose hydrocortisone treatment (1 μg/mL), LDC proliferation was not inhibited (as measured by [3H]thymidine incorporation) in 5 of 8 lesion-derived SMC lines (Figure 2C; LDC sensitivity is defined as >50% inhibition of DNA synthesis). This low dose of hydrocortisone is 10 times higher than the physiological levels of cortisol in the body (average cortisol levels at NY Hospital are 0.12±0.03 μg/mL). Much higher concentrations of hydrocortisone (10 μg/mL; 100-fold higher than physiological), were needed to produce any detectable inhibition of cell proliferation in 5 of 8 LDC lines. At the highest tested dose, 3 of 8 cell lines remained resistant to the antiproliferative effects of hydrocortisone (Figure 2C). Only 2 cells lines, E12sc (a clone of small cells) and late passage E137, were inhibited strongly (75%) with high-dose hydrocortisone treatment (Figure 2A), a result comparable with the sensitive rat SMCs. In a third line, E47sc (also clonal), hydrocortisone inhibited DNA synthesis by 50% (Figure 2A).

Our laboratory previously has shown that LDCs are resistant to the inhibitory effects of TGF-β1. Figure 2D confirms that 6 of 8 LDC lines were resistant to the effects of TGF-β1. The majority of these resistant cell lines were also resistant to hydrocortisone (ie, E60, E64, E63, E145, and E137 early). Two hydrocortisone-sensitive cell lines, E47sc and E137 late passage, were also extremely sensitive to TGF-β1, exhibiting 75% to 90% inhibition of DNA synthesis (Figure 2B).

GC-Induced Apoptosis

GCs have been reported in the literature to induce apoptosis in fibroblasts and lymphocytes; however, their effect on apoptosis of human vascular SMCs has not been reported. Exposure to hydrocortisone (10 μg/mL) for 48 hours in a normal human neonatal SMC line (CRL1999) causes a small increase in the rate of apoptosis compared with untreated control cells (Figure 3A). A small increase in cell death was also observed in the E12sc and E47sc LDC lines treated with hydrocortisone. However, the E85 LDC line, after 48 hours of high-dose hydrocortisone treatment (10 μg/mL), had the greatest increase of cell death with a 3-fold increase above basal levels (Figure 3A).

The MTT assay was used as a second method of measuring cell death. After 20 hours of exposure of the normal CRL1999 SMC line to increasing concentrations of hydrocortisone, a dose of 10 μg/mL hydrocortisone decreased cell survival by 30% (Figure 3B). Both E12sc and E47sc LDC lines were resistant to the hydrocortisone-induced effects on
cell death (Figure 3B). E85 was very sensitive to the apoptotic effects of hydrocortisone, with a 30% increase in cell death at 10 μg/mL hydrocortisone, a similar pattern as that seen with the TUNEL assay (Figure 3A). In general, the TUNEL assay underestimates the number of apoptotic cells because cells detach and are lost in subsequent washing steps. The MTT assay is more accurate as only live cells reduce the MTT, but is confounded by the effect of proliferation in nonapoptotic cells.

Pro-Collagen III Production by LDCs
A major antifibrotic effect of GCs is a decrease in type I and III collagen synthesis in many cell types, including fibroblasts\textsuperscript{12,13} and ureteral SMCs.\textsuperscript{43} However the effects of GCs on cells derived from atherosclerotic lesions are unknown. Given the resistance of most LDC lines to HC, the effect on protein and collagen synthesis was examined.

After 5 days’ treatment of the E47sc LDCs with either hydrocortisone alone or in the presence of TGF-β1, the cells were counted and the supernatants removed for the pro-collagen III RIA (PCNPIII). Both TGF-β1 (1 ng/mL) and hydrocortisone (10 μg/mL) inhibited cell proliferation by up to 50% (Figure 4A). Analyzing the supernatants from these cells for pro-collagen III synthesis, hydrocortisone (10 μg/mL) increased pro-collagen III production by 2-fold (Figure 4B). This effect was further exaggerated with the addition of TGF-β1. To confirm this increase in collagen synthesis, proline incorporation (an indirect measure of collagen production) was increased markedly in TGF-β1- and hydrocortisone-treated cells (Figure 4C). Related studies observed similar increases in proline incorporation independent of changes in cell number (not shown). These results suggest that GCs enhance, not reduce, collagen production in LDCs.

Analysis of the GR mRNA in LDCs by RT-PCR
To determine the molecular basis for the resistance to hydrocortisone, RNA extracted from LDCs was analyzed for the presence of the human GR by RT-PCR. The primers were directed to exon 2, giving a 428-bp product, and after 35 cycles of PCR, mRNA for the GR was absent in 10 of 14 LDC lines (Figure 5A; only 6 cell lines shown). LDCs from patient E137 expressed the GR in early (E137E) but not late (E137L) passage. Only 3 other lines were observed with detectable GR mRNA levels, E12sc, E145, which expressed the GR mRNA in early (E137E) but not late (E137L) passage. The sense controls were positive LDC line (E12sc) and a negative cell line (E47sc).

Figure 4. Proliferative and fibrotic responses to hydrocortisone in LDCs. A, Cell numbers after 20 hours of treatment with TGF-β (1 ng/mL) alone or TGF-β plus hydrocortisone (0.1 to 10 μg/mL), or hydrocortisone alone (10 μg/mL) in E47sc LDCs. B, The supernatants from A were analyzed by RIA for pro-collagen type III (n=3) and corrected for differences in cell number. C, [3H]Proline incorporation was determined after 20 hours of hydrocortisone treatment (n=3) in the presence of TGF-β (1 ng/mL).

positive LDC line (E12sc) and a negative cell line (E47sc). Figure 5C illustrates positive staining for the GR (anti-sense) in the E12sc LDC line but not E47sc. The sense controls were negative both in E12sc and E47sc, suggesting the positive hybridizations were not caused by nonspecific annealing of probe.

Presence of Heat Shock Protein 70 But Not Heat Shock Protein 90 in LDCs
The unliganded GR is part of a multiprotein complex, comprising 2 molecules of heat shock protein 90 (Hsp 90), 1 molecule of Hsp 70, 1 molecule of Hsp 56, and 1 molecule of Hsp 26. Abnormal Hsp 70 and Hsp 90 have been reported in steroid-resistant acute lymphoblastic leukemia,\textsuperscript{31} suggesting that if these proteins are absent or dysfunctional, the GR multiprotein complex is unable to form and enter the nucleus, rendering the cells resistant to GCs. RT-PCR analysis of 6 LDCs and 1 normal SMC line (IMA) indicated that Hsp 90 (Figure 6, top) was detected only faintly in 1 line (E85) and was absent in all other lines (182-bp product). Hsp 70 (Figure 6, middle) was strongly positive in 5 of the cell lines (443 bp) but undetectable in E137 and E47sc. The presence of the type
I receptor for TGF-β (Alk5) was detected in all the LDC lines as a positive control for the integrity of the mRNA and is consistent with prior publications demonstrating loss of the type II, but not type I, receptor in LDCs. Absence of the GR in LDCs by Western Blot Analysis

Whole-cell lysates were made from LDCs, and 30 µg of protein was separated on an SDS-PAGE gel and probed with an anti-GR antibody directed at the immunogenic N-terminal modulating region (Novacastra Laboratories) for Western blot analysis. The E12sc LDC line was positive for the GR protein (94 kDa; Figure 7), confirming the positive signal seen for the GR mRNA (Figure 5). E85 LDCs had a low level of the GR protein, though the mRNA was not detected by RT-PCR, and 2 different protein preparations of E47sc confirmed an absence of the GR (Figure 7). As controls, the M199 culture media was negative for the GR protein and the Jurkat T-lymphocyte cell line was positive.

RT-PCR Analysis of the Mineralocorticoid Receptor and 11β-Hydroxysteroid Dehydrogenase Type II mRNA

The same LDC RNA used to detect the GR mRNA (Figure 5) was analyzed for both the mineralocorticoid receptor (MR) and 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), the enzyme required for the metabolism of cortisol to cortisone. Because both mineralocorticoids and GCs are able to signal through the MR with the same affinity, the specificity of MR is conferred by 11β-HSD2, which protects the MR from the large excess of GCs. Figure 8A represents the RT-PCR analysis for MR mRNA (426 bp). In all LDC lines that have been tested (n = 6), the MR is present. The E85 cell line has an approximately 150-bp-smaller PCR product than the predicted PCR product size of 432 bp. The PCR primers anneal to exons 7 and 9, spanning exon 8 (157 bp), suggesting that the E85 cells lack a key region of the ligand-binding domain of the MR.45 11β-HSD2 mRNA (Figure 8B; 305 bp) is abundantly present in E12sc, a cell line that has the GR by RT-PCR (Figure 5) and Western blot analysis (Figure 7). The E85 cell line, which has no GR mRNA by RT-PCR (Figure 5) but a low level of the GR protein (Figure 7), has a low level of 11β-HSD2 mRNA. Interestingly, the E47sc LDC line does not express detectable 11β-HSD2 mRNA by RT-PCR (Figure 8B), nor does it have a GR by RT-PCR (Figure 5) or

Absence of the GR in LDCs by Western Blot Analysis

Whole-cell lysates were made from LDCs, and 30 µg of protein was separated on an SDS-PAGE gel and probed with an anti-GR antibody directed at the immunogenic N-terminal modulating region (Novacastra Laboratories) for Western blot analysis. The E12sc LDC line was positive for the GR protein (94 kDa; Figure 7), confirming the positive signal seen for the GR mRNA (Figure 5). E85 LDCs had a low level of the GR protein, though the mRNA was not detected by
The staining seen in the mouse IgG control section is only showed very little positive immunoreactivity (Figure 9C). An antibody to detect macrophages in the lesion (HAM56) staining for macrophages identified almost all cells in this field as SMC/myofibroblasts as indicated by the smooth muscle-actin staining identified almost all cells in this field as SMC/myofibroblasts (Figure 9A).

Immunohistochemical Analysis of the GR in Endarterectomy Lesions

Immunohistochemical analysis of endarterectomy lesion specimens from 3 patients demonstrated that the GR was expressed strongly by cells within the tunica media (Figure 9A). In this patient, GR staining was also strongly positive around a small branching blood vessel. However, within the atherosclerotic lesion, there was only scattered and relatively weak immunoreactivity for the GR. This confirms the in vitro observation of low levels of GR expression detected by RT-PCR and Western blot analysis. The majority of the GR-positive cells were SMC/myofibroblasts as indicated by the smooth muscle-actin staining of adjacent sections of the same patient (Figure 9B). An antibody to detect macrophages in the lesion (HAM56) showed very little positive immunoreactivity (Figure 9C). The staining seen in the mouse IgG control section is only the hematoxylin counterstain (Figure 9D).

Discussion

Severely narrowed carotid or coronary vessels are commonly revascularized by surgical endarterectomy or balloon angioplasty, respectively. Although initially successful in restoring flow in almost all patients, the major limitation of these procedures is fibroproliferative restenosis of the arteries during the 6-month period after the procedure. The findings of this paper illustrate that the majority of cell lines derived from human endarterectomy lesions are resistant to the antiproliferative effects of hydrocortisone, because of decreased expression of the GR, as determined by RT-PCR, Western blot analysis, and immunohistochemistry of the GR in endarterectomy lesions.

Prior studies have shown that cells derived from arteries of aged rats and from human atherosclerotic lesions are both resistant to the inhibitory effects of TGF-β1. This resistance could be attributed to the loss of the type II TGF-β signaling receptor, and in a subset of patients, this loss was caused by microsatellite instability in the TGF-β type II receptor gene. Although TGF-β1 is one of the key activating and suppressing factors in fibroproliferative disease, the GC system is also recognized as an important fibrosuppressive pathway. Extensive clinical and experimental evidence have suggested that hydrocortisone and its synthetic analogs (ie, dexamethasone and prednisone) suppress fibroproliferative diseases by acting as antiproliferative agents, antifibrotic agents that reduce collagen production, and apoptotic factors.

Given these diverse suppressive effects on fibroproliferative pathways, GCs should suppress restenosis, yet published clinical trials indicated that high-dose steroid therapy does not influence the overall rate of restenosis. Given these diverse suppressive effects on fibroproliferative pathways, GCs should suppress restenosis, yet published clinical trials indicated that high-dose steroid therapy does not influence the overall rate of restenosis. Stone et al randomly assigned 102 patients with documented restenosis after a prior successful coronary angioplasty to receive treatment during repeat coronary angioplasty at the restenotic site. Treated patients received 125 mg methylprednisolone intramuscularly the evening before and the morning of angioplasty and then oral prednisolone (60 mg/d) for 7 days after coronary angioplasty. Angiographic follow-up at 6 months revealed the steroid-treated group had a 59% restenosis rate compared with the no-steroid control group, which had a 56% restenosis rate. A second randomized clinical trial examined 915 patients in total. Treated patients (n = 215) received a single dose of methylprednisolone (1 g IV; 2 to 24 hours before coronary angioplasty). Again, angiography at 6 months’ follow-up showed a 40% restenotic rate in the steroid-treated group compared with 39% in the control group.

One possible explanation for the lack of a clinical benefit is insufficient local concentration of the drug at the site of injury. Intramural administration of hydrocortisone incorporated into polymer microspheres caused a significant reduction in post-angioplasty intimal hyperplasia in rabbits. Likewise, local delivery of dexamethasone via adventitial cuffs around the rat carotid artery produced a 75% reduction in balloon catheter–induced intimal hyperplasia. However, local delivery of dexamethasone by a novel polymer-coated eluting stent in the porcine coronary injury model did not decrease intimal hyperplasia.

An alternate explanation for the clinical resistance to steroid treatment may be offered by the relative resistance of human LDCs to the antiproliferative effects of hydrocortisone. Analogous to TGF-β1 resistance caused by loss of the type II receptor, these steroid-resistant cell lines typically lack key elements of the GR complex. Restenotic lesions have a lower rate of apoptotic SMCs and macrophages compared with primary lesions, implicating reduced apoptosis as an important mechanism in restenotic lesion formation. The resistance of some LDCs to the apoptotic effects of hydro-


cortisone is another mechanism by which the lesion might fail to regress. At higher concentrations of hydrocortisone, some LDCs exhibited cell death, possibly explaining the antiproliferative effect of high doses of hydrocortisone. Interestingly, Sato et al.\textsuperscript{46} reported that human vascular SMCs isolated from IMAs decreased their GR expression after 1 hour’s treatment with Lp(a), a risk factor for atherosclerosis.\textsuperscript{46} These data, coupled with the present studies, suggest lesion cells may be less sensitive to the suppressive effects of steroids in an atherosclerotic environment.

Voisard et al.\textsuperscript{28} cultured cells from human atherosclerotic lesions and found, in vitro, an antiproliferative effect of corticosteroids. However, high doses of hydrocortisone (1250 \( \mu \mathrm{g/ml} \)), or its synthetic analogs, prednisolone (750 \( \mu \mathrm{g/ml} \)) and dexamethasone (40 \( \mu \mathrm{g/mL} \)), were required to inhibit SMC proliferation by up to 70\% after 5 days in culture. Correcting for the relative potency of these agents, the effective dose was approximately 10 000-fold higher than physiological concentrations. Such high-dose steroid therapy, in vivo, could only be achieved for short periods by local administration. Lesion cells that expressed a complete GR complex, such as E85, required a dose of only 10 \( \mu \mathrm{g/mL} \) of hydrocortisone to significantly inhibit DNA synthesis by up to 80\%, whereas most LDCs lacking the GR complex were dramatically resistant to steroid therapy. Although it is possible that cells cultured from these plaques represent a selected subset of cells with resistance to GCs, 3 facts discount in vitro factors as the sole explanation for the resistance: (1) although serial subpassage can be associated with acquired resistance (Figure 1, R13), other lines (R8) show no decrease in sensitivity, suggesting the resistance is caused by the expansion of a preexisting subset of resistant cells; (2) the LDCs exhibited resistance as soon as it was possible to test, typically within 2 to 4 passages; and (3) immunostaining suggests the relative absence of the GR in histological sections of human lesions (Figure 8).

Published evidence has indicated that GCs can act via stimulation of TGF-\( \beta \) activity,\textsuperscript{52} a possibility that would explain the parallel resistance to these 2 factors in almost all rat (Figure 1) and human (Figure 2) lines examined. A notable exception was the E12sc LDC line, which is partially hydrocortisone-sensitive, but TGF-\( \beta \)-resistant, suggesting that hydrocortisone can act via TGF-\( \beta \)-independent pathways. An alternate explanation for the parallel resistance to these 2 inhibitory systems is that a common factor is capable of modulating both the type II TGF-\( \beta \) receptor and members of the GR complex. Such a factor would probably exert its effects at the transcriptional level, because steady-state mRNA levels of both receptors are decreased.

The loss of the GR in the majority of endarterectomy cell lines typically correlated with resistance to the antiproliferative effects of GCs. However, 1 cell line (E47sc), was extremely sensitive to the inhibitory effects of hydrocortisone but lacked detectable levels of the GR. This cell line was positive for the MR but not the enzyme 11\( \beta \)-HSD2, which protects the MR from the relative excess of circulating GCs. In the absence of 11\( \beta \)-HSD2, GCs bind to the MR with similar affinities as aldosterone.\textsuperscript{50} 11\( \beta \)-HSD2 metabolizes cortisol (hydrocortisone) to cortisone, which is inactive and cannot bind to the MR.\textsuperscript{39} Thus, in the absence of 11\( \beta \)-HSD2, as in E47sc, hydrocortisone should readily signal through the MR, thus explaining the functional responses to hydrocortisone in a GR-negative line. The relationship between hydrocortisone, 11\( \beta \)-HSD2, and the MR or GR complex is shown schematically in Figure 10.

Typically, collagen synthesis is inhibited by GCs\textsuperscript{11–13}; however, in vascular SMCs isolated from either bovine or human aortas, GCs enhance collagen protein synthesis,\textsuperscript{51,52} although the GR status of these SMCs was not determined. The present data raise the possibility that the relative loss of the GR signaling complex, or reduced expression of 11\( \beta \)-HSD2, would cause the GCs to interact with the MR and behave as mineralocorticoids. A fibrotic response would result because MR activation typically results in strong induction of type I and type III collagen synthesis.\textsuperscript{32,53}

The human GR gene contains a total of 10 exons and is approximately 80 kb. There are 2 GR isoforms, hGR\( \alpha \) and hGR\( \beta \), which originate by alternative splicing of exon 9.\textsuperscript{44} hGR\( \alpha \) is fully functional and hGR\( \beta \) has no hormone-binding activity.\textsuperscript{54} These 2 alternatively spliced forms of the GR are able to form heterodimers but are inactive compared with hGR\( \alpha \) homodimers, and thus hGR\( \beta \) is thought to act as an endogenous inhibitor of GC action.\textsuperscript{55} The LDC lines that did express the GR only expressed detectable levels of the active form, hGR\( \alpha \); therefore, the resistance to hydrocortisone in these LDCs is probably not caused by hGR\( \beta \) inhibiting the actions of hGR\( \alpha \).

The unliganded GR is part of a multiprotein complex, comprising 2 molecules of Hsp 90 and 1 each of Hsp 70, Hsp 56, and Hsp 26 (Figure 10). Hsp 90 molecules dissociate from the receptor complex on ligand binding,\textsuperscript{56} whereas Hsp 56 is required for directing the receptor complex to the nucleus,\textsuperscript{53} and Hsp 70 is a molecular chaperone for the GR entering the nucleus.\textsuperscript{54} Abnormal Hsp 70 and Hsp 90 have been reported in steroid-resistant acute lymphoblastic leukemia.\textsuperscript{31} The absence of the Hsp 90 complex in the LDC lines, and Hsp 70 in Figure 10. Schematic model of steroid–steroid receptor interactions. Hydrocortisone (HC) does not typically interact with the MR because of rapid inactivation of HC to cortisone by 11\( \beta \)-HSD2. Normally, HC interacts with the GR as a member of a multiprotein cytoplasmic complex that includes Hsp 90, Hsp 70, Hsp 56, and Hsp 26. Hsp 90 dissociates from the GR on HC binding, and the remaining complex traverses the nuclear membrane to exert transcriptional effects leading to suppression of collagen synthesis, induction of proliferation, and induction of apoptosis. However, in the absence of the GR, it is hypothesized that HC levels can overwhelm 11\( \beta \)-HSD2 and signal through the MR. Likewise, in the absence of 11\( \beta \)-HSD2, HC readily signals through the MR, leading to profibrotic effects including the induction of collagen gene expression.
E47sc, suggests the resistance to hydrocortisone in these cells is caused not only by an absent GR, but potentially by an absence of other components of the GR multiprotein complex.

To the extent that cells proliferating from human lesions may reflect the properties of cells that respond to vascular injury, such as angioplasty, the results of the present study suggest restenosis may be another disease involving acquired resistance to GCs. The resistance of these LDCs to the antiproliferative and antifibrotic effects of hydrocortisone may contribute to excessive wound repair in atherosclerosis and restenosis. These results may have therapeutic implications if the GR can be replaced in GR-deficient vessels. Steroids are the first line, and in many cases the only line, of therapy for a broad spectrum of inflammatory and fibrotic diseases. Thus, the ability to restore steroid responsiveness might be adapted to treat steroid-resistant fibrosis in other situations such as congestive heart failure, pulmonary fibrosis, keloids, and arthritis.

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References


Glucocorticoid Resistance Caused by Reduced Expression of the Glucocorticoid Receptor in Cells From Human Vascular Lesions

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