Atherosclerotic lesions may progress due to a “failure to die” by vascular repair cells. Egr-1, a zinc finger transcription factor, is elevated more than 5-fold in human carotid lesions relative to the adjacent tunica media. Lesion cells in vitro also express 2-3-fold higher Egr-1 mRNA and protein levels but express much lower levels of the transforming growth factor-β (TGF-β) Type II receptor (TβR-2) and are functionally resistant to the antiproliferative effects of TGF-β. Lesion cells fail to express a TβR-2 promoter/chloramphenicol acetyltransferase (CAT) construct but overexpress an Egr-1-inducible platelet-derived growth factor-A promoter/CAT construct. Transfection of Egr-1 cDNA represses TβR-2/CAT constructs but induces PDGF-A/CAT. Egr-1 transfection reduces the levels of TβR-2 and confers resistance to the antiproliferative effect of TGF-β. Egr-1 can interact directly with both the −143 Sp1 site and the positive regulatory element 2 (PRE2) (ERT/ets) region of the TβR-2 promoter. Thus, although activating a family of stress-responsive genes, Egr-1 also transcriptionally represses one of the major inhibitory pathways that restrains vascular repair.

It is thought that atherosclerotic changes in the vessel wall are initially due to injury from shear stress, hypertension, hypercholesterolemia, homocysteinemia, smoking, or viral/bacterial pathogens (1). However, it is evident that the major arteries tolerate these injuries for decades, successfully repairing the injury to maintain both vascular integrity and patency. After chronic injury, vascular repair cells, phenotypically similar to both smooth muscle cells and myofibroblasts (2), eventually accumulate in the vessel wall and occlude the vessel by progressive fibroproliferative remodeling. Atherosclerotic lesions commonly show a strong hyperplastic reaction to angioplasty or surgical endarterectomy, suggesting that their response to defined injuries is exaggerated. Vascular repair cells in the late lesion may suffer from a failure to die phenotype that allows the cell to respond to injury but disables its ability to undergo apoptosis as a natural part of wound regression (3).

Cells cultured from human atherosclerotic lesions initially show a high rate of apoptosis as they encounter in vitro conditions (4), but then a substantial subset of cells emerge that are resistant to apoptosis induced by factors such as TGF-β (5) and glucocorticoids (6).

The resistance to the antiproliferative and apoptotic effects of TGF-β is principally due to a selective age-related loss of the Type II receptor for TGF-β (TβR-2) (5, 7, 8). TβR-2 is required for conveying the TGF-β signal (9) to the SMAD family of transcription factors (10), thus leading to fibrotic, antiproliferative, and apoptotic responses in human lesion cells (11). Transfection of cDNA for TβR-2 partially restores the antiproliferative response to TGF-β in lesion cells (5, 7). TβR-2 is expressed in early atherosclerotic lesions but is essentially undetectable in late lesions, except in discrete foci adjoining inflammatory regions (7, 12). A small subset of patients can be defined in which acquired mutations in the TβR-2 contribute to the receptor loss, although the majority of cases cannot be explained by mutations in the receptor (13).

Using cDNA arrays to profile the mRNA transcripts of carotid artery lesions relative to the adjacent media, it was observed that the transcription factor Egr-1 was 5-fold higher in the human atherosclerotic lesion than in the adjacent media (14). Semi-quantitative reverse transcription-polymerase chain reaction confirmed that Egr-1 levels were 4–8-fold higher in individual lesions relative to the adjacent media. Control studies rejected the possibility that the differences in Egr-1 were induced ex vivo because 1) this should induce Egr-1 in both media and lesion equally, and 2) Egr-1 mRNA levels in lesion and media were stable ex vivo for an hour after surgery. The Egr-1 appeared to be transcriptionally active in the human lesion, because a high percentage of Egr-1-inducible genes were also elevated in the lesions relative to the adjacent media. Furthermore, in hypercholesterolemic mice, Egr-1 levels were found to parallel the development of the atherosclerotic lesion (14).

Egr-1 was identified by a number of different groups in widely divergent areas ranging from PDGF-induced mitogenesis to NGF-induced differentiation of neuronal cells. Egr-1 is potentially activated by a variety of cellular stressors: growth factors (15), oxidized lipoproteins (16), shear stress (17), sphingosine 1-phosphate (18), angiotensin II (19), endothelin, and hypoxia (20). In the vascular setting, both proliferation and...
migration after in vitro injury is Egr-1-dependent and blocked by antisense oligonucleotides (21). Egr-1 is rapidly induced upon injury to the rat carotid (22) or after partial stenosis of the rat carotid (17). Elevated Egr-1 in the latter model was associated with elevated tissue factor levels in the artery, suggesting that the Egr-1 had transcriptional effects. Inhibition of Egr-1 via DNA enzymes that degrade the mRNA blocks intimal hyperplasia in injured rat arteries (23).

Elevated Egr-1 activates the transcription of several important gene families that would influence vascular repair and cell survival. Egr-1 is a major activator of PDGF-A chain synthesis (24) and is directly involved in angiotensin II-dependent activation of PDGF and TGF-β expression (19, 25). Egr-1 is considered a major transcription factor for other key repair systems: angiogenic factors, such as vascular endothelial growth factor; procoagulants, such as tissue factor (17); cytokines (interleukin-2 (26) and TNF-α (27)); receptors (Flt-1/28)), apoptotic factors (Fas (29), Fas ligand (30)); cell cycle factors (cyclin D1 (31), p15, p21, p53); metabolic factors (5-lipoxygenase (32), multidrug resistance factor 1 (33), thymidine kinase (34), superoxide dismutase (35), adhesion molecules (ICAM-1 (36), fibronectin (37)), and proteases (urokinase-type plasminogen activator, matrix metalloproteinase type 1).

The present studies examined whether the highly elevated Egr-1 observed in human lesions was retained by the cells that proliferated in vitro and whether Egr-1 contributed to their known resistance to TGF-β. The results indicate that Egr-1 is a transcriptional repressor of TβR-2, presumably by interacting directly with both an Sp1 site and an ets-like ERT site in the proximal promoter of the TβR-2 gene. Thus, although activating a family of stress-responsive genes, Egr-1 also suppresses one of the major inhibitory pathways that restrain vascular repair.

EXPERIMENTAL PROCEDURES

Tissue Specimens—Vascular specimens were obtained during surgical revascularization procedures at The New York Presbyterian/Cornell Medical Center or as waste surgical specimens under institutional review board-approved protocols. Surgical endarterectomy of carotid artery disease produces full diameter lesions of 2–5 cm in length that commonly contain lesion and tunica media without adventitia. The medial tissue can be cleanly dissected from the overlying lesions for cell culture or RNA purification. Internal mammary arteries and radial arteries were obtained as excess waste from coronary artery bypass (CABG) operations.

Cell Culture—Human vascular specimens were typically received and processed within 30 min of surgical excision. Carotid lesions, mammary arteries, and radial arteries were opened longitudinally and gently scraped free of endothelium. Carotid lesions were dissected into the most luminal regions of the fibrous cap and the stripped tunica media, then cultured separately by explanting on serum-coated flasks in M199 with 20% FBS and antibiotics (penicillin/streptomycin). Mammary and radial arteries were scraped extensively on the adventitial side to remove extraneous tissue and then cultured by explant as above. The phenotype and growth properties of these cells have been previously described (5, 7). Approximately 30% of patients produce cultures that can be sustained for 5–10 passages, with an approximate doubling time of 4 days. CCL-64 cells (MV 1 Lu, NBL-7), a mink lung epithelial cell line (ATCC), was cultured in M199 with 10% FBS and antibiotics.

RNA Purification—Total RNA was purified from lesion-derived or medial cells using RNAzol B with minor modifications of the manufacturer’s method. RNA quantity and quality was assessed by gel electrophoresis and whether Egr-1 contributed to their known resistance to TGF-β. The results indicate that Egr-1 is a transcriptional repressor of TβR-2, presumably by interacting directly with both an Sp1 site and an ets-like ERT site in the proximal promoter of the TβR-2 gene. Thus, although activating a family of stress-responsive genes, Egr-1 also suppresses one of the major inhibitory pathways that restrain vascular repair.

Western Blot Analysis—For comparison of Egr-1 protein levels in mouse carotid lesion cells, cells were placed in 35-mm wells (6 well) at 1 × 10^5 cells/well in M199 with antibiotics 24 h before use. For analysis of growth factor effects on Egr-1 protein levels, cells were plated at 6-well plates at 2 × 10^5 cells/well in M199 with 5% FBS and antibiotics. After an overnight plating period, the cells were washed with serum-free media and changed to serum-free M199 with antibiotics for 24 h before treatment with growth factors. FGF-2 (R&D Systems, human, 10 ng/ml) or NGF (Harlan Bioproducts, murine salivary gland, 100 ng/ml) were then added in fresh serum-free media for the specified times before harvest of all the wells as follows.

Cellular lysates were prepared by washing the cell monolayer twice with phosphate-buffered saline and then scraping the cells into ice-cold lysis buffer (40 mM Tris-HCl, 1% Triton X-100, 2 mM MgCl_2, 0.05 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 0.5 mM benzamidine, and 1 μM peptatin) on ice and then centrifuging at 15,000 × g for 10 min to remove particulate. Protein content was determined by the BCA method (Pierce), and 20–30 μg of protein was separated on a 10% polyacrylamide gel under reducing conditions. After electrophoretic transfer of the proteins to a nitrocellulose membrane, the blot was probed with specific antibodies that were detected by peroxidase-la- beled secondary antibodies and chemiluminescent reporters (ECL or ECL+; Amersham Pharmacia Biotech). Antibody titers ranged from 1:100 for Egr-1 (Santa Cruz Biotechnology, sc-110), 1:200 for antibody to TβR-2 (Santa Cruz, L21), to 1:2000 for β-actin (Sigma, clone AC-15). Molecular weights were determined from pre-stained standards (Bio-Rad).

Nuclear Protein Preparation—Nuclear proteins were prepared from lesion or medial cells by minor modifications of standard methods. Cells (1 × 10^6/75 cm^2) were scraped into 4 ml of cold phosphate-buffered saline, pelleted at 450 × g, resuspended in 3 volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl_2, 10 mM KCl, 0.1 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1.5 μg/ml peptatin A, 0.2 mM levamisole, 10 mM β-glycerophos- phate, 0.5 mM benzamidine, 0.5% Nonidet P-40), and chilled on ice for 10 min. The sample was centrifuged at 10,000 × g, and the supernatant, containing cytosolic proteins, was frozen at −70 °C for later analysis. The pellet was resuspended in 2 volumes of buffer A and 2 volumes of buffer B (1.5 mM MgCl_2, 20 mM HEPES, 420 mM NaCl, 0.2 mM EDTA, and DTT, 0.2% leupeptin, aprotinin, pepstatin, levamisole, β-glycerophosphate, and benzamidine as in buffer A). The pellet was vortexed and homogenized 10 times through a sterile 25-gauge needle. The suspension was pelleted at 10,000 × g, and the supernatant, containing soluble nuclear protein, was mixed with an equal volume of buffer C (buffer A except: 100 mM KCl, no Nonidet P-40, 0.2 mM EDTA, and 20% glycerol). Aliquots were snap-frozen in an ethanol/dry ice slurry and stored at −70 °C.

Electrophoretic Mobility Shift Assays—For probes for electrophoretic mobility shift assays were synthesized by commercial sources as complementary, single-stranded DNAs and then mixed in equimolar ratios, heated to 75 °C for 2 min, and then chilled on ice. Sp1 and Egr-1 consensus probes were purchased from Santa Cruz. DNA sequences used were (5′ to 3′, + strand): PDGF, GGGGGGGGCGGGGGGGGCGGGGCGGGGCGGGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGGGGCGG.
Cloning of Human Egr-1—Human Egr-1 was cloned from human lesion-derived mRNA by reverse transcription-polymerase chain reaction using primers directed against nucleotides 131–149 and 2115–2131 of human Egr-1 (GenBank™ accession number M62839 (38)). The polymerase chain reaction product was cloned into pCR2.1 (Invitrogen), and the orientation was established by restriction analysis. The insert was subcloned into pcDNA3.1/zeo at the HinXbaI sites. The identity of the clone was confirmed by sequencing approximately 700 base pairs from each end. Coupled in vitro transcription/translation (TnT, Promega) indicated that the cDNA produced an 82-kDa protein.

**Transfection of Transcription Factors and Promoter/Reporter Constructs**—For expression of promoter/reporter constructs in lesion or medial cells, cells were plated at 105 cells/well at least 24 h before a brief wash with M199 and transfection with 4 μl of LipofectAMINE R-2 promoter/reporter constructs. Cells were incubated for 4 h in M199, and then the cells were changed to normal growth media for 48 h before harvest for determination of CAT or luciferase levels. In some studies, the cells were transfected first with Egr-1 (human or murine), Sp1 (murine vectors kindly provided by Dr. John Schuetz, St. Jude’s Children’s Research Center, Memphis, TN), or Egr-1 (human or murine), Sp1 (murine vectors kindly provided by Dr. Seong-Jin Kim (NIH/NCI) (39). The transfection was carried out using a small set of genes that differed. Table I contains genes that were increased or decreased in lesion cells and might be relevant to a resistant phenotype. Among these genes is Egr-1, which was 1.9 times higher in lesion cells (L) than in autologous medial cells (M) in culture. In contrast, Sp1 mRNA levels in lesion cells were decreased to one-half the level in medial cells, so that the Egr-1/Sp1 mRNA ratio was effectively 4 times higher in lesion compared with medial cells.

**Levels of Egr-1 Protein in Medial and Lesion Cells**

Whether these elevated Egr-1 mRNA levels translated to increased Egr-1 protein levels was examined by Western blot on eight lesion/media cultures, two of which had been tested in the cDNA arrays. In all eight lesion/media-matched cultures (E196, E197, E221, E243, E246, E278, E281, E291), the lesion cells (L) expressed markedly higher Egr-1 protein antigen than did the cells derived from the adjacent media (M) (Fig. 1, four patients shown). The same blots were reprobed with an antibody to β-actin, and equal levels were observed between samples (Fig. 1). Quantitation of the Egr-1 band intensities by densitometric scanning of film or by Storm chemiluminescence yielded similar results, both indicating that the level of Egr-1 antigen level was 2.6 times higher in lesion cells compared with medial cells (L/M ratio = 2.6, p < 0.0002). Thus, the elevated Egr-1 levels observed in the atherosclerotic lesion are retained by the lesion cells in culture and lead to elevated Egr-1 protein levels.

**Levels of TβR-2 Protein and Antiproliferative Response to TGF-β1 in Medial and Lesion Cells**

Although the lesion cells demonstrated elevated Egr-1 levels, levels of the TβR-2 protein were significantly lower in lesion cells than in medial cells (L/M ratio = 0.38, p < 0.01). This is consistent with prior data indicating both reduced 125I-TGF-β cross-linking to the TβR-2 and reduced TβR-2 mRNA by reverse transcription-polymerase chain reaction in lesion cells (5). Thus, there was a consistent pattern of elevated Egr-1 and reduced TβR-2 in lesion-derived cells relative to their medial counterparts (Fig. 2A). Furthermore, the reduced levels of the TβR-2 were associated with functional resistance to the antiproliferative effect of TGF-β1 in the lesion cells. The ability of TGF-β1 to inhibit DNA synthesis in two of the matched lesion/medial cultures is shown in Table II, and two examples are illustrated in Figs. 2B and 2C. Normal medial cells were unresponsive to TGF-β1, whereas lesion cells were partially responsive to low levels of TGF-β1 at 1 hour after exposure (Fig. 2B). As expected, lesion cells were much more responsive to TGF-β1 at 24 hours compared with medial cells (Fig. 2C). The increased responsiveness of lesion cells to TGF-β1 is consistent with higher Egr-1 levels in lesion compared with medial cells, and it suggests that Egr-1 is at least partly responsible for the increased TGF-β1 sensitivity of lesion cells.
The expression of TβR-2, Egr-1, and antiproliferative responses to TGF-β in lesion (L) and medial (M) cells. A, protein was harvested as in Fig. 1. Western blots were probed for Egr-1 and TβR-2 using specific antibodies and chemiluminescent detection. Band intensity was semi-quantified by densitometric scanning of the film or by Storm imaging of the chemiluminescence. Four patients, representative of eight patients examined, are shown. B, antiproliferative response to TGF-β in two of the autologous pairs used in panel A. Cells were plated at 1 x 10^5 cells/well for 24 h before treatment with the specified doses of TGF-β. The rate of DNA synthesis was determined 20 h later by a 4-h incorporation of [3H]thymidine into DNA. Data points are mean ± S.E., n = 3.

Medial cell lines is shown in Fig. 2B. Although cells from the media are inhibited 50–60% by 1 ng/ml TGF-β1 in 24 h, cells derived from the adjacent lesion are stimulated 50–60% under identical conditions. The ability of TGF-β to stimulate DNA synthesis is probably due to the induction of soluble mitogens such as PDGF (40) and connective tissue growth factor (41) by a subset of cells possessing TβR-2. The mitogens can then diffuse in the culture flask to stimulate cells regardless of their TβR-2 status.

Modulation of Egr-1 Levels by FGF-2 and NGF

Egr-1 expression is rapidly stimulated by growth factors such as PDGF, FGF, and NGF. To determine whether Egr-1 levels could be induced by growth factor in medial cells, which have low Egr-1 levels, medial cells were subjected to serum withdrawal for 24 h and then stimulated with FGF-2 (10 ng/ml) or NGF (100 ng/ml) for specified periods of time up to 4 h. Both FGF-2 and NGF led to a rapid increase (30 min) in Egr-1 protein (82–85 kDa) to a level twice the base-line level in medial cells (Fig. 3, A and B). The effect was partially reversed at 1–2 h after growth factor treatment, remaining 50% above the unstimulated level. Based on parallel wells in which the media was changed, but without growth factor (4 h), the increase was not due to nonspecific stimulation of the cells. Although NGF was able to stimulate Egr-1 levels in medial cells (E196M is shown in Fig. 3), NGF had no mitogenic effect on this cell line, as determined by [3H]thymidine incorporation 24 h after stimulation. Lesion-derived cells, which had very high basal levels of Egr-1 protein (E197L), did not show any further stimulation by NGF (Fig. 3A). In lesion cells with lower base-line levels (E196L), stimulation of Egr-1 levels by FGF-2 was detectable.

TβR-2 Promoter Activity in Lesion and Medial Cells

In light of the association of high Egr-1 levels with low TβR-2 levels and resistance to TGF-β, the expression of TβR-2 promoter/reporter constructs was examined in lesion versus medial cells. As shown in Fig. 4 the CAT reporter plasmid is essentially inactive in the absence of a promoter (CAT null). The introduction of the TβR-2 promoter from −274/+50 or −8–47/−50 causes an increase to 8–10-fold above base-line reporter activity in medial cells but not in lesion cells (n = 3 matched cell lines, mean ± S.E.). Conversely, the PDGF-A chain promoter, a prototype of Egr-1-inducible promoters (15), is about 2-fold more active in lesion cells than in medial cells, whereas the SV40-luciferase construct is more variable but unchanged.

Egr-1 Suppresses Transcription of TβR-2

Transient Transfection—Prior studies indicated that the Type II promoter is partially controlled by 2 Sp1 sites at −143 and −25 relative to the transcriptional start site (Fig. 5). Two other PREs were identified by deletion analysis, either 5′ to the Sp1 sites or 3′ to the predicted transcriptional start site (PRE2) (42). Conventionally, Egr-1 is thought to activate transcription at overlapping Egr-1/Sp1 sites (24). However, Egr-1 can interfere with Sp1 to block the activation of some genes such as the rat plasma membrane-glycoprotein/multidrug resistance gene 1B (33), murine adenosine deaminase (43), and the macrophage colony-stimulating factor gene (44). To determine the effects of elevated Egr-1 on transcription of TβR-2, expression vectors containing Egr-1 or Sp1 under the control of the cytomegalovirus promoter (pcDNA3) were transfected into several cell types, and then 48 h later, other plasmids containing regions of the TβR-2 promoter (−274/+50 or −47+/+50) driving the CAT reporter gene were retransfected into the same cultures. Control studies transfecting either empty vector, human Egr-1, or murine Egr-1 into E12 cells and then harvesting the cells at specific time points for Western blot indicated that both human and murine Egr-1 expression vectors increased Egr-1 antigens to levels 3–5 times the control levels by 48 h and that these levels were sustained at 96 h post-transfection. Egr-1 expression via transient transfection strongly suppressed the transcription from the −274/+50 region of the TβR-2 promoter in 3 different cell types: CCL64 (mink lung epithelial), E12 (lesion-derived), and HCT116 (human colon cancer, TβR-2 receptor mutant) (E12 shown in Fig. 6A, 97% reduction). Transcription of the −47+/+50 TβR-2/CAT construct was also suppressed in CCL64 and E12 cells (75% reduction), but it was essentially unaffected by Egr-1 in the HCT116 cells. Conversely, Sp1 transfection slightly increased the transcription of the TβR-2/CAT constructs (30%), consistent with both constructs having functional Sp1 sites (Fig. 6A).

Stable Transfection—These studies have been confirmed using stable transfections of Egr-1 or Sp1 into the HCT116 cells. After selection for stable expression, the cells were re-transfected transiently with TβR-2/CAT constructs, PDGF-A pro-
moter-CAT, or SV40-luciferase. As shown in Fig. 6B, plasmid expression of Egr-1 markedly suppressed the TβR-2/274 construct (96% reduction) but had only a small effect on TβR-2/47/CAT (27% reduction). Sp1 increased transcription of the TβR-2/47 construct (71% increase). As previously reported, the PDGF-A promoter was strongly induced by Egr-1 (277% increase) (24). Thus, Egr-1 suppresses the TβR-2 promoter but activates the PDGF-A promoter in the context of a chromatin environment.

Egr-1 Reduces the Cellular Levels of the TβR-2 and Confers Resistance to TGF-β

The prior promoter data suggest that the endogenous TβR-2 gene would be suppressed by overexpression of Egr-1. Among the cell lines in which transfection studies are feasible, TβR-2 is not expressed by HCT116 cells due to homozygous mutation or in the E12 cells, and consequently, both cells are resistant to TGF-β. CCL64 cells are mink lung epithelial cells that express naturally high levels of TβR-2, consistent with their high sensitivity to TGF-β. CCL64 cells were transiently transfected with empty vector, human Egr-1, or murine Egr-1 48 h before examining their levels of TβR-2 and their antiproliferative response to TGF-β. Both human Egr-1 cDNA (huEgr-1) and mouse Egr-1 (moEgr-1) increased Egr-1 antigen levels to 4 to 7 times the level in vector transfectants. Concurrently, TβR-2 levels were decreased to 18–37% that of control levels (Fig. 7, panel B). Transfection of human Egr-1, which reduced the receptor to 18% of control, conferred resistance to all doses of TGF-β, and at lower doses (0.1–0.5 ng/ml),
DNA synthesis was stimulated up to 60% above baseline, an effect almost identical to the response of lesion-derived cells (Fig. 2B).

Parallel studies in smooth muscle cells isolated from a grossly normal human radial artery (RA-1) further indicated that transfection of Egr-1 could almost completely suppress the antiproliferative response to TGF-β1. RA-1 cells transfected with the pcDNA3 expression vector were inhibited 70% by 1 ng/ml of TGF-β, whereas Egr-1-transfected cells were essentially unaffected by TGF-β treatment. Western blot analysis confirmed the concurrent decrease in TβR-2 levels. Combined with the prior data, this strongly suggests that Egr-1 decreases the TβR-2 and confers functional resistance to the antiproliferative effect of TGF-β. However, it is possible that Egr-1 has additional effects upon mitogen production that might also influence TGF-β responses.
Egr-1 Interacts with the −143 Sp1 Site

At least two models of Egr-1 suppressive action in lesion cells can be proposed from prior studies. 1) Egr-1 interacts directly with the TβR-2 promoter or induces a factor, such as Sp3, that directly binds to and represses the TβR-2 promoter, or 2) Egr-1 does not interact with the TβR-2 promoter but instead interacts with Sp1 or ERT/ets factors to sequester or “squelch” the factor and block the transactivation of the promoter. These models can be distinguished easily because the former “repression” model predicts increased binding to the TβR-2 promoter sites in lesion cells, whereas the latter, “sequestration” model predicts decreased binding to the promoter. Using nuclear proteins extracted from stably transfected HCT116 cells (used in Fig. 6B), the binding to radiolabeled, double-stranded oligo (30 base pairs) matching the −143 Sp1 site was examined by gel shift assay (Fig. 8). A series of studies using specific antibodies to Egr-1, Egr-2, Egr-3, Sp1, and Sp3 could readily identify the protein-probe complexes. The complexes migrate in 2 sets that probably correspond to dimeric complexes that migrate as the protein (Egr-1 = 82 kDa; Sp1 = 90 kDa; Sp3 = 103 kDa) plus the labeled probe (18 kDa) and then as a higher molecular weight complex identical to the TβR-2 promoter. The smaller band in each set is eliminated by a blocking antibody to Egr-1 (C19, Santa Cruz). One band in each set is supershifted by an antibody to Sp3, a known inhibitor of Sp1 activity, and the other band is shifted by anti-Sp1. Antibodies to Egr-2 and Egr-3 had no effect. After transfection with Egr-1, the Egr-1-probe complex is strongly up-regulated and blocked by antibody to Egr-1 (Fig. 8). Sp1 and Sp3 were slightly increased in the transfected cells.

Thus, Egr-1 can interact directly with the −143 site in the TβR-2 promoter.

This was further examined by using recombinant Egr-1 protein produced by in vitro transcription/translation. To examine the potential competition with Sp1-like factors, HCT116 cells stably transfected with Sp1 were used for nuclear protein preparation. Under these conditions, each of the Sp1, Sp3, and Egr-1 bands are apparent. The addition of an excess of cold −143 probe markedly reduced binding to all 3 protein complexes. The addition of Egr-1 protein strongly increased both of the Egr-1 bands and reduced the upper Sp1 and Sp3 bands to undetectable levels, suggesting that elevated Egr-1 competitively displaces Sp1 and Sp3 from the −143 site (Fig. 8). Neither the in vitro transcription/translation reaction buffer nor Sp1 protein produced by the same method had the effect of Egr-1 in the gel shift assay.

TβR-2 Promoter Binding Activity in Medial and Lesion Cells

To determine whether a direct interaction of Egr-1 with the TβR-2 promoter was observable in lesion cells, a series of three autologous media/lesion cultures were examined. The binding of the nuclear proteins to double-stranded, radiolabeled oligomers identical to the TβR-2-143 Sp1/Egr-1 site was examined by electrophoretic mobility shift assays. The results, shown in Fig. 9, indicate that binding of Egr-1 to the −143 site was consistently stronger in the lesion than in medial cells (Fig. 9A). The binding of the same nuclear proteins to the PDGF-A chain Sp1/Egr-1 site (Fig. 9B) was generally similar to the TβR-2-143 site, although cells from patient E281 media (M) showed greater binding than lesion (L) cells. In contrast, binding of the same nuclear proteins to a consensus Sp1 probe

**Fig. 8.** Interaction of Egr-1 with the TβR-2-143 Sp1 binding site. Left panel, HCT116 cells were transfected with Egr-1 and then selected with G418. Nuclear proteins from untransfected (C) or Egr-1-transfected cells (Egr-1 Tfect) were incubated with 32P-oligo probe homologous to the −143 Sp1 site in the TβR-2 promoter. Protein-oligo complexes were separated by polyacrylamide gel electrophoresis, and the identity of the complexes was determined by a blocking antibody to Egr-1, antibody to Sp1, or antibody to Sp3. Right panel, HCT116 cells were incubated with the radiolabeled TβR-2-143 probe (C) and then blocked with unlabeled probe (cold probe, +), or recombinant Egr-1 protein was added (Egr-1 prot.) to the nuclear lysate with labeled probe, followed by analysis with polyacrylamide gel electrophoresis. The known positions of specific protein-probe complexes is shown to the left of the autoradiograms. Molecular mass was determined by prestained markers, shown on the right.

**Fig. 9.** Comparison of TβR-2-143 promoter binding in nuclear proteins from medial- and lesion-derived cells. Nuclear proteins were purified from cells cultured from the media (M) or lesion (L) of three human atherosclerotic lesions (E291, E292, E281). Nuclear proteins were incubated with one of three 32P-oligo probes: TβR-2-143 (panel A), PDGF-A (panel B), or Sp1 (panel D). The position of the protein-probe complexes is shown beside each autoradiogram. Cytoplasmic proteins from the same cells were analyzed by Western blot for Egr-1 (panel C).
indicated relatively stable levels of Sp1 binding, with some patients (E292) showing slightly lower Sp1 binding in lesion cells versus medial cells. Nuclear proteins were prepared and co-incubated with ^32P-probe as in Fig. 9, except the PRE2 region of the TβR-2 was used. Medial (M) and lesion (L) cells were additionally incubated with blocking antibody to Egr-1 or supershift antibody to Sp1, as indicated by (+) below the lanes.

**Interaction with PRE2 Site**

The Egr-1 and TβR-2 promoter co-transfection studies indicated that the elevated levels of Egr-1 that are observed in human atherosclerotic lesions in vivo are retained by the lesion-derived cells in vitro. Egr-1 is rapidly induced by mitogens such as PDGF, FGF, and EGF as well as by modified lipoproteins, shear/mechanical stresses, and free radicals. Thus, it is reasonable that Egr-1 would be elevated in atherosclerotic lesions, particularly in inflammatory areas. However, when the cells are removed from the lesion and cultured in vitro under identical conditions, it was expected that the levels of Egr-1 would equalize between medial and lesion cells. Likewise, Egr-1 levels could be expected to increase in both cell types due to activation and increased proliferative rates in vitro. Surprisingly, however, the overall mRNA levels, by array analysis, decreased as the cells were cultured, but the relative difference between medial and lesion cells were largely retained. This suggests that the elevated Egr-1 levels are at least partially due to an intrinsic dysregulation of the Egr-1 gene or that the stimulus for Egr-1 production is intrinsic to the cell. In medial cells, Egr-1 protein is transiently elevated within 1 h after FGF or NGF stimulation. Normally, Egr-1 is thought to exhibit negative feedback to its own promoter, thus ensuring its transient expression after stimulation.

**DISCUSSION**

The present data indicate that the elevated levels of Egr-1 are retained by the lesion-derived cells in vitro. Egr-1 is rapidly induced by mitogens such as PDGF, FGF, and EGF as well as by modified lipoproteins, shear/mechanical stresses, and free radicals. Thus, it is reasonable that Egr-1 would be elevated in atherosclerotic lesions, particularly in inflammatory areas. However, when the cells are removed from the lesion and cultured in vitro under identical conditions, it was expected that the levels of Egr-1 would equalize between medial and lesion cells. Likewise, Egr-1 levels could be expected to increase in both cell types due to activation and increased proliferative rates in vitro. Surprisingly, however, the overall mRNA levels, by array analysis, decreased as the cells were cultured, but the relative difference between medial and lesion cells were largely retained. This suggests that the elevated Egr-1 levels are at least partially due to an intrinsic dysregulation of the Egr-1 gene or that the stimulus for Egr-1 production is intrinsic to the cell. In medial cells, Egr-1 protein is transiently elevated within 1 h after FGF or NGF stimulation. Normally, Egr-1 is thought to exhibit negative feedback to its own promoter, thus ensuring its transient expression after stimulation.

Presently, the stimulus for the elevated Egr-1 levels in lesion cells is not known, but possibilities include: (a) genetic or methylation defects in the Egr-1 promoter, (b) increases in cellular free radicals that might induce Egr-1, (c) constitutive activation of protein kinase Cs or mitogen-activated protein kinases, (d) alterations in cellular sphingolipid balance, or (e) failure in the negative feedback pathway for Egr-1 transcription. Transcript profiling suggests reduced levels of extracellular signal-regulated kinase 1 (ERK1), extracellular signal-regulated kinase 1 (MKK4/SEK1), and c-Jun NH2-terminal kinase 2 (JNK2) in lesion cells, kinases that might be involved in Egr-1 activation in lesion cells (Table I). Although further studies will be required to evaluate the role of kinases, transcript profiling does not indicate an obvious activation of these pathways.

The present data further indicates that elevated Egr-1 can suppress transcription of the TβR-2. Previously Egr-1 was principally observed to activate gene expression by displacing Sp1, which commonly serves as a weaker transcription factor. Prior publications have identified two general mechanisms by which Egr-1 could suppress transcription: direct repression of the promoter via DNA binding (46) or squelching of transcription via interactions with Sp1, independent of DNA binding (44). Using both cells transfected with Egr-1 and medial/lesion cultures, which express different Egr-1 levels, it appears likely that the reduction in TβR-2 transcription is associated with an increase in protein binding to both the −143 Sp1 site and the PRE2 region. The interaction of Egr-1 with the −143 Sp1 is consistent with known Egr-1/Sp1 hybrid sites, although it is possible that Egr-1 and Sp1 bind in an adjacent, non-competitive manner to this region (Fig. 5). The binding of Egr-1 to the PRE2 sites may define a new type of negative regulatory function at some ets-like sites. The PRE2 region contains two negative strand, ets-like ERT sites. The predicted Egr-1 site, also negative strand, would likely mask at least one of the ERT sites. It is also possible that Egr-1 induces other factors such as Sp3, which then have a repressor activity on the TβR-2 promoter. However, the main factor in lesion cells appears to be Egr-1, and in general, Sp3 mRNA levels tend to be markedly lower in lesion that medial cells (Table I).

The present studies indicate that the elevated levels of Egr-1 in lesion cells is a potential explanation for the resistance of these cells to inhibition by TGF-β. Although the TGF-β1 gene is potently transactivated by Egr-1 (47), the present data indi-
cates that, paradoxically, the Type II receptor for TGF-β is probably suppressed by high, sustained levels of Egr-1. It is interesting to speculate that a component of sustained cellular activation may be suppression of inhibitory pathways such as the TGF-β pathway. In cases where production of TGF-β is elevated, as in chronic repair/inflammation, it might be necessary to target the TGF-β-producing cell from the autocrine inhibitory effects of TGF-β.

The current results may have some relevance to the progression of tumors as well. Almost all tumor cell lines examined have acquired resistance to the antiproliferative and apoptotic effects of TGF-β (48). Only in relatively rare cases such as the RER+ phenotype of familial colon carcinomas, are acquired mutations in TβR-2 a sufficient explanation for the resistance (49). In most other cases, the resistance to TGF-β is associated with reduced levels of TGF-β, but not due to detectable mutations or promoter hypermethylation (50), and thus, transcriptional repression of TβR-2 may be a significant cause of resistance to the TGF-β tumor suppressor pathway (51). Notably, some oncogenic rearrangements, such as the EWS/Fli1 rearrangement, create repressors for TβR-2 transcription (52).

Accumulating evidence indicates that atherosclerosis is associated with both reduced levels of TGF-β (53) and with acquired resistance to the apoptotic effects of TGF-β in lesion cells (5, 7). Based on the current data, it is reasonable to speculate that chronic expression of Egr-1 is a major contributing factor to acquired resistance to TGF-β. Acquired resistance to inhibitors might then be a major factor determining the failure to die phenotype that describes the advanced atherosclerotic lesion. In vitro and in vivo data both suggest that blocking Egr-1 expression via antisense or DNAzymes inhibits smooth muscle cells proliferation and migration and blocks injury-induced intimal hyperplasia (21, 23). Combined, the evidence indicates that chronically elevated Egr-1 in lesions cells is 1) a marker of a highly activated phenotype, which includes functional resistance to inhibitory agents, 2) a direct repressor of the TGF-β pathway, and 3) a potential target for intervention in cases of excessive vascular repair.

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Elevated Egr-1 in Human Atherosclerotic Cells Transcriptionally Represses the Transforming Growth Factor-β Type II Receptor
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