Early Growth Response-1 Promotes Atherogenesis
Mice Deficient in Early Growth Response-1 and Apolipoprotein E Display Decreased Atherosclerosis and Vascular Inflammation

Evis Harja, Loredana G. Bucciarelli, Yan Lu, David M. Stern, Yu Shan Zou, Ann Marie Schmidt, Shi-Fang Yan

Abstract—Early growth response-1 (Egr-1) regulates expression of proinflammatory and procoagulant genes in acute cell stress. Experimental evidence suggested that Egr-1 transcripts were upregulated in human atherosclerotic plaques versus adjacent unaffected tissue. To test the impact of Egr-1 in chronic vascular stress, we examined its role in a murine model of atherosclerosis. Real-time PCR analysis of aortae retrieved from apoE−/− mice demonstrated increased Egr-1 transcripts in an age-dependent manner, compared with aortae retrieved from C57BL/6 control animals. Therefore, homozygous Egr-1−/− mice were bred into the apoE−/− background. Homozygous double-knockout mice (Egr-1−/−/apoE−/−) in the C57BL/6 background were maintained on normal chow diet. At age 14 and 24 weeks, atherosclerotic lesion area and complexity at the aortic root were strikingly decreased in mice deficient in both Egr-1 and apoE compared with mice deficient in apoE alone. In parallel, transcripts for genes regulating the inflammatory/prothrombotic response were diminished in Egr-1−/−/apoE−/− aortae versus apoE−/−. In vitro, oxidized low-density lipoprotein (OxLDL), a key factor inciting atherogenic mechanisms in the vasculature, upregulated Egr-1 expression in monocytes via the MEK-ERK1/2 pathway. We conclude that Egr-1 broadly regulates expression of molecules critically linked to atherogenesis and lesion progression. (Circ Res. 2004;94:333-339.)

Key Words: transcription factor ■ oxidized low-density lipoprotein ■ signaling pathway ■ hypercholesterolemia ■ atherosclerosis

Atherosclerosis is a chronic inflammatory disease in which lipid and inflammatory pathways drive pathological expansion of the neointima in the vessel wall. Atherosclerotic lesions in human cardiovascular disease are enriched in smooth muscle cells (SMCs) and mononuclear phagocytes (MPs) as lesion development progresses. Experimental evidence strongly supports the premise that low-density lipoproteins (LDL) and particularly their oxidatively modified species (OxLDL) play key roles in atherogenesis. However, the precise mechanisms that evoke chronic inflammatory processes implicated in plaque formation and progression, and, essentially, plaque rupture and thrombosis, remain incompletely defined.

The immediate early gene, early growth response-1 (Egr-1), is a zinc-finger transcription factor linked to maladaptive host response mechanisms in settings characterized by acute cellular perturbation, such as that induced by hypoxia/hypoxemia. Previously, we demonstrated that induction of hypoxia in vitro6-7 and in vivo8 upregulates transcripts and protein for Egr-1 and increases its nuclear translocation, particularly in MPs and SMCs. Indeed, studies in vivo highlighted the novel observation that Egr-1 functioned as a "master switch," regulating diverse pathways in the host response to ischemic stress. Compared with wild-type mice, homozygous Egr-1−/− mice failed to display enhanced expression of a diverse range of procoagulant/proinflammatory genes in the lung on ischemia/reperfusion (I/R) injury. In other studies, blockade of Egr-1 in a rodent model of isogeneic orthotopic lung transplantation using antisense oligonucleotides enhanced survival and suppressed transcripts for IL-1β, tissue factor (TF), and plasminogen activator inhibitor-1 (PAI-1). In parallel, fibrin deposition and neutrophil recruitment, key components of tissue injury, were diminished.

Experimental evidence is emerging to link Egr-1 to chronic vascular and inflammatory stress in vivo. In human emphysematous lung sampled at the time of lung reduction surgery and subjected to microarray expression analyses, transcripts for Egr-1 were consistently upregulated compared with levels of transcripts prepared from normal lung tissue. Together with the demonstration that transcripts for Egr-1 were strikingly upregulated in both human and murine atherosclerotic lesions compared with adjacent unaffected tissue, these findings strongly suggested that roles for Egr-1 in chronic vascular diseases such as atherosclerosis were likely.

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From the Division of Surgical Science, Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, NY.
Correspondence to Dr Shi-Fang Yan, BB1705, Department of Surgery, College of Physicians and Surgeons of Columbia University, 630 W 168th St, New York, NY 10032. E-mail sy18@columbia.edu
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To explore the potential role of Egr-1 in atherogenesis, homozygous Egr-1−/− mice were bred into the apoE−/− background. ApoE−/− mice develop spontaneous atherosclerotic lesions in the arterial vasculature driven by elevated levels of cholesterol with a pattern similar to that observed in the human. In the present study, we evaluated the effect of genetic deficiency of Egr-1 in the apoE−/− background. In vitro, we tested the premise that oxidized LDL (OxLDL) modulated expression of Egr-1 in MP, cells importantly involved in atherogenesis and lesion progression.

Materials and Methods

Animal Studies
Homozygous Egr-1−/− mice7,16 were backcrossed >6 generations into C57BL/6 in our laboratory before crossing with apoE−/− mice, the latter purchased from Jackson Laboratories (Bar Harbor, Maine). All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of Columbia University. Homozygous Egr-1−/− mice were intercrossed with apoE−/− mice to generate breeding pairs with heterozygous deficiency of Egr-1 and apoE (Egr-1+/−/apoE−/−), which sired Egr-1−/−/apoE−/− and Egr-1+/−/apoE−/− littermate offsprings. All of the mice were fed normal chow.

Quantification of Atherosclerotic Lesion Area
After the mice were fasted for 4 hours and then anesthetized, their blood was withdrawn from the inferior vena cava into heparin and plasma was stored for analysis. For quantitative PCR studies, aortae were retrieved and rapidly frozen in liquid N2 and stored at −80°C before analysis. For histology studies, the aorta and heart were harvested and stored in buffered formalin (10%). Cryostat sections were prepared after hearts were sequentially embedded in gelatin at temperatures of 5% 10%, and 25%. Serial sections, 10 μm in thickness, were cut from the level of the aortic valve leaflets up to about 480 μm above the leaflets in the aortic sinus; alternate sections were retrieved and placed onto Superfrost Plus glass slides (Fisher Scientific); four sections were placed on each slide for a total of six slides. Sections were then stained with Oil Red O and counterstained with hematoxylin/light green (Sigma). Atherosclerotic lesion areas were quantified on one section from each slide (beginning at the side where three distinct valves first appear) using a Zeiss microscope and image analysis system: mean lesion area from slides two through five is reported. Two of the investigators, blinded to the experimental conditions, analyzed the slides and performed the morphometric analysis.

RNA Extraction and Real-Time PCR
Total RNA (1 μg) was extracted from frozen single aorta using Trizol reagent (Life Technologies Inc.), and was processed directly to cDNA synthesis using the TaqMan Reverse Transcription Reagents kit (Applied Biosystem) according to the manufacturer’s protocol. All PCR primers and TaqMan probes were designed using software PrimerExpress (Applied Biosystem). The sequences of forward, backward primers, and probes for target genes (mouse Egr-1, JE/MCP-1, IL-1β, TF, PAI-1, VCAM-1, and ICAM-1) and housekeeping gene (β-actin) were selected based on published sequence data from the NCBI database as shown in Table 1. Primers were synthesized, and TaqMan probes for target genes or β-actin were labeled with the reporter dye TAMRA or VIC in the 5’ end, respectively, and quencher dye TAMRA in both of the 3’ end from Applied Biosystem. All reactions were performed in triplicate in ABI PRISM 7900HT Sequence Detection System. β-Actin was used as an active and endogenous reference to correct for differences in the amount of total RNA added to reaction and to compensate for different levels of inhibition during reverse transcription of RNA and during PCR. Data are calculated by 2−ΔΔCT method19 and are presented as fold induction of transcripts for target genes in apoE−/− mice or in Egr-1−/−/apoE−/− mice normalized to β-actin, compared with C57BL/6 mice (defined as 1.0-fold in each case).

Immunohistochemistry
Aortic arch was harvested, fixed in formalin (10%), and embedded in paraffin. Sections were stained with the following primary antibodies: rabbit anti-Egr-1 IgG (8 μg/mL; Santa Cruz), rat F4/80 monoclonal antibody (10 μg/mL; PharMingen), mouse smooth muscle-actin monoclonal antibody (10 μg/mL; Sigma), or nonimmune IgG, respectively. Secondary antibodies (affinity-purified alkaline phosphatase-conjugated anti-rabbit, anti-rat, or anti-mouse IgGs) and substrates were used.7

Biochemical Analyses
Levels of total cholesterol and triglyceride were determined in fasted mice using chromogenic assays (Sigma). Glucose levels were determined from samples of tail vein blood using a glucometer (Roche Diagnostics).

Preparation of Oxidized Low-Density Protein (OxLDL)
Human plasma was purchased from the American Red Cross. Plasma LDL were prepared by sequential ultracentrifugation with a density cut-off of d=1.019 to 1.063 g/mL and oxidized in the presence of 2.5 mmol/L CuCl2 at 37°C for 48 hours, and dialyzed extensively followed by assessment of TBARS (thiobarbituric acid reactive substances) to verify the degree of oxidation. The protein concentration of LDL or Ox-LDL was determined using the Lowry assay.

Cell Culture
RAW 264.7 cells, a murine macrophage cell line (ATCC), were grown to 70% to 80% confluence, serum-starved for 24 hours, and
then incubated with OxLDL for the indicated time periods. Where indicated, cells were pretreated with MEK-ERK1/2 inhibitor 0.05 (Figures 2A through 2C). Egr-1−/−/apoE−/− animals displayed no atherosclerotic lesions (not shown). At 24 weeks of age, similar results were observed, however, the differences between double-null and single-apoE−/− mice were even more striking: 7-fold decrease in mean atherosclerotic lesion area was observed in Egr-1−/−/apoE−/− mice (29 252.50±9390 μm², n=16) versus littermate apoE−/− bearing Egr-1 (15 285.58±4547 μm², n=10); P=0.05 (Figures 2A through 2C). Egr-1−/−/apoE−/− animals displayed no atherosclerotic lesions (not shown). At 24 weeks of age, similar results were observed, however, the differences between double-null and single-apoE−/− mice were even more striking: 7-fold decrease in mean atherosclerotic lesion area was observed in Egr-1−/−/apoE−/− mice (29 252.50±9390 μm², n=16) versus littermate apoE−/− animals (205 169.5±243 243 μm², n=10); P<0.0001 (Figure 2D through 2F). At both ages, Oil Red O staining of histological sections prepared at the aortic root revealed larger and more numerous lesions in the mice bearing Egr-1 in the apoE−/− background. In parallel with decreased atherosclerotic lesion area at the aortic root, the lesion complexity index was determined. This index was quantified by the ratio of complex lesions to the total number of lesions. Complex lesions were defined as those characterized by fibrous caps, cholesterol clefts, or lesion necrosis. The complexity index was significantly reduced in Egr-1−/−/apoE−/− mice versus littermate apoE−/− mice at age of 24 weeks; (0±0 versus 0.733±0.136); P=0.0003 (Figure 2G).

Effects of Genetic Deletion of Egr-1 on Activation of Proinflammatory/Procoagulant Mediators in Atherosclerosis

To begin to elucidate the molecular mechanisms underlying these observations, we performed real-time quantitative PCR on RNA prepared from the aortae of double-null and single-apoE−/− female mice. Transcripts for genes implicated in the inflammatory and procoagulant response were significantly higher in apoE−/− mice versus Egr-1−/−/apoE−/− animals: JEMCP-1, 5.7-fold, P=0.012; IL-1β, 5.6-fold, P<0.0001; TF, 4.8-fold, P=0.0002; PAI-1, 3-fold, P=0.015; VCAM-1, 3.3-fold, P=0.0002; and ICAM-1, 5.5-fold, P<0.0001 (Figure 3).
Deletion of Egr-1 Does Not Affect Levels of Cholesterol/Triglyceride or Glucose in ApoE\(^{-/-}\) Mice

These studies suggested that deletion of Egr-1 substantially reduced the extent of atherosclerosis in apoE\(^{-/-}\) mice; a key question to address, however, was whether deletion of Egr-1 implicated on classic risk factors for atherosclerosis in apoE\(^{-/-}\) mice. Because the most striking risk factor driving atherosclerosis in the apoE\(^{-/-}\) model is the elevation of cholesterol and triglyceride, we examined these parameters in the mice at 14 and 24 weeks of age. At 14 weeks of age, the levels of total cholesterol and triglyceride in plasma from Egr-1\(^{-/-}\)/apoE\(^{-/-}\) mice and apoE\(^{-/-}\) mice were not different (Table 2). Similarly, at 24 weeks of age, there were no differences in levels of these factors between the two genotypes (Table 2). Furthermore, we found that the levels of plasma glucose were not different between the two genotypes (not shown).

Molecular Mechanisms by Which Egr-1 Contributes to Atherosclerotic Lesion Initiation/Progression

Because modified lipoproteins are enriched in apoE\(^{-/-}\) mice before the activation and upregulation of Egr-1, and because our data demonstrated that transcripts for Egr-1 were elevated in the vasculature of these animals before the development of frank atherosclerotic lesions, we tested the potential impact of (modified) lipoproteins on Egr-1 expression in macrophages, key cells linked to the initiation and progression of atherosclerosis. To test these concepts, we prepared oxidized LDL from human plasma in the presence of low concentrations of Cu\(^{2+}\) and exposed OxLDL (1 to 20 \(\mu \text{g/mL}\)) to cultured RAW 264.7 cells, a murine macrophage line, for 1 hour. Compared with normal media, OxLDL significantly increased the expression of Egr-1 antigen by Western blotting in RAW cells in a dose-dependent manner (Figure 4A), with a peak effect observed at 5 \(\mu \text{g/mL}\) (\(\sim 7.3\)-fold; \(P<0.0001\)).

To identify the molecular pathways by which OxLDL mediated upregulation of Egr-1, we tested the role of the MEK-ERK 1/2 MAP kinase pathway. Incubation of RAW cells with OxLDL, 5 \(\mu \text{g/mL}\), resulted in a time-dependent increase in phosphorylation of p44/p42 MAP kinases. Peak
phosphorylation of these species by OxLDL was observed at 15 and 30 minutes incubation (≈7.4- and 8.1-fold compared with untreated RAW cells; *P*<0.0001) (Figure 4B). In contrast, there were no changes in the expression of nonphosphorylated forms of ERK1/2 in the presence of OxLDL (Figure 4B). To determine if the MEK-ERK1/2 MAP kinase pathway was linked to OxLDL-mediated regulation of Egr-1, RAW cells were pretreated with PD98059, 10 μmol/L, for 60 minutes before incubation with OxLDL. Compared with cells treated with OxLDL alone, RAW cells exposed to PD98059 and OxLDL, 5 μg/mL, displayed a significantly reduced expression of Egr-1 antigen by Western blotting of ≈75%, *P*<0.0001 (Figure 4C). These results suggested that the MEK-ERK1/2 MAP kinase pathway importantly contributes to OxLDL-mediated induction of Egr-1 expression.

**Discussion**

The Egr-1 gene product, also called Zif268, NGF1-A, Krox24, or TIS8, was first characterized as an “immediate early gene” secondary to its pattern of expression after exposure of cells to mediators associated with growth and differentiation. On response to a triggering event, Egr-1 is rapidly induced within minutes of the stimulus, followed by decay within hours.21 In vivo, these facets of Egr-1 biology were evident in the response to acute oxygen deprivation. The central importance of Egr-1 in the regulation of genes mediating inflammatory and procoagulant consequences in these settings was underscored by the enhanced survival and lung function observed in homozygous Egr-1−/− mice subjected to unilateral ischemia/reperfusion lung injury.9

In atherosclerosis, both initiating and progression factors may yield acute cardiovascular events if left unchecked. Our studies show that the Egr-1 pathway likely impacts to a greater extent in disease progression. A highly significant difference was observed in mice doubly null for both Egr-1 and apoE at 24 weeks versus mice solely deficient in apoE; the distinctions between double- and single-null animals at age 14 weeks were also significant but not striking. Although multiple proinflammatory genes were regulated at least in part by Egr-1 at age 14 weeks in these animals, it is very likely that other transcription factors, such as NF-κB and AP-1 also played critical roles in these events.22,23 Our data have shown that oxidized lipoproteins such as OxLDL may upregulate Egr-1 in MP; these findings suggest that in early atherosclerosis, OxLDL use a range of molecular pathways to initiate vascular perturbation. It is important to note that other studies have suggested that enzymatically degraded low-density lipoproteins may be more potent inducers of Egr-1 mRNA than either OxLDL or native LDL.24 In those experiments, studies were limited to the use of Mono-Mac-6 cells; thus, the direct relevance to the system studied here is not fully clarified. Further, in those studies, the signaling pathways by which such modified species of LDL modulated Egr-1 expression were not addressed. In other studies, it has been shown that native and modified LDL induce expression of Egr-1 in endothelial cells and smooth muscle cells, although the precise signaling mechanisms were not elucidated.25,26 Thus, our studies provide the first demonstration of a link between oxidized LDL and Egr-1 regulation in MP, at least in part via the MEK-ERK1/2 pathway.

Other species likely to be important especially at early stages of atherogenesis are oxidized phospholipids. Evidence suggests that these species may activate genes linked to early proinflammatory events in atherosclerosis, in part, by activation of Egr-1. Studies by Kadl and colleagues27 indicate that oxidized 1,α-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholin (OxPAPC) upregulated Egr-1 and MCP-1 in cultured human umbilical vein endothelial cells, and in liver and heart when infused into C57BL/6 mice. Furthermore, the isoprostane 8-iso-PGE2 has been shown to stimulate monocyte binding to endothelial cells in part via Egr-1−/− dependent mechanisms.28 Beyond proteins/lipids modified or generated by oxidative pathways, recent studies have implicated upregulation of Egr-1 to a specific pathway by which infectious agents, especially *Chlamydia pneumoniae*, may augment vascular stress and, long-term, accelerate atherosclerosis.29,30 Thus, it is very likely that Egr-1 is a final common pathway recruited by a range of biochemically modified species and biological agents in the vasculature in atherosclerosis. However, it is clear that in early disease multiple distinct pathways contribute to an important degree.

In later stages of atherosclerosis, our findings link Egr-1−/− dependent mechanisms to disease progression. At later stages of disease, age 24 weeks, mice deficient in both Egr-1 and apoE displayed a marked decrease in atherosclerosis versus mice deficient in apoE alone. These findings provide mechanistic insights into the description by McCaffrey and colleagues32 that Egr-1 mRNA was highly enriched, approximately 5-fold, in fibrous caps versus adjacent media of atherosclerotic lesions retrieved from 13 human subjects undergoing carotid endarterectomy. In addition to proinflammatory mediators, Egr-1−/− dependent genes include genes such as tissue factor (TF) and PAI-1 that mediate prothrombotic events. Indeed, the essential role of Egr-1 in regulation of the tissue factor gene provided the first clue to the importance of this transcription factor in vascular biology.6 The finding that Egr-1−/− mice displayed diminished upregulation of tissue factor and PAI-1, in parallel with decreased

| Table 2. Levels of Plasma Cholesterol and Triglyceride |
|-------------|-------------|-----------|-----------|
| Age, wk  | Genotype | Cholesterol | Triglyceride |
|           |           | n mg/dL | P          | n mg/dL | P          |
| 14        | −/−       | 13 195.50±18.62 | 0.15 | 17 47.35±2.91 | 0.95 |
| 24        | −/−       | 9 256.74±40.78 | 0.14 | 9 47.69±6.08 |
|           | +/+       | 11 302.42±38.62 | 0.14 | 9 97.01±14.03 | 0.09 |
|           | −/−       | 14 360.10±15.26 | 0.14 | 14 127.44±10.30 |
fibrin deposition in the lungs of mice subjected to ischemia/reperfusion, has direct implications for chronic vascular diseases in which activation of procoagulant mechanisms and generation of tissue factor are fundamental characteristics of progressive disease. Central roles for tissue factor in atherosclerosis have been described, particularly in the context of plaque instability and rupture.31

In this context, recent observations have suggested roles for Egr-1 in regulation of diverse pathways linked to atherosclerosis. For example, Egr-1 (as well as activator protein-1 and NF-κB) mediates CD40 ligand–induced expression of tissue factor in human endothelial cells. In addition, a potential role for Egr-1 in transcriptional activation of peroxisome proliferator–activated receptor gamma 1 (PPAR-γ) in vascular smooth muscle cells has been suggested.34 Furthermore, elevated Egr-1 in human atherosclerotic cells transcriptionally represses transforming growth factor-β type II receptor, thereby providing a mechanism to suppress vascular repair pathways.35 Finally, recent studies suggested that administration of simvastatin suppressed expression of tissue factor in apoE-null mice with advanced atherosclerosis, in parallel with decreased vascular lesion expression of Egr-1.36 When these concepts were probed in tissue culture, these investigators found that mouse macrophages displayed decreased lipopolysaccharide-induced binding of nuclear proteins to the Egr-1 consensus DNA sequence after pretreatment with simvastatin.36 Taken together, these considerations underscore the concept that multiple proatherogenic forces may converge at the level of Egr-1, thus elucidating critical roles for Egr-1 as an amplification step in the progression of atherosclerosis and vascular injury.

Indeed, further evidence for the inducibility of Egr-1 under stress conditions of acute vascular injury emerged from studies in denuding arterial injury in the rat aorta. Egr-1 and a number of its target genes were induced at the wound margins in the endothelium.37,38 The first in vivo evidence of a specific role for Egr-1, and not only an association, in the response to arterial injury, came from experiments using catalytic oligodeoxynucleotide that specifically target Egr-1 mRNA. Application of this technology in rats subjected to carotid artery injury blocked neo-intima formation.39 Studies in Egr-1−/− mice are now required to conclusively link this pathway to acute neo-intimal expansion triggered by physical denuding injury to the endothelium.

Taken together, our studies provide definitive mechanistic support for the link between Egr-1 and the pathogenesis of atherosclerosis. Because cardiovascular disease remains a major cause of morbidity and mortality worldwide, we propose that delineation of the contribution of Egr-1 to atherosclerotic lesion initiation and progression may provide novel insights into the pathogenesis of this complex disorder and elucidate new strategies for therapeutic intervention.

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Figure 4. Oxidized low-density lipoprotein (OxLDL) induces expression of Egr-1 in RAW264.7 mouse macrophages via the MEK-ERK1/2 pathway. A, RAW cells were incubated with the indicated concentrations of OxLDL for 1 hour and Western blotting for Egr-1 antigen was performed. B, RAW cells were incubated with OxLDL as indicated and Western blottings for phospho- and total ERK1/2 MAP kinase were performed. C, RAW cells were incubated with OxLDL in the presence or absence of PD98059, and Western blotting for Egr-1 or Sp1 was performed.


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