Increased Expression of the Transforming Growth Factor-β Signaling Pathway, Endoglin, and Early Growth Response-1 in Stable Plaques

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Background and Purpose—Unstable atherosclerotic plaques are characterized by increased macrophages and reduced smooth muscle cells (SMCs) and collagen content. Endoglin, an accessory transforming growth factor-β (TGFβ) receptor, is a modulator of TGFβ signaling recently found to be expressed on SMCs in atherosclerotic plaques. Its function in plaque SMCs and plaque development is unknown. Early growth response-1 (EGR-1), a transcription factor downstream of TGFβ, stimulates SMC proliferation and collagen synthesis. In atherosclerotic lesions, it is mainly expressed by SMCs. Therefore, we studied the TGFβ, endoglin, and EGR-1 pathway in advanced atherosclerotic plaques in relation to plaque phenotype.

Methods—Human carotid atherosclerotic plaques (n=103) were collected from patients undergoing carotid endarterectomy. Histologically, plaques were analyzed for plaque characteristics, ie, collagen, macrophage and SMC content, and intraplaque thrombus. Intraplaque endoglin, pSmad (indicative for TGFβ signaling), EGR-1, and TGFβ levels were analyzed using Western blots and enzyme-linked immunosorbent assays, respectively.

Results—Higher endoglin and EGR-1 protein levels correlated positively with increased plaque collagen levels, increased smooth muscle cell content, and decreased intraplaque thrombi as well as TGFβ signaling (pSmad). Although EGR-1 overexpression in vitro stimulated collagen synthesis, inhibiting endoglin resulted in lower EGR-1 levels, decreased SMC proliferation, and decreased collagen content.

Conclusions—TGFβ in human atherosclerotic plaques is active and signals through the TGFβ/Smad pathway. For the first time, we show a strong association between endoglin and EGR-1, increased collagen and SMCs expression, decreased levels of intraplaque thrombosis, and a stable plaque phenotype. (Stroke. 2009;40:439-447.)

Key Words: collagen ■ EGR-1 ■ endoglin ■ plaque stability ■ smooth muscle cells

Atherosclerosis is a chronic inflammatory response in the arterial wall.1 Stable plaques contain more collagen and smooth muscle cells (SMCs), whereas unstable plaques have more macrophages and contain a large lipid core covered with a thin fibrous cap.2 Unstable plaques are more likely to rupture and cause clinical symptoms, eg, cerebrovascular accidents or myocardial infarction.3,4 Therefore, insights into the mechanisms that determine plaque stability are crucial.

Transforming growth factor-β (TGFβ) plays an important role in atherogenesis.5 TGFβ regulates important cellular processes influencing the development and progression of atherosclerosis such as remodeling of the extracellular matrix and stimulation of SMC proliferation.6 TGFβ transduces its signal by binding to TGFβ Type I and II serine/threonine kinase receptors leading to phosphorylation of Smad proteins.7 On ligand binding, the Type II receptor will form a complex with ALK5 phosphorylating Smad2/3 or ALK1 and phosphorylate Smad1/5.8 Endoglin (CD105) is an accessory transmembrane TGFβ receptor and a modulator of TGFβ signaling.9-13

In human atherosclerotic lesions, the TGFβ signaling components are detectable in endothelial cells, SMCs, and macrophages and rapidly upregulated during vascular injury.14 Although TGFβ is highly expressed in human atherosclerotic lesions, its contribution to lesion development and progression is still unclear.5,6,15 Inhibition of TGFβ signaling in an athero-
sclerotic mouse model significantly inhibits collagen synthesis in atherosclerotic plaques, suggesting an important role for TGFβ in plaque stabilization. TGFβ-induced collagen synthesis is mediated through increased expression of the transcription factor early growth response-1 (EGR-1). Both endoglin and EGR-1 are highly induced shortly after balloon catheter injury and are expressed by SMCs and endothelial cells in advanced atherosclerotic lesions, whereas expression levels in healthy arteries are low.

Although increased collagen production should improve plaque stabilization, the delicate balance between synthesis and degradation of collagen is more important than synthesis alone. Extracellular matrix metalloproteinase (MMP) inducer (EMMPRIN) stimulates the production of different MMPs and degradation of collagen is more important than synthesis. The role of endoglin and EGR-1 in this context and the correlation with matrix degradation and EMMPRIN expression yet remains unknown. In addition to their effect on collagen synthesis, TGFβ and EGR-1 both stimulate SMC proliferation, although the role of TGFβ in this context is controversially discussed, because TGFβ is known for its inhibitory effect on endothelial cell proliferation. Interestingly, endoglin reduces the inhibitory effect of TGFβ on endothelial cell proliferation.

We therefore hypothesized that stable human atherosclerotic plaques contain increased expression levels of endoglin and EGR-1, which mediate the beneficial effects of TGFβ on plaque stability through induction of collagen synthesis and stimulation of SMC proliferation.

**Materials and Methods**

**Study Design**

Athero-Express is a multicenter study combining a biobank of carotid endarterectomy material and patient follow-up data. The study was approved by the local medical ethics committees of each participating center and performed in accordance with institutional guidelines. Informed written consent was obtained from all patients. Baseline characteristics of the patients are depicted in the Table.

**Immunohistochemistry**

Of each patient (n=103), the culprit lesion was divided into sections of 5 μm and each plaque was stained for histological determination as described previously. The adjacent segment was used to isolate total protein. Atherosclerotic plaques were stained for endoglin (613134, BD), von Willebrand factor (A0082; Dako), SMC (A2547; Cell Signaling Technology), and EGR-1, which mediate the beneficial effects of TGFβ on plaque stability through induction of collagen synthesis and stimulation of SMC proliferation.

**Protein Expression Analysis**

Endoglin, EGR-1, pSmad2 (3104; Cell Signaling Technology), pSmad1/5 (9516; Cell Signaling Technology), and EMMPRIN (SC9753, Clone K-20; Santa Cruz Biotechnology) levels were determined by Western blotting. Twenty micrograms of total protein was loaded on a reduced polyacrylamide gel. Quantification of the bands was carried out using densitometric analysis software, Quantity One (Gel doc; Bio-Rad). Expression levels were correlated to the expression of βactin (CloneAC-74; Sigma). A reference sample was loaded on each blot allowing the comparison between runs. Total MMP activities were measured using the Biotrak activity assay kits RPN2631 for MMP-2 and RPN2634 for MMP-9 (Amersham Biosciences). Total TGFβ1 levels were measured using enzyme-linked immunosorbent assay (DB100B; R&D Systems), applying 20 μg of total Tripure (Boehringer Mannheim, Indianapolis, Ind) isolated protein (n=96).

**Effect of mRNA Inhibition of Endoglin on Smooth Muscle Cell Proliferation and Transforming Growth Factor-β Signaling**

Human aortic SMCs (HA-SMCs, CRL-1999; American Type Culture Collection) were cultured according to the manufacturer. HA-SMCs were infected with adenoviruses expressing Endoglin RNAi or LacZ control at a multiplicity of infection of 250. After 16 hours, the cells were washed and allowed to recover for 24 hours, starved overnight, and stimulated with TGFβ (1 ng/mL) for the indicated times. Cells were washed and total protein was isolated using Tripure Isolation Reagent. Endoglin, EGR-1, pSmad1/5, pSmad2, pSmad3, procollagen-I (SPL1D6), and Ki-67 (Clone MIB-1; Dako) expression levels were studied using Western blotting as described previously. Migration was measured using a modified Boyden chamber as previously described.

**Early Growth Response-1 Affects Collagen and CAGA-Reporter Activity**

Human embryonic kidney-293 cells (CRL-1573; American Type Culture Collection) were cultured in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin. At approximately 80% confluence, cells were washed twice with phosphate-buffered saline and 0.4 mL serum-free DMEM was added. Human embryonic kidney cells were transfected (Lipofectamine Plus; Invitrogen) with 0.1 ng/mL TGFβ (1 ng/mL) for the indicated times in the presence of different concentrations of an EGR-1 expression plasmid (kindly provided by Dr Yutaka Inagaki) or 0.1 μg CAGA-Luc, an artificial plasmid binding sequence reporter construct, in the absence or presence of different concentrations of a TGFβ1 expression plasmid (kindly provided by Professor Dr J. Milbrandt). PKG-LacZ was used as transfection control. After 3 hours, 0.25 mL of 1% FCS, 1% penicillin/streptomycin DMEM medium was added. Two days after transfection, cells were washed twice with phosphate-buffered saline and incubated at room temperature for 15 minutes in 0.2 mL of Lysis buffer (10 mmol/L DTT, 10 mmol/L CDTA, 50% glycerol, 5% Triton X-100, 125 mmol/L Tris, pH 7.8). Fifty microliters of lysate was transferred to a 96-well plate and 50 μL of 2X βGal assay buffer (200 mmol/L sodium phosphate, pH 7.3, 2 mmol/L MgCl2, 100 mmol/L βmercaptoethanol, 1.33
 Luciferase activity was determined by analyzing 10 μL of lysate (Promega) in a Berthold Lumat LB 9507 (Berthold Technologies).

### Statistical Analysis

Data are presented as mean and 95% CI. Because our data were not normally distributed, we used the nonparametric Mann-Whitney (for comparison of 2 categories) or Kruskal-Wallis test (for comparison of 3 or more categories). Correlations were analyzed with the Spearman rank test. Student t test was used for the in vitro experiments when we compared 2 conditions. A probability value of <0.05 was considered significant.

### Results

#### Early Growth Response-1 and Plaque Composition

EGR-1 was mainly expressed by SMCs (Figure 1A) and to a lesser extent by macrophages (Figure 1D). EGR-1 positively associated with a more fibrous plaque (Figure 2A). Plaques with a high collagen content showed significantly higher EGR-1 levels compared with plaques with no or moderate collagen staining (Figure 2B). EGR-1 protein levels show significant positive correlation with intraplaque α-SMC actin levels (correlation coefficient: 0.280, P=0.049; Figure 2C). Plaques with positive SMCs/macrophages ratios showed higher EGR-1 expression when compared with plaques with higher macrophage content (Figure 2D). Furthermore, high EGR-1 levels positively correlated with low levels of the leukocyte chemoattractant interleukin-8 (correlation coefficient: –0.423, P=0.00002; Figure 2E). Interestingly, EGR-1 levels negatively correlated with the presence of intraplaque thrombi, indicative of unstable plaques (Figure 2F). Representative Western blots of our protein targets are depicted in Figure 2G.

### Endoglin and Plaque Composition

Endoglin was expressed in some of the intraplaque vessels and SMCs within the lesion and to some extent by macrophages (Figures 1B and E). pSMAD expression was observed in SMCs, endothelial cells (not shown), and macrophages (Figures 1C and F). However, not all endothelial cells and SMCs expressed endoglin. Interestingly, endoglin expression was increased in plaques with more α-SMC actin (R²=0.285; P=0.035; Figure 2H). Endoglin and macrophage content of the plaque did not correlate (P=0.280, not shown). Endoglin expression was higher in plaques containing high levels of collagen (Figure 2I), indicative of a stable plaque less prone to rupture. Furthermore, endoglin positively correlated with less thrombi in the plaque (Figure 2J).

### Intraplaque Transforming Growth Factor-β Signaling

The level of phosphorylated (p)-Smad within a lesion is as measure for TGFβ signaling. Endoglin positively correlated with pSmad2 and pSmad3 (P<0.001 and P=0.003, respectively; Figure 3A–B) levels in atherosclerotic plaques but did not correlate with pSmad1 (correlation coefficient=0.173; P=0.24), suggesting the presence of an endoglin-TGFβ-Smad2/3 signaling pathway within the lesion. Furthermore, pSmad2, but not pSmad1, was positively associated with SMCs (R²=0.289; P=0.03). In vitro, TGFβ has been demonstrated to induce EGR-1 expression, which was mediated through pSmad2/3. Accordingly, TGFβ levels were associated with a significantly increased expression of EGR-1 (Figure 3C). In addition, EGR-1 expression was associated with TGFβ signaling illustrated by increased pSmad2 (correlation coefficient=0.464, P=0.001) as well as endoglin expression levels (correlation coefficient=0.363, P=0.009; Figure 3D–E) but not pSmad1 (Figure 3F).

Because TGFβ signaling is influenced by endoglin, we analyzed the causal interaction between EGR-1 and endoglin. Endoglin-RNAi expressing vascular SMC showed no upregulation of EGR-1 expression after TGFβ stimulation, whereas LacZ-transduced cells demonstrated a 1.3-fold increase (Figure 4A–B; Supplemental Figure 1A, available online at http://stroke.ahajournals.org). Transfection of EGR-1 resulted in a dose-dependent decrease in luciferase activity of an artificial SMD3 luciferase reporter showing a possible interference of EGR-1 on TGFβ signaling (Figure 4F).
Figure 2. EGR-1 expression was significantly higher in fibrous plaques compared with plaques with a fibroatheromatous or atheromatous phenotype (*P = 0.011, correlation coefficient = 0.288, **P = 0.003; A). Plaques with heavy collagen staining showed a significant higher expression of EGR-1 compared with plaques with no/moderate staining of collagen (B). EGR-1 levels correlated significantly to (Continued)
and MMP-2 (Figure 5A–B), pSmad1 levels negatively associated with TGF\(\beta\)/H\(9252\) and MMP-9 activity, \(\text{P} < 0.017\) and \(\text{P} < 0.014\), respectively; Figure 5C–D). TGF\(\beta\) \(\text{P} = 0.019\), pSmad2 \(\text{P} = 0.027\), and endoglin strongly associated positively with EMMPRIN 45 kD \(\text{P} = 0.008\), which has been associated with a stable plaque phenotype, but not between pSmad1 and EMMPRIN 45 kD (Figure 5E–H). pSmad1 levels were higher with decreased expression of EMMPRIN 58 kD. Furthermore, increased EGR-1 levels were associated with a significant increase in EMMPRIN 45 kD (Figure 5I). There was no significant association between EGR-1 and pSmad1 with EMMPRIN 58 kD or MMP-2, nor did EGR-1 correlate with MMP-9 levels (not shown), suggesting that TGF\(\beta\) influences MMP activity through the 2 separate cascades, ALK5/Smad2/MMP2 and ALK1/Smad1/MMP9.

**Endoglin Influences Smooth Muscle Cell Proliferation and Extracellular Matrix Metalloproteinase Protein Inducer Levels In Vitro**

Endoglin knockdown in SMCs using RNAi did not affect TGF\(\beta\)-induced Smad1/5 phosphorylation, but did inhibit pSmad2 (Figure 4C). On TGF\(\beta\) stimulation, Ki-67 expression

Figure 3. Endoglin expression strongly correlated with pSmad2 levels \(\text{P} = 0.000001\), correlation coefficient \(0.640\)) and pSmad3 \(\text{P} = 0.003\), correlation coefficient \(0.314\)) (A–B). EGR-1 levels correlated positively to TGF\(\beta\) \(\text{C} = 0.039\), correlation coefficient \(0.221\)), endoglin (D) levels \(\text{P} = 0.001\), correlation coefficient \(0.346\), and pSmad2 \(\text{P} = 0.001\), correlation coefficient \(0.341\)). No significant correlation between EGR-1 and pSmad1 was observed (F). (G) Representative Western blots. \(\text{Spearman test. Kruskal-Wallis test.}\)

Figure 2 (Continued). \(\alpha\)-smooth muscle cell actin levels (correlation coefficient \(0.288\), \(\text{P} = 0.049\); C) and to a positive SMC/macrophage ratio (D). Interleukin-8 levels were significantly lower in plaques containing high levels of EGR-1 (E). The amount of intraplaque thrombus staining correlated negatively to EGR-1 levels (correlation coefficient \(-0.225\), \(\text{P} = 0.022\)). Endoglin and \(\alpha\)-smooth muscle cell actin levels correlated significantly \(\text{P} = 0.006\), correlation coefficient \(0.368\); Figure 3H). Endoglin expression was increased in plaques with high amounts of collagen, which points to a potential contributory role for endoglin toward stabilizing atherosclerotic plaques (Figure 3I). We observed a negative association between endoglin expression and intraplaque thrombus (correlation coefficient \(-0.277\), \(\text{P} = 0.007\); Figure 3J). G, Representative Western blots. \(\text{Spearman test. Kruskal-Wallis test.}\)
was induced in LacZ-transduced cells indicating dividing cells, but not in endoglin knockdown cells (Figure 4D, Appendix, Supplement 1C). Furthermore, 5 days after knockdown, there were significantly fewer SMCs (1.53e6/1.1006 vs 0.27e6/1.1006 in LacZ versus 0.27e6/1.1006 in RNAi; P<0.05; n=3).

Furthermore, although TGFβ inhibited SMC migration in LacZ-transduced cells toward fetal calf serum, it was ameliorated in endoglin RNAi cells (45.3%/1.1006 vs 73.9%/1.1006 for LacZ versus 73.9%/1.1006 in RNAi; P<0.05; n=3). Analyzing EMMPRIN 45kD levels showed that although TGFβ stimulation slightly increased EMMPRIN 45 kD levels, knocking-down endoglin resulted in severely reduced EMMPRIN 45 kD expression after TGFβ addition (Figure 4E; Appendix, Supplement 1B). In the absence of endoglin, TGFβ failed to induce procollagen I production. TGFβ stimulation caused an average 1.44-fold (±0.096) increase in collagen I levels in LacZ-transduced SMCs, whereas there was an average 0.42-fold decrease in procollagen I expression in endoglin knockdown cells (data not shown).

**Correlation of Early Growth Response-1 With COL1A2-Promoter Activity**

EGR-1 overexpression in human embryonic kidney-293 cells resulted in 2.4-fold increase in EGR-1 mRNA levels. Furthermore, EGR-1 transfection resulted in a significant dose-dependent increase in COL1A2 promoter activity when compared with empty vector controls (P=0.011; Figure 4G).

**Discussion**

TGFβ signaling, involving endoglin, pSmad2, and EGR-1, is associated with plaque stability. Besides significant correla-
tions with a fibrous plaque phenotype and increased collagen levels, these factors were increased in SMC-rich plaques and correlated with decreased amounts of intraplaque thrombus. Previously, a small study reported higher TGFβ levels to be associated with a stable plaque phenotype.\textsuperscript{28} In these stable fibrous plaques, the expression of Smad2/3 was restricted to SMCs.\textsuperscript{29} Furthermore, TGFβ plasma levels may have prognostic significance in coronary artery disease. They are generally decreased in patients with coronary atherosclerosis and correlate inversely with the presence of myocardial ischemia.\textsuperscript{30–32} TGFβ signals through phosphorylation of Smad proteins. Detection of pSmads showed active TGFβ signaling within atherosclerotic lesions in SMC and endothelial cell. The correlations among endoglin, EGR-1, and pSmad2 suggests that endoglin modulates the TGFβ/Smad2 pathway within the plaque affecting expression of the down-

\begin{figure}
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\caption{Endoglin and pSmad2 positively correlate with MMP-2 activity (A; $P=0.001$, correlation coefficient=0.477 and $P=0.003$, correlation coefficient=0.451). TGFβ and pSmad1 negatively correlate with MMP-9 activity ($P=0.017$, respective correlation coefficients=−0.380 and −0.432; C–D). This coincided with significant correlation of TGFβ, pSmad2, and endoglin with EMMPRIN 45 kD ($P=0.019$, correlation coefficient=0.374, $P=0.002$, correlation coefficient=0.462 and $P=0.004$, correlation coefficient=0.440), whereas no association between EMMPRIN 45 kD and pSmad1 was observed (not shown) or endoglin and EMMPRIN 58 kD ($P=0.576$; E–H). EGR-1 was correlated to EMMPRIN 45 kD ($P=0.026$) and not to EMMPRIN 58 kD ($P=0.67$; Figure 6I). \textsuperscript{S}Spearman test.}
\end{figure}
stream transcription factor EGR-1. Knocking-down endoglin expression reduces pSmad2 and EGR-1 levels, whereas it hardly affects pSmad1/5 levels, further indicating a relation between endoglin and TGFβ/Smad2/EGR-1 in atherosclerotic lesions.

TGFβ influences SMC proliferation, although its effect is not clear. TGFβ stimulates vascular SMC proliferation at low concentrations\(^1\) and knockdown of TGFβ stimulates SMC growth.\(^3\) SMCs from normal arteries are growth-inhibited by TGFβ in vitro; those derived from atherosclerotic and restenotic lesions are resistant to the antiproliferative effect of TGFβ.\(^6\) Furthermore, SMCs in stable lesions express more TGFβ than in unstable lesions.\(^28\) Also, SMCs within the fibrous cap differ in their responsiveness to TGFβ.\(^29\) In contrast to SMCs of the normal vessel wall, endoglin expression is readily detectable in SMCs in atherosclerotic lesions. We show that endoglin is easily detectable in proliferating cultured SMCs. Endoglin knockdown resulted in decreased proliferation. Endoglin knockdown ameliorated the TGFβ-induced inhibition in cell migration. Therefore, we suggest that endoglin-expressing SMCs within the plaque are resistant to TGFβ-induced growth inhibition and migration.

Santiago et al\(^19\) found significantly decreased SMC proliferation and migration after blocking EGR-1 expression. We found a positive relation among endoglin, SMC content of the plaque, and EGR-1 expression suggesting a plaque-stabilizing role for endoglin and EGR-1. EGR-1 inversely correlated with interleukin-8 expression and macrophage content. Additionally, both EGR-1 and endoglin negatively correlated with intraplaque thrombi, further arguing for a stabilizing role of EGR-1 and endoglin in atherosclerosis. Furthermore, collagen-rich plaques showed significantly higher EGR-1 expression. In vitro experiments confirmed the involvement of EGR-1 in increasing intraplaque collagen levels and is coherent with previous studies showing EGR-1-induced collagen production in fibroblasts.\(^17\) Atherosclerotic plaque progression is a dynamic process with continuous remodeling of the extracellular matrix. MMPs, together with the inducer of MMP expression, EMMPRIN, mediate different processes within the atherosclerotic lesion, including matrix degradation, cell infiltration, and migration. TGFβ is known to modulate the extracellular matrix and was found to inhibit EMMPRIN expression in human uterine fibroblasts.\(^38\) We found a significant positive correlation between TGFβ and EMMPRIN 45 kD and a negative correlation between TGFβ and MMP-9, pointing toward a more stable plaque phenotype. Interestingly, although MMP-9 activity was associated with pSmad1, MMP-2 activity correlated with pSmad2. Endoglin expression correlated significantly with EMMPRIN expression. EMMPRIN stimulates MMP-9 in monocytes and MMP-2 in SMCs.\(^39\) EMMPRIN 45 kD is expressed by plaque SMCs, and we previously showed that EMMPRIN 45 kD associated with SMC presence, MMP-2, and a fibrous plaque. We also observed a positive correlation between endoglin and MMP-2. TGFβ and pSmad1 but not endoglin inversely correlated with EMMPRIN 58 kD and MMP-9, both associated with inflammatory unstable plaques. Furthermore, in vitro downregulation of endoglin in SMCs resulted in reduced EMMPRIN 45 kD after TGFβ stimulation.

Therefore, endoglin potentially stabilizes plaques through EMMPRIN 45 kD regulation, thereby modulating MMP-2 levels and matrix deposition. Induction of the TGFβ/EGR-1 pathway not only enhances collagen synthesis, but also inhibits its degradation, resulting in an increased collagen deposition and thus a more stable fibrous cap.

TGFβ appears to protect against the development of unstable atherosclerotic lesions by stimulating vascular SMC proliferation and modification of the extracellular matrix. Most of these effects appear to be mediated through the ALK5/TGFβRII pathway and EGR-1. Expression of EGR-1 and endoglin are important for this stabilizing effect, resulting in increased SMC content, more fibrous lesions with increased collagen contents, and fewer intraplaque thrombi.

**Conclusion**

We propose a TGFβ signaling pathway in atherosclerotic plaques, in which endoglin modulates the downstream effects of TGFβ regulated by pSmad2/3 and increasing EGR-1 expression, resulting in increased collagen production, less matrix degradation, more SMC proliferation, and a reduction in inflammatory cells by inhibition of inflammatory cytokine production, in other words, in a more stable plaque phenotype. This nomimates the TGFβ/endoglin/EGR-1 pathway as a potential new target for plaque stabilization therapy.

**Disclosures**

None.

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