Increased Expression of Transforming Growth Factor-β1 as a Stabilizing Factor in Human Atherosclerotic Plaques

Francesco Cipollone, MD; Maria Fazia, PhD; Gabriella Mincione, PhD; Annalisa Iezzi, MD; Barbara Pini, MD; Chiara Cuccurullo, MD; Santì Ucchino, MD; Francesco Spigonardo, MD; Marcello Di Nisio, MD; Franco Cuccurullo, MD; Andrea Mezzetti, MD; Ettore Porreca, MD

Background and Purpose—Transforming growth factor-β (TGF-β) is a growth factor/cytokine involved in vascular remodeling and atherogenesis. Recent studies in apolipoprotein E-deficient mice have demonstrated a pivotal role of TGF-β in the maintenance of the balance between inflammation and fibrosis in atherosclerotic plaques. Furthermore, inhibition of TGF-β signaling has been shown to accelerate plaque formation and its progression toward an unstable phenotype in mice. However, if this mechanism is operative also in humans is still unknown. The aim of this study was to characterize the expression of TGF-β1 in human carotid plaque and to correlate it with the extent of inflammatory infiltration and collagen content with the clinical signs of plaque instability.

Methods—Plaques were obtained from patients undergoing carotid endarterectomy and divided into symptomatic and asymptomatic according to clinical evidence of recent transient ischemic attack or stroke. Plaques were analyzed for TGF-β1 expression by Immunocytochemistry, Western, and Northern blotting analysis. Immunocytochemistry was used to identify CD68+ macrophages, CD3 T lymphocytes, HLA-DR+ cells, and α-smooth muscle cells. Procollagen and interstitial collagen content were analyzed by immunohistochemistry and Sirius Red staining, respectively.

Results—Plaque TGF-β1 mRNA was increased up to 3-fold in asymptomatic as compared with symptomatic plaques. Plaques from asymptomatic group had fewer (P<0.0001) macrophages and T lymphocytes compared with symptomatic plaques. TGF-β1 gene was transcriptionally active as demonstrated by increased (P<0.0001) TGF-β1 protein expression in asymptomatic plaques. Immunohistochemistry showed that TGF-β was mainly expressed in plaque shoulder and was associated with a comparable increase (P<0.0001) in plaque procollagen and collagen content.

Conclusions—In conclusion, this study demonstrates the higher expression of TGF-β1 in human asymptomatic lesions and provides evidence that TGF-β1 may play an important role in the process of plaque stabilization. (Stroke. 2004;35:2253-2257.)

Key Words: atherosclerosis ■ carotid stenosis ■ growth factors ■ inflammation

There is increasing evidence that inflammation plays a central role in the cascade of events that eventually results in plaque erosion and fissuring. In particular, it is now well established that the balance between synthesis and degradation of interstitial collagen is a key event in the evolution of atherosclerotic plaque toward instability.

Transforming growth factor-β (TGF-β) is a multifunctional growth factor/cytokine involved in many physiological and pathological processes, such as vascular remodeling and atherogenesis. In fact, it is well known that TGF-β may control vascular smooth muscle cell proliferation and extracellular matrix deposition, and it has been suggested to play an antiinflammatory role during the progression of atherosclerosis. Furthermore, TGF-β expression has been demonstrated in human atherosclerotic plaques, and the presence of different isoforms and receptors is well described. Recent studies in apolipoprotein E-deficient mice have demonstrated the involvement of TGF-β in the maintenance of the balance between inflammation and fibrosis in atherosclerotic plaques, and inhibition of the TGF-β signaling by anti–TGF-β antibody has been shown to accelerate the development of atherosclerotic lesions. In addition, Lutgens et al have recently confirmed that treatment with recombinant soluble receptor TGF-β, which inhibits TGF-β signaling, accelerates plaque progression by increasing inflammatory component and decreasing collagen content in the vascular wall.

However, the precise role of TGF-β in the evolution of human atherosclerotic plaque toward instability is still unknown. Thus, in the present study, we set out to investigate the possible role of TGF-β1 in the pathophysiology of atherosclerotic plaque instability in humans.
We studied 24 of 40 consecutive, not previously examined, surgical patients enlisted to undergo carotid endarterectomy for extracranial high-grade internal carotid artery stenosis (>70% luminal narrowing). The degree of luminal narrowing was determined by repeated Doppler echography and intra-arterial cerebral angiography using the criteria of the North American Symptomatic Carotid Endarterectomy Trial (NASCET). Recruitment was completed when 2 predetermined equal groups of 12 patients according to clinical evidence of plaque instability were achieved. The first group included 12 patients (7 mol/L; 5 females; age 72±2 years) who presented with clinical symptoms of cerebral ischemic attack (symptomatic patients, group 1). Endarterectomy was performed 10 to 40 days after the onset of symptoms in these patients. The second group included 12 patients (6 mol/L; 6 females; age 72±3 years) who had an asymptomatic carotid stenosis (asymptomatic patients, group 2). Asymptomatic carotid stenosis was detected on the basis of systematic clinical examination of patients with coronary or peripheral disease. Percentage of carotid diameter reduction (70% to 93%), procedural methods, concomitant therapy, and risk factors did not differ between the 2 groups (Table). By the time of surgery, all patients were using chronic aspirin therapy (100 mg/daily). The study was approved by local ethics review committees and performed in accordance with institutional guidelines. Written informed consent was obtained from all patients before each examination. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Immunohistochemistry

After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen. Then, serial sections were prepared as previously described and incubated with specific antibodies anti-CD68, anti-CD3, anti-HLA-DR, anti-α-smooth muscle actin (Dako Corporation, Carpenteria, Calif), anti-TGF-β1 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), and anti-procollagen type I (Takara Shuzo Co, Shiga, Japan). All antibodies were used with streptavidin–peroxidase complex and diaminobenzidine as final chromogen. Finally, sections were counterstained with Gill hematoxylin (Sigma Chemical Co, St Louis, MO). Omission of primary antibodies and staining with type-matched and class-matched irrelevant immunoglobulin served as negative controls. The specimens were analyzed by an expert pathologist (intra-observed variability 6%) blinded to the patient’s therapy. Accurate quantitative analysis was performed with a computer-based image analysis system (AlphaEase 5.02; Alpha Innotech Corp) as previously described.

**Double Immunofluorescence Microscopy**

Plaque sections were fixed in cold acetone at −20°C for 10 minutes and double immunofluorescence was performed as previously described. After 3 washing steps with 0.1 mol/L phosphate-buffered saline for 10 minutes, nonspecific binding was blocked with 5% bovine serum albumin (Sigma Chemical Co). The sections then were incubated with the primary antibody anti–TGF-β1 (Santa Cruz Biotechnology Inc) for 1 hour at room temperature and then washed twice in phosphate-buffered saline for 5 minutes. Anti-rabbit IgG R-Phycocerythrin (Sigma Chemical Co) was used as secondary antibody for 1 hour at room temperature. Then, sections were incubated with the following primary antibodies (Dako Corp): anti-α smooth muscle actin, anti-CD68, and anti-CD3. Anti-mouse IgG FITC (Sigma Chemical Co) conjugate was used as secondary antibody. Omission of primary antibodies and staining with isotype-matched control Ig served as negative controls. Labeled specimens were examined by confocal microscopy using a Zeiss LSM 510 Meta instrument (Carl Zeiss).

**Sirius Red Staining for Collagen Content**

Frozen tissue sections were rinsed with distilled water and incubated with 0.1% Sirius red (Sigma Chemical Co) in saturated picric acid for 90 minutes, as previously described. Sections were rinsed 2 times with 0.01 N HCl for 1 minute and then immersed in distilled water. After dehydration with 70% ethanol for 30 seconds, the sections were observed under polarized light after cover-slipping. The sections were photographed with identical exposure settings for each section.

**Western Blot Analysis**

TGF-β1 protein was detected by Western blot as previously described. Briefly, frozen tissues from atheromatous carotid plaques were homogenized and lysed; protein concentration for each tissue extract was determined at 595 nm with bovine serum albumin standard (Biorad protein assay, Melville, NY). Tissue extracts were electrophoresed on SDS-polyacylamide gels; resolved proteins were transferred onto 0.45 μm nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) and then incubated with rabbit polyclonal TGF-β1 antibody (Santa Cruz Biotechnology Inc) overnight. After washing, the blots were incubated with a horseradish peroxidase-linked anti-rabbit antibody (Amersham Biosciences). Bands were quantified by computer-assisted densitometry (AlphaEase 5.02) and expressed as densitometric units (DU). Immunodetection of β-actin (Sigma Chemical Co) was performed to assure equal gel loading.

**RNA Extraction and Northern Blot Analysis**

Total RNA was isolated using a NucleoSpin RNA kit (Macherey-Nagel) and Northern analysis was performed as previously described. Human TGF-β1 cDNA was kindly provided by Dr M.L. McGiedy (Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, Md). The membrane was rehybridized with human GAPDH probe as an internal standard.

**Statistical Analysis**

For clinical data and histological examination, variables were compared by use of the χ² test. The significance of difference in enzyme expression and inflammatory cell infiltration between symptomatic and asymptomatic patients was analyzed by Student t test. Data were
expressed as percentage or mean±SD. All calculations were performed using the SPSS 11.0.1 computer program.

Results

Histological Analysis
Plaque ulceration was significantly more common in the symptomatic plaques (7 of 12 [60%]) than in the asymptomatic lesions (3 of 12 [25%]; \( P < 0.05 \)). In contrast, no significant differences were observed with regard to intraplaque hemorrhage (Table 1).

Cellular Composition
Immunohistochemistry revealed inflammatory infiltration in all specimens examined, which was more evident in the shoulder of symptomatic plaques. Macrophages and T-lymphocyte infiltration occurred coincidentally and was most prominent in the shoulder of the lesions and in the immediate vicinity of the atheromatous core of the lesions. Plaque area occupied by macrophages and T cells was significantly greater \( (P < 0.0001) \) in symptomatic than in asymptomatic plaques. In addition, the inflammatory cells in the symptomatic group were characterized by a strong expression of the HLA-DR antigen, a feature that contrasted with the low expression of HLA-DR in asymptomatic plaques. Finally, a small, nonsignificant reduction in smooth muscle cells was observed in symptomatic plaques (Table).

TGF-\( \beta \)1 Is Expressed in Macrophages and Smooth Muscle Cells of Plaque Shoulder
Atherosclerotic lesions contained immunostainable TGF-\( \beta \)1. Interestingly, TGF-\( \beta \)1 was more abundant in asymptomatic lesions, as confirmed by quantitative analysis (18.2±2% versus 9±3.5%, \( n=12, \ P < 0.0001 \)) (Figure 1A and 1B). TGF-\( \beta \)1 accumulated in the shoulder region and in the periphery of the lipid core. TGF-\( \beta \)1 staining pattern indicated its localization in the activated macrophages and smooth muscle cells. Localization of TGF-\( \beta \)1 in plaque cellular components was also evaluated by immunofluorescence double-labeling experiments, which showed a strong expression of TGF-\( \beta \)1 in macrophages (Figure 1C) and smooth muscle cells (Figure 1D), and a very weak coinmunoreactivity of TGF-\( \beta \)1 and CD3 \( ^- \) T cells.

TGF-\( \beta \)1 Is Expressed in Higher Amounts in Asymptomatic Plaques
Western blot analysis revealed higher TGF-\( \beta \)1 expression in all the asymptomatic plaques but only weak expression in the

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**Figure 1.** A and B, Immunostaining \(( \times 10 \)\) for TGF-\( \beta \)1 in asymptomatic (A) and symptomatic (B) plaques. C and D, Double labeling immunofluorescence \(( \times 20 \)\) on asymptomatic plaque sections showing localization of TGF-\( \beta \)1 (red staining) in macrophages (green staining; panel C) and smooth muscle cells (green staining; D). Yellow staining indicates colocalization areas. Similar regions of the plaque are showed. These results are typical of 12 symptomatic and 12 asymptomatic plaques.

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**Figure 2.** Northern blot (A) and Western blot (B) for TGF-\( \beta \)1. These results are typical of 12 symptomatic and 12 asymptomatic plaques.
symptomatic plaques (Figure 2). By quantitative analysis, levels of TGF-β1 in asymptomatic plaques significantly exceeded those in symptomatic plaques (3780±309 DU, n=1568±12, P<0.0001).

**TGF-β1 Is Modulated at Transcriptional Level**

We performed Northern blot followed by quantitative analysis to determine whether TGF-β1 is modulated at the transcriptional level in the atherosclerotic plaques. Accordingly, with protein analysis, TGF-β1 mRNA expression was significantly more abundant in asymptomatic plaques than in symptomatic plaques (4309±682 versus 1809±349 DU, n=12, P<0.0001) (Figure 2).

**Procollagen and Collagen Content**

Immunohistochemistry analysis of procollagen type I showed markedly lower immunoreactivity for this protein in symptomatic plaques as compared with asymptomatic ones (8.7±3.4% versus 19.6±3.9%, n=12, mean±SD; P<0.0001) (Figure 3). Levels of procollagen were always associated with comparable levels of TGF-β1. Similarly, Sirius Red polarization showed considerably lower content of interstitial collagen in the tissue sections of symptomatic plaques compared with asymptomatic plaques (8.9±3.5% versus 17.4±3.1%, n=12, mean±SD; P<0.0001).

**Discussion**

It has been supposed that the response to TGF-β1 within vascular lesions is an important aspect of atherosclerosis progression toward clinical expression in humans. In fact, atherosclerosis is considered a chronic inflammatory disease, and the balance between degradation and deposition of the extracellular matrix is thought to be important for the maintenance of plaque stability in humans. TGF-β is a growth factor that exerts many regulatory actions. These functions are best reflected in several antiinflammatory effects on vascular cells and in a strongly positive effect on extracellular matrix production.

In the present study, we found that TGF-β mRNA levels are increased up to 3-fold in asymptomatic as compared with symptomatic plaques; TGF-β gene was transcriptionally active as demonstrated by the parallel increase in TGF-β1 protein expression at immunocytochemistry and Western blot analyses. Notably, enhanced TGF-β1 staining at immunohistochemistry and expression at Western blot analysis were observed in asymptomatic plaques despite a significant reduction in the overall number of macrophages, thus ruling out any hypothesis that enhanced TGF-β1 in asymptomatic plaques is merely a consequence of higher macrophage infiltration. Finally, enhanced TGF-β1 expression in asymptomatic plaques was associated with a comparable increase in collagen content, thus providing a tangible mechanism of plaque stabilization. It is worth noting that high TGF-β1 levels were associated with increased procollagen expression, which accounts for the plaque collagen content. Thus, these findings, taken together, provide evidence for the key role of TGF-β1 in the progression of atherosclerotic lesions toward a more stable phenotype.

Our results are also in agreement with previous studies that demonstrated that inhibition of TGF-β signaling accelerates atherosclerotic progression and induces an unstable phenotype in mice, and within animal studies demonstrating the association of a stable plaque phenotype with increased TGF-β immunoreactivity. Thus, in the present study we expand previous data in humans showing for the first time to our knowledge a differential TGF-β1 expression in asymptomatic versus symptomatic atherosclerotic plaques, with greater TGF-β1 expression found in plaque regions characterized by low inflammatory infiltration and increased collagen content.

A potential limitation of the present study arises from the fact that sensitive immunohistochemical staining procedures used for detection of TGF-β1 antigen does not allow direct conclusions with respect to TGF-β activity and its specific receptor. In this light, it should be noted that a TGF-β receptor dysfunction characterized by a decreased type II/type I receptor ratio has been demonstrated in cells derived from human atherosclerotic lesions, with consequent switch from an antiproliferative to profibrotic response to TGF-β. Thus, although in this study TGF-β overexpression in asymptomatic plaques was associated with increased collagen content and decreased inflammatory infiltration, we cannot exclude that other mechanism(s) regulating TGF-β receptor expression could play a role in the characterization of the final plaque phenotype.

Another limitation of the present work is that we did not measure systemic levels of TGF-β1 in patients who entered in this study and thus we could not correlate the localized expression of TGF-β1 with its plasma or serum levels of TGF-β1. This correlation would be important because it would suggest a systemic process in these patients rather than a local phenomenon directly related to plaque rupture.

The mechanisms responsible for TGF-β1 modulation in atherosclerotic plaque are not totally clear and therefore need further investigation. Nevertheless, previous studies have demonstrated that inflammatory mediators such as CD40 ligand, critically involved in atherogenesis, may downregulate TGF-β1 expression and collagen production, thus orienting atherosclerotic plaques toward instability.

In conclusion, this study addresses the missing link between TGF-β1 overexpression and plaque stability by providing evidence that synthesis of TGF-β1 is inversely associated with transient ischemic attack and stroke, possibly by

![Asymptomatic vs Symptomatic Plaques](image-url)
matrix deposition promoting plaque strength. These findings raise the possibility that future drugs able to selectively modulate TGF-β1 expression might provide a novel form of therapy for plaque stabilization.

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