Increased Rate of Apoptosis in Intimal Arterial Smooth Muscle Cells Through Endogenous Activation of TNF Receptors


Abstract—Intimal proliferation of smooth muscle cells (SMCs) is a key event in the vascular response to injury, including the early stages of atherosclerosis and restenosis after angioplasty. Tumor necrosis factor-α (TNF-α) has been reported to stimulate growth of cultured human SMCs, but activation of TNF receptors is also known to induce cell death by apoptosis. We report here that SMCs isolated from the neointima of injured rat aortas are characterized by increased expression of TNF-α in response to interleukin-1β and γ-interferon compared with medial SMCs. Basal and serum-stimulated DNA synthesis was higher in intimal than in medial SMCs. In contrast to previous findings on human SMCs, exposure to interleukin-1β/γ-interferon or TNF-α did not affect the growth of rat medial SMCs, inhibited DNA synthesis, and decreased cell numbers in cultures of intimal SMCs. Incubation of intimal SMCs with these cytokines also resulted in induction of terminal dUTP nick end-labeling positivity and caspase-3 expression, suggesting cell death by apoptosis, whereas medial cells were markedly less sensitive in this respect. Cytokine-induced apoptosis in intimal cells was effectively inhibited by treatment with antibodies against TNF receptors. These findings suggest that endogenous activation of TNF receptors may represent a way to limit accumulation of SMCs in injured arteries. This mechanism may also be important in SMC death in advanced atherosclerotic plaques. (Arterioscler Thromb Vasc Biol. 2001;21:1909-1914.)

Key Words: smooth muscle cells ■ apoptosis ■ injury ■ cell replication

Activation of medial smooth muscle cells (SMCs), which migrate into the intima to proliferate and produce extracellular matrix proteins, is a key event in the vascular response to injury. The recruitment of SMCs into early fatty, inflammatory atherosclerotic lesions is believed to involve a similar mechanism.1 There is evidence that neointimal SMCs are different from the majority of medial cells. For example, SMCs isolated from rat aortic neointima 15 days after endothelial denudation show increased cell replication, decreased α-actin expression, and a more epithelioid morphology than do medial SMCs.2,3 This finding is also in accordance with the observation that vascular activation of transcription factors and growth-regulatory genes by injury is restricted to the media in the immediate phase but thereafter takes place mainly in intimal cells.4 Cells that, during development, lose their functional role are removed by apoptosis.5,6 Apoptosis also is believed to be important for remodeling processes in the adult organism, such as in repair after tissue injury.7 In experimental animals, the induction of intimal SMC replication by mechanical injury is accompanied by neointimal SMC apoptosis.2,8,9 The latter is likely to modulate the repair process and to facilitate the return to normal tissue structure. A high rate of SMC cell death by apoptosis also occurs in advanced atherosclerotic plaques.10–13 The pathophysiological role of this phenomenon remains to be fully understood but may be of considerable importance for loss of plaque stability and rupture.

Tumor necrosis factor-α (TNF-α) is a pluripotent mediator of inflammation14 and is expressed in atherosclerotic plaques.15,16 It is also expressed by medial and neointimal SMCs after balloon injury but is absent in the normal vasculature.17,18 In culture, TNF-α stimulates the migration and replication of human SMCs,18 suggesting that it may play a role in the recruitment of SMCs during plaque formation. However, when given together with interleukin-1β (IL-1β) and γ-interferon (IFN-γ), TNF-α has also been shown to induce apoptosis in SMCs.19 Accordingly, TNF-α may contribute to the development, tissue turnover, and stabilization of atherosclerotic lesions by regulating both SMC proliferation and cell death during different stages of atherogenesis. The aim of the present study was to investigate the role of TNF-α in activation of proliferation and cell death in intimal versus medial SMCs.

Methods

Cell Culture

SMC cultures were isolated from the media of thoracic aortas of adult rats and the neointimal layer of thoracic aortas 2 weeks after...
balloon catheter deendothelialization, as previously described. In brief, aortas and carotid arteries were excised 14 days after injury. Tissue of the neointimal layer was carefully isolated from the media under the dissecting microscope and collected from 8 vessel segments of 3 independent experiments. Intimal SMCs were harvested from explants of the neointimal tissue. At passage 2, the intimal SMCs were assessed by immunostaining for α-smooth muscle actin, and possible contamination by macrophages or endothelial cells was excluded by immunostaining with monoclonal antibodies to ED2 (clone MCA342, Serotec) and von Willebrand factor (clone 2F2-A9, Pharmingen), respectively. Cell cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/10% FCS, 1 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells from passages 5 to 10 were used for the present studies. Cells were grown in DMEM/F12 medium supplemented with 10% FCS and 100 U/mL penicillin/streptomycin ( Gibco). To reach comparable cell densities at 48 hours, intimal SMCs were always seeded at a slightly lower density than were medial cells. Each experiment was performed with an independent analysis of 3 different batches of intimal or medial SMCs. All experiments that compared intimal and medial SMCs were performed in parallel.

**Analysis of TNF-α Expression**

SMCs were seeded at a density of 15 000 to 20 000 cells per well in 24-well plates (Nunc) and grown to subconfluent density. Great care was taken to adjust these conditions so that there were similar cell densities in medial and intimal cells when serum starvation was started. The cells were serum starved in 0.1% FCS–supplemented F12/DMEM medium for 48 hours and subsequently exposed to rat IFN-γ (1 ng/mL, Genzyme), human IL-1β (100 U/mL, R&D Systems), rat TNF-α (10 ng/mL, R&D Systems), or control medium (0.1% FCS–supplemented F12/DMEM) for 24, 48, and 72 hours. Inhibition assay was performed by the addition of a 1:100 dilution of (0.1% FCS–supplemented F12/DMEM) to the cell cultures. To reach comparable cell densities at 48 hours, intimal SMCs were always seeded at a slightly lower density than were medial cells. Each experiment was performed with an independent analysis of 3 different batches of intimal or medial SMCs. All experiments that compared intimal and medial SMCs were performed in parallel.

DNA Synthesis

SMCs were seeded and grown on 13-mm glass coverslips in 24-well plates as previously described. After 48 hours of serum starvation, the cells were incubated in medium containing 2 μCi/mL of Net-355 thymidine 6-H (NEN Life Science Products Inc) and different concentrations of FCS or cytokines. After 24 hours, the cells were rinsed with PBS, fixed overnight in 3% glutaraldehyde in 0.1 mol/L sodium cacodylate/0.05 mol/L sucrose in PBS, and dehydrated for 10 minutes in 70% ethanol and 95% ethanol. The slides were then dipped in Kodak NTB2 emulsion, air dried, exposed at 4°C minutes in 70% ethanol and 95% ethanol. The slides were then resuspended SMCs with the Medonic CA470 cell counter. At the beginning of each such measurement, random measurements of cell number were also manually performed in a Bürker chamber.

**RT-PCR Analysis**

For reverse transcription–polymerase chain reaction (RT-PCR) analysis, RNA extracts were prepared and purified with the total RNA isolation system (Promega). For RNA extraction, cells were washed twice with ice-cold PBS and transferred to a vial after being scraped into and resuspended in 0.6 mL denaturing solution containing citrate/sarcosine/β-mercaptoethanol and guanidine thiocyanate. To each sample, 0.06 mL of 2 mmol/L sodium acetate, pH 4.0, and 0.6 mL phenol/chloroform/alkohol were added, mixed thoroughly between each addition, and chilled for 15 minutes. The sample was then centrifuged at 10 000 rpm for 20 minutes at 4°C, the top aqueous phase was removed, and to this an equal volume of isopropanol was added. The RNA was precipitated at −20°C overnight, pelleted by centrifugation at 10 000 rpm for 15 minutes at 4°C, resuspended in 0.5-mL denaturing solution, reprecipitated in isopropanol, pelleted again, washed in ice-cold ethanol, recovered by centrifugation, and resuspended in 0.1 mL RNase-free water. Reverse transcription of 1.5 μg of RNA was performed using SuperScriptII RNaseH− reverse transcriptase (Gibco). After the reaction, the samples were stored at −20°C.

RT-PCR amplification and quantification were performed on a Perkin-Elmer Cetus 3500 thermal cycler model 9600. The following primers and probes were used: (1) primer/probe for rat TNF-α; 0.05 U AmpliTaggold™ uracil N-glycosylase; 0.01 U Amp-Erase™ uracil N-glycosylase; 0.05 U Ampli Taq Gold™ (Perkin Elmer/Applied Biosystems); and 0.1 mmol/L probe. The reactions were performed in Micro Amp optical 96-well plates. The following primers and probes were used: (1) primer/probe for rat TNF-α amplification primers (sense sequence, ATGTATCGCTACCAACGGTGGA and antisense sequence, GCTGTCGCAAGATGGGTTCAG) were a kind gift of Prof Y. Shen (Roberts Research Center Sun Health Research Institute, Sun City, Arizona). Analysis of specific rat TNF-α and β-actin mRNAs by RT-PCR was accomplished by using a Clontech amplimer set. For our purpose, the rat TNF-α amplimer set (5500-3) and rat β-actin amplimer set (5506-1) were used, and PCR amplification was performed by adding 2 μL of cDNA to 50 μL of reagent mixture (0.2 mmol of dNTP, 2 U Taq polymerase, 1.5 mmol/L MgCl₂, buffer, and 0.4 mmol/L of each primer) and amplified with a 27- to 30-step cycle program after denaturation at 94°C for 3 minutes. The type 1 (p55) TNF receptor cDNA amplification step cycle included 94°C for 1 minute, 56°C for 2 minutes, and 72°C for 3 minutes; the rat TNF-α and rat β-actin amplification step cycle included 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes. The number of step-cycles was determined by finding the logarithmic phase of cDNA amplification. This step is crucial for a semiquantitative evaluation of mRNA amount. A 15-μL aliquot of each reaction was electrophoresed on a 2% agarose gel, and the bands were analyzed by ethidium bromide staining. Quanification of the bands was performed by using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Kodak). The results are expressed as band intensity ratios: TNF receptor/β-actin and TNF-α/β-actin.

**Real-Time Quantitative RT-PCR (Tag-Man PCR)**

For real-time quantitative RT-PCR, rat intimal and medial SMCs (passages 7 to 9) were cultured and seeded in duplicate or triplicate at a density of 15 000/well in 24-well plates. Cells grown in DMEM with 0.1% FCS for 24 hours were then treated with 1 ng/mL IFN-γ plus 100 U/mL IL-1β for the indicated time. Total RNA was extracted from cells with an Ultraseq reagent (Nordic BioSite AB). The total RNA (1 μg) was incubated with 50 pmol/μL hexanucleotides (pdN6, Pharmacia Biotech AB) at 70°C for 3 minutes and subsequently reverse-transcribed at 25°C for 10 minutes, 42°C for 3 minutes, and 72°C for 3 minutes; the rat TNF-α and rat β-actin amplification step cycle included 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes. The number of step-cycles was determined by finding the logarithmic phase of cDNA amplification. This step is crucial for a semiquantitative evaluation of mRNA amount. A 15-μL aliquot of each reaction was electrophoresed on a 2% agarose gel, and the bands were analyzed by ethidium bromide staining. Quanification of the bands was performed by using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120 (Kodak). The results are expressed as band intensity ratios: TNF receptor/β-actin and TNF-α/β-actin.
medial cells were activation of DNA synthesis in close to 50% of the cells with low concentrations of serum (0.1 to 1% FCS) resulted in SMCs were characterized by increased proliferative capacity.

In accordance with previously published studies, 22,23 intimal P 0.05 was considered significant.

Statistics

Values are given as mean±SD or mean±SEM. Between-group analyses were made with ANOVA and post hoc testing. A value of P<0.05 was considered significant.

Results

In accordance with previously published studies, 22,23 intimal SMCs were characterized by increased proliferative capacity compared with medial SMCs. Stimulation of intimal SMCs with low concentrations of serum (0.1 to 1% FCS) resulted in activation of DNA synthesis in close to 50% of the cells during a 24-hour period, whereas the corresponding values in medial cells were <20%. Also, in the presence of higher serum concentrations, DNA synthesis was more abundant in intimal than in medial SMCs (data not shown).

Growth-arrested intimal and medial SMCs contained none or only minor amounts of TNF-α mRNA transcripts, as assessed by RT-PCR (data not shown). Exposure of the cells to IFN-γ/IL-1β induced a marked increase in TNF-α mRNA levels in both intimal and medial SMCs. However, this increase was significantly greater in intimal than in medial cells (Figure 1).

The media from serum-starved control cultures of intimal and medial SMCs contained low amounts of TNF-α as determined by ELISA (8±12 and 10±25 pg/mL, respective-

 levels in both intimal and medial SMCs. However, this increase was significantly greater in intimal than in medial cells (Figure 1).

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levels in both intimal and medial SMCs. However, this increase was significantly greater in intimal than in medial cells (Figure 1).
In cultures kept in medium containing 0.1% FCS for 48 hours, there was no significant difference in cell numbers between medial and intimal cells. Exposure of the cells to TNF-α/H9251 or IFN-γ/H9253/IL-1β/H9252 markedly decreased cell numbers in intimal cell cultures (19 ± 7 × 10^4 and 16 ± 3 × 10^4 cells per well, respectively, vs 29 ± 4 × 10^4 cells per well in controls; P < 0.05), but did not affect cell number in cultures of medial cells. To analyze the possible activation of apoptosis in response to cytokines, the TUNEL technique was used. In intimal as well as medial SMCs grown in medium containing 0.1% FCS for 48 hours, the frequency of TUNEL-positive cells was < 5%. Exposure of intimal SMCs to IFN-γ/IL-1β or TNF-α resulted in a > 5-fold increase in the rate of apoptosis, whereas cytokines induced only little apoptosis in medial cells (Figure 4). Addition of blocking antibodies specific for the TNF receptors markedly reduced cytokine-induced TUNEL positivity in intimal cells (Figure 4). In intimal SMC cultures, exposure to IFN-γ/IL-1β or TNF-α also resulted in cell shrinkage and detachment, adding further support to the argument for ongoing cell death by apoptosis (Figure 5), whereas such changes were markedly less frequent in medial SMCs exposed to cytokines.

The MTT assay of metabolic activity was used as an additional technique for analysis of the effect of cytokines on SMC viability. Exposure of intimal SMCs to IFN-γ/IL-1β for 48 hours reduced MTT levels to 70.9 ± 2.5% of that in control cultures (P < 0.0001), whereas no decrease was seen in medial cells.

Figure 2. Expression of p55 TNF receptor (TNFR) mRNA in intimal and medial cells exposed to cytokines. A, Ethidium bromide staining after RT-PCR with the use of the rat p55 TNF receptor and β-actin–specific primers on RNA isolated from (1) intimal cells exposed to IFN-γ/IL-1β, (2) intimal cells exposed to control medium, (3) medial cells exposed to IFN-γ/IL-1β, and (4) medial cells exposed to control medium. B, Results are expressed as the ratio of the intensity of p55 TNF receptor to β-actin bands of at least 3 independent analyses on different batches of intimal and medial SMCs.

Figure 3. Activation of DNA synthesis in intimal and medial SMCs stimulated with cytokines. Serum-starved intimal (△) and medial (□) SMCs were exposed to medium containing 3H-labeled thymidine and various concentrations of TNF-α for 24 hours. The fraction of labeled nuclei was determined by autoradiography. The results are presented as mean ± SD of at least 3 independent analyses on different batches of intimal and medial SMCs.
cells. Addition of antibodies against the p55 TNF receptor completely inhibited the MTT-reducing effect of IFN-γ/IL-1β (103.6±6.1% of controls) in intimal SMCs, whereas nonimmune IgG was unable to reverse the decrease in MTT activity caused by cytokines (68.0±4.7%, P<0.0001, compared with control cultures).

To further analyze the possible activation of apoptosis in SMCs exposed to cytokines, activation of the protease caspase-3 was determined. Exposure of intimal SMCs to IFN-γ/IL-1β or TNF-α resulted in a significant increase in caspase-3 activity as determined by ELISA, whereas only a minor increase was observed in medial cells (Figure 6).

Discussion

The present study demonstrates that rat neointimal SMCs have an increased capacity to synthesize TNF-α and express TNF receptors in response to cytokine stimulation compared with medial SMCs. TNF-α activates cell death by apoptosis in neointimal SMCs, and studies with blocking antibodies suggest that apoptosis induced by IFN-γ/IL-1β involves an autocrine stimulation of TNF receptors. TNF-α does not stimulate DNA synthesis in rat neointimal SMCs and does not appear to be involved in the increased proliferative capacity of these cells. A similar but significantly lower activation of TNF-α and TNF receptor expression, as well as apoptosis, was also observed in medial SMCs exposed to these cytokines.

Balloon injury of rat arteries results in activation of medial SMCs that migrate to the intima, where by proliferation and synthesis of extracellular matrix, they form a thickened neointima.25 There is evidence that SMCs involved in this repair process are recruited from a distinct subpopulation of medial cells. SMCs isolated from the neointima 15 days after injury show decreased smooth muscle α-actin and myosin expression, epithelioid morphology, and increased replicative capacity compared with medial cells.2,22,23,26 Cell lines with similar characteristics have been isolated from rat aortic media.2,23

Experiments with blocking antibodies suggest that fibroblast growth factor plays a role in activation of DNA synthesis in the immediate phase after arterial injury27 and that platelet-derived growth factor is involved primarily in the activation of migration.28 TNF-α is transiently expressed by medial SMCs after injury and subsequently more abundantly by cells in the neointima.17,29 The observation by Tanaka et al17 of a correlation between intimal SMC TNF-α expression and DNA replication suggests that TNF-α may contribute to intimal cell growth. This notion is also supported by the fact that TNF-α is a potent growth factor for human SMCs.30 However, in the present study, we found no evidence that TNF-α is a mitogen for rat SMCs. On the contrary, TNF-α inhibited neointimal SMC DNA synthesis. Interestingly, this effect appeared to be specific for neointimal SMCs and was not observed in cultures of medial cells.

In the neointima of injured rat arteries, the initial phase of cell replication is followed by occurrence of cell death by apoptosis.2,8,9 Our findings indicate that neointimal SMCs are characterized by an increased sensitivity to cytokine-induced apoptosis mediated by increased endogenous TNF-α expression and activation of TNF receptors that may contribute to limit SMC accumulation in the intima.

The present findings add further support to the notion that TNF-α has an important role in atherosclerosis. TNF-α is expressed in atherosclerotic plaques15,16,31 and has been demonstrated to be functionally important for the development of transplant atherosclerosis.32 It has several biological properties that may influence the development of atherosclerosis. It is a potent activator of endothelial adhesion molecule expression and activates the proinflammatory activities of macrophages.14 In contrast to the present findings, TNF-α functions as a mitogen for growth-arrested human SMCs.30 Interestingly, other studies have shown that TNF-α may stimulate the growth of quiescent cells while inducing apoptosis in rapidly proliferating cells.53 This observation is well in line with the present finding that TNF-α activates apoptosis more in rapidly proliferating neointimal cells than in more slowly replicating medial cells.

The apoptotic effect of TNF-α is mediated by the TNF receptors that activate caspases through the TNF-α receptor—
associated death domain protein and the Fas protein associated with the death domain.\(^{34}\) Accordingly, TNF-α, as well as IFN-γ/IL-1β, in concentrations that have been shown to activate endogenous TNF-α expression was found to activate caspase-3 in intimal SMCs. The finding that antibodies against TNF receptors inhibit induction of TUNEL positivity and cause a decrease in MTT levels caused by IFN-γ/IL-1β suggest that this pathway is also involved in cytokine-induced apoptosis of intimal SMCs. Exposure of intimal SMCs to cytokines resulted in a 5-fold increase in TUNEL-positive cells but only a 40% increase in caspase-3 activity. Although no direct linearity may be expected between caspase-3 and TUNEL positivity, this difference may indicate that other apoptotic pathways are also involved. Using a selective inhibitor of the caspase-3 family, Obara et al\(^{35}\) have demonstrated that caspase-3 activation mediates induction of apoptosis in human vascular SMCs. They have also demonstrated that inhibition of nuclear factor-κB by overexpression of I-κB dramatically induces apoptosis in SMCs. Accordingly, differences in nuclear factor-κB activation by TNF-α may also be involved in the differences observed between intimal and medial SMCs in the present study.

In summary, the present study demonstrates an increased TNF-α expression in neointimal SMCs stimulated with IFN-γ and IL-1β. This activation of TNF-α is associated with an increased rate of apoptosis.

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**References**

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