Protective Function of Transcription Factor TR3 Orphan Receptor in Atherogenesis
Decreased Lesion Formation in Carotid Artery Ligation Model in TR3 Transgenic Mice

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Background—Smooth muscle cells (SMCs) play a key role in intimal thickening in atherosclerosis and restenosis. The precise signaling pathways by which the proliferation of SMCs is regulated are largely unknown. The TR3 orphan receptor, the mitogen-induced nuclear orphan receptor (MINOR), and the nuclear receptor of T cells (NOT) are a subfamily of transcription factors belonging to the nuclear receptor superfamily and are induced in activated SMCs. In this study, we investigated the role of these transcription factors in SMC proliferation in atherogenesis.

Methods and Results—Multiple human vascular specimens at distinct stages of atherosclerosis (lesion types II to V by American Heart Association classification) derived from 14 different individuals were studied for expression of these transcription factors. We observed expression of TR3, MINOR, and NOT in neointimal SMCs, whereas no expression was detected in medial SMCs. Adenovirus-mediated expression of a dominant-negative variant of TR3, which suppresses the transcriptional activity of each subfamily member, increases DNA synthesis and decreases p27Kip1 protein expression in cultured SMCs. We generated transgenic mice that express this dominant-negative variant or full-length TR3 under control of a vascular SMC-specific promoter. Carotid artery ligation of transgenic mice that express the dominant-negative variant of TR3 in arterial SMCs, compared with lesions formed in wild-type mice, results in a 3-fold increase in neointimal formation, whereas neointimal formation is inhibited 5-fold in transgenic mice expressing full-length TR3.

Conclusions—Our results reveal that TR3 and possibly other members of this transcription factor subfamily inhibit vascular lesion formation. These transcription factors could serve as novel targets in the treatment of vascular disease. (Circulation. 2002;106:1530-1535.)

Key Words: muscle, smooth ■ atherosclerosis ■ genes

Smooth muscle cells (SMCs) display a dynamic phenotype varying from quiescent fully contractile cells to proliferative cells. This phenotypic modulation is associated with a variety of vascular diseases, ranging from atherosclerosis to restenosis after angioplasty. Detailed knowledge about the molecular mechanisms underlying proliferation as well as phenotypic changes associated with proliferation is crucial in identifying novel targets for intervention. In our search for genes involved in SMC activation in atherogenesis, we have revealed by differential display analysis the induction of TR3 and mitogen-induced nuclear orphan receptor (MINOR) expression by in vitro–activated SMCs. TR3 and MINOR are members of the nerve growth factor–induced gene-B (NGFI-B) family that has also been named nuclear receptor subfamily 4 group A (NR4A) and that contains, so far, 1 additional member, the nuclear receptor of T cells (NOT). These transcription factors contain a similar structural organization of their functional domains: an amino-terminal domain involved in trans-activation, the DNA-binding domain that contains 2 zinc fingers, and a ligand-binding domain at the carboxyl terminus. All 3 subfamily members bind the same response element(s), and they are referred to as orphan receptors, because the ligands that may regulate their transcriptional activity have not yet been identified. The NGFI-B–like factors have been implicated in diverse cellular signaling events, eg, T-cell receptor–mediated apoptosis. In key experiments on T-cell apoptosis, a dominant-negative form of TR3 that lacks the transactivation domain and consequently blocks the transcriptional activity of each subfamily member has been described. Overexpression of this variant under the control of a promoter specific for developing thymocytes in transgenic mice perturbs thymocyte development, resulting in increased circulating T cells.

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The aim of the present study was to determine the expression profiles of TR3, MINOR, and NOT in human vascular lesions and to assess the function of these transcription factors in the modulation of SMC proliferation and, consequently, in atherogenesis. Our results show that TR3, MINOR, and NOT are expressed in human atherosclerotic lesions and that TR3 inhibits SMC proliferation in vivo.

Methods

Human Tissue Specimens

Human tissue samples were obtained from organ donors with informed consent, according to protocols approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam. The specimens were paraffin-embedded, sectioned, and mounted on Superfrost Plus glass slides (Emego). Vascular specimens were characterized by immunohistochemistry to establish the stage of disease according to the American Heart Association classification.10

SMC Culture and RNA Isolation

Human SMCs were explanted from umbilical cord arteries and were used at passages 5 to 7.11 Murine SMCs were explanted from aortas. Briefly, murine aortas were harvested, sliced, and put in gelatin-coated 6-well plates in medium 199 with HEPES and 10% (vol/vol) FBS with penicillin/streptomycin (GIBCO-BRL). Cells were cultured and used for experiments at passages 2 to 4. SMCs were characterized with a monoclonal antibody directed against smooth muscle α-actin (1A4, DAKO) and demonstrated uniform fibrillar staining. Total RNA was isolated with Trizol reagent (GIBCO-BRL).

\[^{3}H\]Thymidine Incorporation

SMCs were seeded at 4 × 10^4 cells per well of a 24-well plate and reached 60% to 70% confluence after 24 hours. Cells were infected with a dominant-negative variant of TR3 (ΔTA) or TR3 adenovirus at 1 × 10^9 plaque-forming units (pfu) for 2 hours.11 SMCs were made quiescent in serum-free medium for 60 hours, stimulated with 10% (vol/vol) serum, and labeled for 18 hours with 0.5 Ci/mL [methyl-\[^{3}H\]]thymidine (Amersham). Incorporated radioactivity was precipitated for 30 minutes at 4°C with 10% (wt/vol) trichloroacetic acid, washed twice with 5% (wt/vol) trichloroacetic acid, and dissolved in 0.5N NaOH (0.5 mL per well); incorporated \[^{3}H\]thymidine was measured by liquid-scintillation counting. All experiments were performed in triplicate and repeated at least twice.

Immunohistochemistry and Western Blotting

TR3 antigen was detected by immunohistochemistry with a rabbit antibody directed against Nur77 (M-210, Santa Cruz Biotechnologies). For Western blotting, another Nur77 antibody was applied,11 and p27\(^{kip}\) and α-tubulin were detected with monoclonal antibodies (BD Biosciences and Cedar Lane, respectively).

In Situ Hybridization and RNase Protection Assay

In situ hybridization and RNase protection assays were performed as described.12 The following probes were synthesized for in situ hybridization: TR3, GenBank No. L13740, base pairs 1221 to 1905; MINOR, GenBank No. U12767, base pairs 1435 to 2172; NOT, GenBank No. X75918, base pairs 119 to 1003; and SM22α, GenBank No. NM-011526, base pairs 330 to 582. Matching sense riboprobes were assayed for each gene and were shown to give another background nor an aspecific signal. The sections were exposed for 2 to 8 weeks. The RNase protection probe for NOT was from base pairs 1434 to 1555.

Double In Situ Immunohistochemistry

In situ hybridization assays were performed as described,12 with minor modifications. Briefly, the proteinase K step was reduced to 1 minute. After probe hybridization and wash steps, immunohistochemistry was performed. Slides were dipped in emulsion, exposed, developed, counterstained with hematoxylin, and embedded in Glycergel (DAKO).

Northern Blotting and Southern Blotting

Northern blotting and Southern blotting were performed as described.3,13 The transgene was detected by using a fragment of human TR3 cDNA (base pairs 864 to 1905).

Mice

Animal care and experimental procedures were approved by the Animal Experimental Committee at our institute. Transgenic mice expressing ΔTA or the full-length TR3 gene were generated in an FVB background (Broekman, Someren, the Netherlands) by injection of DNA into the pronuclei of fertilized oocytes. The DNA that was injected consisted of the 1406-bp EcoRI-SalI fragment of the SM22α promoter as described14 (GenBank No. U36589, base pairs 1393 to 2797), a 203-bp rabbit β-globin intron from the pS1 mammalian expression vector (Promega), either full-length TR3 cDNA (base pairs 1 to 1950) or ΔTA (base pairs 864 to 1950) with a substitution of amino acid 251 from threonine to methionine, and a 622-bp polyadenylation signal of human growth hormone (GenBank No. J03071, base pairs 6699 to 7321). Different founders were raised. Mice were homozygous, as established by Southern blotting, and backcrossed with wild-type mice. At 8 weeks of age, the mice were subjected to carotid artery ligation as described.18 Briefly, mice were anesthetized by intraperitoneal injection of a solution of midazolam (12.5 mg/kg body wt) and Hypnorn (Janssen Pharmaceuticals; 0.01 mL per mouse), and the left common carotid artery was ligated near the distal bifurcation (n=4 to 7). At 2.5 or 4 weeks after ligation, the mice were anesthetized and subsequently perfused via the heart with PBS, and carotid arteries were harvested.

Morphometry

The ligated artery was sectioned from the ligature toward the aortic arch. A standardized reference point was set at which the vessel structure was not distorted by the ligature and the elastic laminae were intact. Cross sections 0.7 mm from the reference point were morphometrically analyzed by using QWin software (Leica Microsystems) on digital images of the vessel, obtained with a Sony DXC-950 3CCD video camera. The circumferences of the lumen, internal elastic lamina, and external elastic lamina were measured, and medial area, neo intimar area, and neointima/media ratio were calculated.

Statistical Analysis

Statistical analyses were performed with SPSS, version 10.0.5, software. Experimental values are expressed as mean±SEM. The significance of differences was determined by using the nonparametric Mann-Whitney 2-tailed U test and expressed as a probability value.

Results

Activated Human Primary SMCs Express TR3, MINOR, and NOT

In agreement with our previous observation that the expression of TR3 and MINOR is induced on the activation of primary human SMCs, we now show that NOT is also upregulated and exhibits kinetics similar to those of TR3 and MINOR (Figure 1A through 1C). All 3 genes displayed typical characteristics of early response genes in SMCs on an atherogenic stimulus.

TR3, MINOR, and NOT Expressed in Human Atherosclerotic Lesions

To evaluate the role of these transcription factors in vascular disease, we determined mRNA-expression profiles of TR3, MINOR, and NOT in multiple human vascular specimens at different stages of the disease (lesion type II to V, according to the American Heart Association), derived from 14 different individuals ranging in age from 17 to 66 years (listed in the
All 3 NR4A members were exclusively expressed in neointimal cells and not in normal medial SMCs (Table). As a typical example of an early lesion, we show a type II lesion of a 40-year-old female (first specimen on Table, Figure 2A and 2B). This lesion consisted mainly of SMCs, but some macrophages were present (Figure 2D and 2E). mRNA expression of TR3, MINOR, and NOT was found in neointimal cells (Figure 2F through 2N). In Figure 2H, 2K, and 2N, in situ hybridization was combined with immunohistochemistry for a macrophage-specific antigen, and these data clearly show that SMCs indeed express these transcription factors. Macrophages also express TR3, MINOR, and NOT (E.K. Arkenbout, unpublished data, 2002). The expression of these transcription factors in lesions was not homogeneous, and the mere fact that not all neointimal SMCs synthesize TR3, MINOR, or NOT may indicate that lesion SMCs have diverse phenotypic characteristics. In addition, we demonstrated that TR3 protein expression was comparable to the typical mRNA pattern (Figure 2O and 2P).

### Increased DNA Synthesis on Adenoviral Infection of SMCs With Dominant-Negative Variant

To assess the function of NGFI-B–like transcription factors in SMCs, we infected cultured human SMCs with adenoviral vehicles encoding either a ΔTA or full-length TR3 or a mock virus and determined the effect on DNA synthesis and expression of the cell cycle inhibitor p27 Kip1. First, infection with ΔTA adenovirus and TR3 virus resulted in the expression of the encoded proteins of 35 and 68 kDa, respectively, in a virus dose-dependent way (Figure 3A). Second, because of ΔTA overexpression, [3H]thymidine incorporation was almost 10-fold increased compared with mock-infected cells (Figure 3B).
probably because mock-infected SMCs show relatively low \(^{[3]H}\)thymidine incorporation, this DNA synthesis was not further diminished on the addition of full-length TR3 adenovirus. Third, \(p27^{kip1}\) expression was substantially decreased in \(\Delta TA\)-overexpressing SMCs (Figure 3C).

### Opposite Effects on DNA Synthesis in Transgenic SMC \(\Delta TA\) and Full-Length TR3

To evaluate the in vivo function of these orphan nuclear receptors in lesion formation, we generated transgenic mice expressing either \(\Delta TA\) or full-length TR3 under control of the SM22\(\alpha\) promoter, which directs the expression of transgenes specific to arterial SMCs.\(^{14,16}\) Independent homozygous transgenic lines (\(\Delta TA-F\), \(\Delta TA-D\), and TR3-A) that differ in the copy number of the transgenes were bred (Figure 4A).

The transgenic mice reproduced normally and were apparently healthy. We observed the expression of the transgenes in SMCs in the arterial vessel wall by radioactive in situ hybridization, whereas no expression was detected in the arterial wall of wild-type mice (Figure 4B). DNA synthesis of SMCs explanted from aortas of \(\Delta TA\)-expressing transgenic mice was 1.5-fold higher for \(\Delta TA-F\) and \(\Delta TA-D\) compared with wild-type murine SMCs (\(P=0.017\) and \(P=0.005\), respectively) and 2-fold lower for SMCs from the TR3-A line (\(P=0.039\)) (Figure 5A).

Importantly, infection of \(\Delta TA-F\) and \(\Delta TA-D\) transgenic SMCs with adenoviral full-length TR3 reverted the enhanced DNA synthesis, as shown by a 80% decrease of \(\Delta TA\) adenovirus and \(\Delta TA\) DNA as well as to endogenous murine TR3 DNA. B, mRNA expression of transgenes was observed in aortas of \(\Delta TA-F\), \(\Delta TA-D\), and TR3-A mice by radioactive in situ hybridization with human TR3-specific sequences (base pairs 1221 to 1905), which do not detect murine TR3. No hybridization was observed in aortas of wild-type mice. Lu indicates lumen. Bar=35 \(\mu\)m.

### Opposite Effects on Lesion Formation on Carotid Artery Ligation in Transgenic Mice Expressing \(\Delta TA\) or Full-Length TR3

Wild-type mice and the transgenic lines were subjected to carotid artery ligation,\(^{15}\) a model in which an SMC-rich neointima is induced. First, SM22\(\alpha\) expression was investigated by in situ hybridization in vessels from wild-type mice ligated for different time periods. Expression of SM22\(\alpha\) mRNA was present in the nonligated carotid artery. Furthermore, medial expression was observed 1 week after ligation; this observation was in contrast to the wire-induced injury model in which downregulation of SM22\(\alpha\) was seen 7 days after injury.\(^{17}\) At 4 weeks after ligation, medial as well as neointimal SMCs expressed SM22\(\alpha\) (Figure 6A). The morphology of the carotid arteries of the different transgenic lines was identical, indicating that transgene expression does not affect the structure of the vessel wall (Figure 6B). Sections of ligated vessels of \(\Delta TA\) transgenic mice (\(\Delta TA-D\)) and wild-type littermates are shown after 2.5 weeks of ligation, during which period wild-type mice developed hardly any lesions (Figure 7A). Lesions of TR3-expressing mice and control littermates are shown after 4 weeks of ligation, during which period wild-type mice developed hardly any lesions (Figure 7A). Lesions of TR3-expressing mice and control mice are shown after 4 weeks of ligation to reveal an inhibition of lesion formation (Figure 7B). The neointima/media ratios were assessed by morphometric analyses and showed that lesions are 3-fold larger in \(\Delta TA\) transgenic mice compared with wild-type mice (\(n=4\) to 6; \(P=0.019\) and \(P=0.014\) for \(\Delta TA-D\) and \(\Delta TA-F\), respectively) (Figure 7A).

As expected, the TR3-expressing mice exhibited the opposite
response, with an almost 5-fold reduction of neointimal formation compared with wild-type mice (n=5 to 8, P=0.008) (Figure 7B).

**Discussion**

Activated SMCs are a hallmark of pathological vascular processes, including atherosclerosis, in-stent restenosis after PTCA, and vein-graft disease. In the present study, we examined the expression profiles of the NGFI-B transcription factor family in human atherosclerotic lesions. Our results clearly show that inhibition of these transcription factors in SMCs results in enhanced lesion formation, whereas TR3 decreases the extent of lesion formation. We hypothesize that NGFI-B–like factors may have a protective function in the initiation and progression of human atherogenesis by preventing excessive SMC proliferation.

We showed that TR3, MINOR, and NOT are expressed by in vitro–activated SMCs and in atherosclerotic lesions in a wide range of individuals at different stages of atherosclerosis. For genes that are expressed exclusively in atherosclerotic lesions but are absent from the normal vessel wall, the inhibition of vascular lesion formation is an unexpected function. However, defense mechanisms may be essential in controlling pathological processes such as atherogenesis and are also operational during the progression of the disease. Furthermore, preliminary data show that TR3 is also expressed in in-stent restenosis material (data not shown); these data suggest that induction of this transcription factor is a general process initiated on SMC activation.

At present, the exact mechanism by which TR3 reduces vascular lesion formation is unknown. TR3 has been associated with the induction of apoptosis during T-cell development.6–9 Moreover, in prostate tumor cells, it has been demonstrated that TR3 translocates, in response to apoptotic agents, from the nucleus to the mitochondria to induce cytochrome c release, resulting in programmed cell death.18 However, our data suggest that apoptosis is not the mechanism by which TR3 mediates its inhibitory capacity. Infection of human SMCs with either mock, TR3, or ΔTA adenovirus does not change the number of apoptotic SMCs, as determined by alterations in nuclear morphology and the generation of activated caspase-3 (data not shown). Moreover, infection of SMCs with ΔTA adenovirus, but not with mock or full-length TR3 adenovirus, results in a decrease of p27Kip1 protein, indicating that the dominant-negative TR3 variant promotes cell cycle progression by downregulating this cyclin-dependent kinase inhibitor. Although further studies are required to reveal the exact underlying molecular mechanisms by which TR3 may inhibit SMC proliferation, we postulate that the TR3-regulated genes constitute cell-cycle regulators.

It is known that NGFI-B family members can form heterodimers, resulting in synergistic effects on their tran-
scriptional activities and changes in target sequence preferences. In addition, both TR3 and NOT form heterodimers with the retinoid X receptor (RXR). Recently, it has been shown that nerve growth factor–induced phosphorylation of TR3 results in translocation of RXR-TR3 heterodimers from the nucleus into the cytoplasm, consequently interfering with RXR-mediated signal transduction. A potential contribution of TR3-RXR dimerization in the process of atherogenesis needs to be addressed.

In summary, we propose that the NGFI-B hormone receptors are inhibitors of vascular lesion formation and that these transcription factors are expressed in atherosclerotic lesions to protect the vessel wall from excessive SMC proliferation. Increased activation of these orphan receptors by an as-yet-unknown ligand(s) may be applied in the treatment of atherosclerosis and restenosis and may lead to targeted therapeutic interventions.

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