Retinoic Acid–Induced Tissue Transglutaminase and Apoptosis in Vascular Smooth Muscle Cells

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Abstract—Retinoids exert antiproliferative and prodifferentiating effects in vascular smooth muscle cells (SMCs) and reduce neointimal mass in balloon-injured blood vessels. The mechanisms through which retinoids carry out these effects are unknown but likely involve retinoid receptor-mediated changes in gene expression. Here we report the cloning, chromosomal mapping, and biological activity of the retinoid-response gene rat tissue transglutaminase (tTG). Northern blotting studies showed that tTG is rapidly and dose-dependently induced in a protein synthesis–independent manner after stimulation with the natural retinoid all-*trans* retinoic acid (atRA). The induction of tTG was selective for atRA and its stereoisomers 9-*cis* and 13-*cis* RA, because little or no elevation in mRNA expression was observed with a panel of growth factors. Western blotting and immunofluorescence confocal microscopy showed an accumulation of cytosolic tTG protein after atRA stimulation. Radiolabeled cross-linking studies revealed a corresponding elevation in in vitro tTG activity. The increase in tTG activity was reduced in the presence of 2 distinct inhibitors of tTG (monodansylcadaverine and cystamine). atRA-induced tTG mRNA and protein expression were followed by a significant elevation in SMC apoptosis. Such retinoid-induced programmed cell death could be partially inhibited with each tTG inhibitor and was completely blocked when both inhibitors were used simultaneously. These results establish a role for atRA in the sequential stimulation of tTG and apoptosis in cultured SMCs. atRA-mediated apoptosis in SMCs seems to require the participation of active tTG, suggesting a potential mechanistic link between this retinoid-inducible gene and programmed cell death. **(*Circ Res*. 2000;87:881-887.)**

Key Words: tretinoin ■ transcription ■ protein-glutamine γ-glutamyltransferase ■ chromosome ■ cDNA

Perturbations in the genetic program of smooth muscle cell (SMC) growth arrest and differentiation are central to the pathogenesis of many vascular occlusive disorders as well as diseases of the airway, gastrointestinal, and urogenital systems. The majority of research investigating the basis for changes in SMC phenotype during disease progression has focused on membrane-bound receptors that effect intracellular signaling cascades involved in SMC growth and differentiation control.[1,2] In recent years, studies have been initiated to examine the role of steroid receptor-binding hormones on SMC phenotype. For example, 17β-estradiol, which binds the estrogen receptors, suppresses SMC growth and migration, stimulates SMC vasodilation through the activation of nitric oxide synthase, and reduces neointimal formation after arterial injury.[3] Ligands for the peroxisome proliferator-activated receptors exhibit similar effects on SMC phenotype.[4] Another family of hormones, the retinoids,[5] recognizes 6 distinct nuclear receptors designated as retinoic acid receptors (α, β, and γ) and retinoid X receptors (α, β, and γ).[6] Previous studies have documented retinoid receptor expression and activity in SMCs[7] and have shown that retinoids, principally atRA, antagonize SMC mitogenesis[7–12] and migration[13] while promoting a SMC-differentiated phenotype.[14–16] In a recent series of corroborative papers, all-*trans* retinoic acid (atRA) was shown to increase luminal diameter after experimental injuries to the vessel wall.[17–23] Collectively, these studies point to a versatile role for retinoids in the control of SMC phenotype and the response of the vessel wall to insult.[24]

Steroid receptors act as ligand-activated transcription factors that mediate changes in a cell's gene expression profile. For example, activated retinoid receptors, bound to discrete *cis* elements called retinoic acid (retinoid X) response elements, recruit critical coactivators around the preinitiation complex of a gene, stimulating the rate at which a gene is transcribed.[25] Several retinoid-responsive target genes have been identified, including growth factors, cytokines, proteases and their inhibitors, cyclin-dependent kinases and their inhibitors, transcription factors (especially homeobox genes), matrix molecules, and apoptotic factors.[26] Presently, there is a paucity of information relating to retinoid-response genes in

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SMCs and even less about the mechanisms underlying retinoid-mediated changes in SMC phenotype.

In a directed screen for retinoid-inducible genes that may mediate the biological effects of atRA in SMCs, we cloned a portion of the rat tissue transglutaminase (tTG) cDNA. tTG is a retinoid-inducible, multifunctional gene implicated in substrate-specific isopeptide bond cross-linking, GTP-mediated signaling, and apoptosis.[27] Although its expression in vascular SMCs has been documented both in vitro and in vivo,[28–30] the precise role of tTG in SMC biology is unclear. Here, we report the cDNA sequence and chromosomal mapping of rat tTG. We show that tTG is induced selectively by retinoids and that such gene activation occurs in the absence of de novo protein synthesis. We additionally demonstrate that the activation of tTG precedes retinoid-induced apoptosis, which can be completely blocked with inhibitors of tTG. These results suggest that one component of atRA's growth antagonism in SMCs may involve tTG-mediated apoptosis.

Material and Methods

Cell Culture

Cultured rat aortic SMCs (RASMCs) were obtained from the thoracic aorta of male Sprague-Dawley rats by a modified explant protocol, as described.[31] These cells and the PAC-1 SMC line[32] were grown in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Both cell types express many of the major SMC-restricted markers.[33]

Cloning and Chromosomal Mapping of Rat tTG

A first-strand cDNA library was generated (Amersham Pharmacia) from cultured RASMCs treated with all-*trans* retinoic acid (kindly provided by Dr Louise Foley, Hoffmann-La Roche, Nutley, NJ). The library was then PCR-screened using a high-fidelity polymerase (TaKaRa; Panvera Corp) with primers to the mouse tTG cDNA (GenBank accession number M55154). Three independent tTG clones were sequenced on both strands (Biotech Thermo Sequenase dye terminator cycle sequencing kit; Amersham Pharmacia) using a Perkin Elmer Applied Biosystems 377 automated sequencing machine. Sequence analysis was performed using the FASTA, pileup, and pretty algorithms within Genetic Computer Group's Wisconsin Package (version 10). All three clones were aligned and analyzed using the GeneDoc program (online at http://www.cris.com/~ketchup/genedoc/shtml). Chromosomal mapping of the rat tTG gene was accomplished with a rat-hamster radiation hybrid (RH) panel (Research Genetics), as described.[34]

Northern Blotting

Cells stimulated with atRA (2×10^{-6} mol/L), 9-*cis* RA (2×10^{-6} mol/L), 13-*cis* RA (2×10^{-6} mol/L), 10% FBS, α-thrombin (2 U/mL), basic fibroblast growth factor (20 ng/mL), transforming growth factor-β_1 (2 ng/mL), epidermal growth factor (10 ng/mL), or an equal volume of dimethylsulfoxide (DMSO control) were harvested for total RNA isolation, as described,[33] and probed with an 800-bp fragment of the rat tTG cDNA. A glyceraldehyde phosphate dehydrogenase cDNA was used as an internal control. In some experiments, we used cycloheximide (CHX; 2.5 μg/mL; Sigma) to assess the requirement for de novo protein synthesis on tTG expression. This concentration of CHX was shown to have minimal toxicity over a 24-hour time period and reduced new protein synthesis by >90% (data not shown).

Western Blotting

Extracts of RASMCs treated with atRA or DMSO were isolated for total protein synthesis and Western blotting, as described.[33] A polyclonal goat antibody raised against rabbit tTG (Upstate Biotechnology) was applied to immobilized proteins at a dilution of 1:1000. An antibody to smooth muscle calponin (clone hCP, Sigma) was used as an internal control. Secondary antibodies conjugated to horseradish peroxidase (1:2000 dilution) were used to reveal immunoreactive protein with ECL Plus reagents (Amersham Pharmacia).

Confocal Microscopy

RASMCs were cultured in chamber slides in the presence of DMSO or atRA for 48 hours. After fixation and permeabilization with acetone and methanol, the cells were incubated with anti-tTG (1:100 dilution) for 1 hour, washed in PBS with 0.1% tween 20, incubated with rabbit antigoat IgG conjugated to biotin (1:100 dilution, Pierce), washed and incubated with streptavidin-FITC (1:100 dilution, Pierce) for an additional 30 minutes, and then washed 2 times with PBS. Cells were then stained with the DNA fluorochrome TO-PRO-3 iodide (Molecular Probes). Slides were cover-slipped under aqueous mounting medium and viewed with an Olympus Fluoview FV 300 confocal microscope. Voltage settings were kept constant between atRA-treated and control cells during imaging.

tTG Activity Assay

tTG activity was measured indirectly in an in vitro assay as the Ca^{2+}-dependent incorporation of ^{3}H-putrescine (0.832 Ci/mmol; New England Nuclear) into *N*,*N*-dimethyl casein (Calbiochem), as described.[35] Monodansylcadaverine (MDC) (Sigma), a competitive substrate inhibitor,[36] and cystamine (Sigma), an active site inhibitor,[37] were tested at varying doses to optimize studies aimed at assessing their inhibitory effects on tTG activity and apoptosis. Trypan blue exclusion was used as a measure of cell viability. tTG activity was expressed as pmol of ^{3}H-putrescine incorporation into *N*,*N*-dimethyl casein per minute per milligram of total protein after subtraction of background levels obtained by substituting EDTA for Ca^{2+}.

Apoptosis Assays

SMCs stimulated for 22 hours with atRA (2×10^{-6} mol/L) or an equal volume of DMSO were fixed and either stained with DAPI or processed for TUNEL, as described.[38]

Statistics

All graphical data are presented as mean±SEM and are representative of at least 2 independent studies. ANOVA with appropriate post hoc testing (GraphPad Prism) was carried out on data pertaining to the tTG activity and the DAPI staining assay for apoptosis. Statistical significance was assumed if $P<0.05$.

Results

Sequence and Amino Acid Homology of Rat tTG

The rat tTG cDNA is at least 3393 nt in length, with 2058 nt of coding sequence yielding a 686 amino acid protein (GenBank accession number AF106325). As anticipated, the rat tTG cDNA is highly homologous within the coding region to the orthologous mouse (92.4%), human (83%), and guinea pig (82.2%) cDNA sequences (data not shown). The deduced amino acid sequence encompassing the active site domain of rat tTG is identical to mouse and evolutionarily conserved across several species (Figure 1). Nucleotide sequence homologies diverge considerably within the 3′ untranslated regions of mouse (77.5%), guinea pig (49.6%), and human (43.7%) tTG (data not shown). We exploited this sequence divergence in the design of PCR primers for RH mapping studies. Rat tTG was RH-mapped to chromosome 3, between D3Got123 and D3Rat4 at >1000:1 odds (data not shown). This region, corresponding roughly to 3q41 to 3q42, is syntenic with human chromosome 20q12, which is where the

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268  GCQQVKYGQCWVFAAVACTVLRCLGIPTRVVTNYNSAH  305  Rat (AF106325)
268  ----------------------------------------  305  Mouse (M55154)
268  ---R------------------------------------  305  Human (M55153)
268  ---R-----------------------------F-----  305  Guinea Pig (M19646)
269  ---P----------------M----V-S----------  306  Chicken (U47273)
335  -VP ----------G--N--S-A----S-T----D---  371  Horseshoe crab (D12593)
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Figure 1. Amino acid homology between rat tTG and several other species of tTG is shown for the active site domain. The critical cysteine residue (amino acid 277) is underlined and labeled in bold. This stretch of homology corresponds to amino acids 268 to 305 of rat, mouse, human, and guinea pig tTG. GenBank accession numbers for each species of tTG are shown parenthetically.

human tTG has been mapped previously.[39] Information regarding the RH framework maps can be found online at http://goliath.ifrc.mcw.edu.

tTG Is an Immediate-Early Retinoid Response Gene

tTG is induced in a variety of cell types on exposure to retinoids, such as atRA.[27] Figure 2A shows similar activation of tTG when SMCs are stimulated with atRA. Increasing steady-state transcript levels of tTG emerge as early as 3 hours after atRA addition, with peak expression at 24 hours. Induction of tTG in SMCs seems to be highly restricted to atRA, because little or no activation is observed on treatment with serum and a panel of growth factors (Figure 2B). 13-*cis* RA, which has more favorable pharmacokinetic and toxicological profiles than atRA and readily isomerizes to both atRA and 9-*cis* RA,[24] induces tTG expression (Figure 2B), as does the 9-*cis* RA stereoisomer itself (data not shown). The induction of tTG with atRA is dose-dependent (Figure 2C) and occurs in the absence of de novo protein synthesis (Figure 2D). A consistent superinduction of tTG mRNA is observed in the presence of CHX, suggesting the existence of a labile mRNA binding protein involved in the destabilization of the tTG transcript (Figure 2D). The mRNA kinetics,

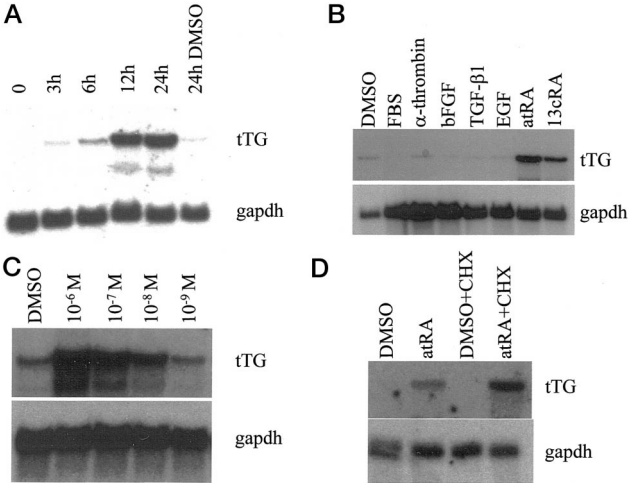

Figure 2. tTG mRNA expression studies. Representative Northern blots showing time course expression of tTG after 2×10^{-6} mol/L atRA stimulation for the indicated times or 0.1% DMSO for 24 hours (A); specific induction of tTG with 2×10^{-6} mol/L atRA or 13-*cis* RA (B); dose-dependent increase in tTG after 24 hours atRA stimulation (C); and protein synthesis–independent induction of tTG after 24-hour treatment with 2×10^{-6} mol/L atRA in the absence or presence of 2.5 μg/mL CHX (D).

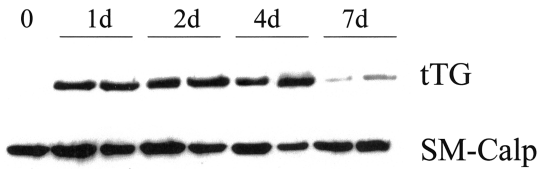

Figure 3. Representative Western blot demonstrating elevated tTG protein in cultured RASMCs after atRA treatment. Duplicate cultures of RASMCs were incubated for 24 hours in 0.25% FBS and then stimulated for the indicated times with 2×10^{-6} mol/L atRA. Smooth muscle calponin (SM-Calp) confirmed cell identity, equivalent protein loading, and specificity in retinoid-responsiveness.

protein synthesis–independent expression, and superinduction with CHX indicate that tTG is an immediate-early retinoid response gene.

Protein Expression and Activity of tTG

To determine whether atRA-induced tTG mRNA led to its enhanced translation, we measured steady-state protein levels by Western blotting. tTG is clearly increased within 24 hours after atRA stimulation (Figure 3). tTG protein remains elevated up to 4 days, with levels returning toward baseline at 7 days (Figure 3). Shorter time-course studies reveal increases in tTG protein as early as 6 hours after atRA administration (data not shown). Little, if any, tTG protein was detected in the culture medium, indicating that the protein is not actively secreted under our culture conditions (data not shown). Confocal microscopy demonstrates low levels of immunoreactive tTG in the cytosol of control RASMCs (Figure 4A). At the same voltage setting, cultured RASMCs show a dramatic increase in cytosolic tTG after 48 hours of atRA stimulation (Figure 4B). Consistent with previous reports in other cell types,[40] a small percentage of tTG is noted within the nucleus (Figures 4A and 4B).

We next used a well-defined in vitro assay to assess 2 inhibitors of tTG cross-linking activity. We used an indirect assay that measures the amount of cross-linking activity on addition of Ca^{2+} (see Materials and Methods). Peak tTG protein levels (48 hours) coincided with a 6-fold elevation in tTG cross-linking activity (control, 0.0105 ± 0.001 versus atRA, 0.0625 ± 0.009; $P<0.001$). This induced activity could be partially inhibited with the active site inhibitor cystamine (0.0395 ± 0.003); however, inclusion of both cystamine and MDC caused a consistent and significant inhibition of in vitro tTG activity (0.0625 ± 0.009 versus 0.009 ± 0.003, $P<0.001$) with no evidence of overt cytotoxicity. Similar tTG expression and activity results were found in the PAC-1 SMC line (data not shown). These results establish tTG protein induction with atRA treatment and demonstrate the utility of combining 2 inhibitors to block tTG cross-linking activity in cultured RASMCs.

atRA-Induced SMC Apoptosis Is tTG-Dependent

Several studies (mainly in cancer cell lines) have shown that atRA stimulates the expression of tTG and elicits apoptosis.[27,41] To determine whether a mechanistic link exists between atRA-stimulated tTG expression and programmed cell death, we evaluated the tTG inhibitors studied above for their

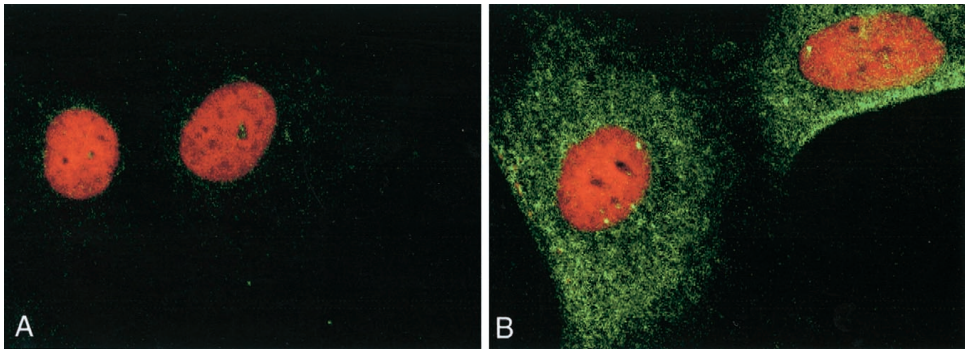

Figure 4. Intracellular localization of tTG protein after atRA treatment. Cultured RASMCs were incubated for 24 hours with 0.25% FBS and then stimulated for 48 hours with 0.1% DMSO (A) or 2×10^{-6} mol/L atRA (B). Confocal microscopy revealed low, cytosolic staining of immunoreactive tTG in DMSO control cells, which was greatly elevated in cells exposed to atRA. FITC stain (green) shows tTG, whereas TO-PRO-3 iodide (red) reveals the nucleus. Magnification is ×200.

effects on atRA-induced apoptosis in SMCs. The results depicted in Figure 5 show a 7-fold increase in apoptotic SMCs (DAPI stained nuclear morphology) with exposure to atRA. The number of atRA-induced apoptotic SMCs ranged from 5% to 8% of the cell population. This level of apoptosis is considerably less than the 20% observed in RASMCs stimulated with 200 μmol/L H_2O_2 (data not shown). Each tTG inhibitor shows partial, but statistically insignificant, inhibition of apoptosis when added individually (Figure 5). However, consistent with the in vitro activity data above, combining both tTG inhibitors (nontoxic by trypan blue exclusion) results in near complete inhibition of atRA-stimulated apoptosis (Figure 5). TUNEL staining confirmed the inhibition of atRA-induced apoptosis with both tTG inhibitors (Figure 6). These results document atRA-mediated apoptosis in RASMCs and suggest that tTG is an important determinant of such programmed cell death.

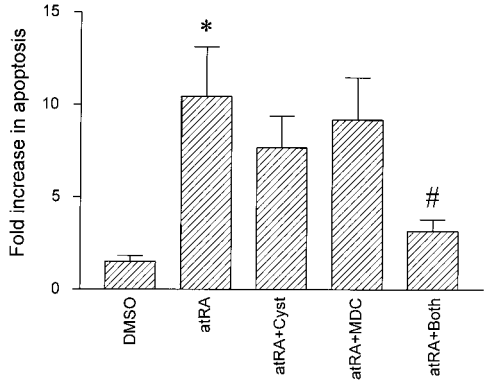

Figure 5. atRA-induced SMC apoptosis. Cultured RASMCs were incubated in 35-mm dishes for 24 hours in 0.25% FBS and then stimulated with 2×10^{-6} mol/L atRA for an additional 22 hours in the absence or presence of tTG inhibitors (150 μmol/L). Later time points (eg, 48 hours) could not be evaluated because of the difficulty in distinguishing apoptotic cells from necrotic cells. atRA elicited a statistically significant elevation in the apoptotic index (*$P<0.05$ vs DMSO control). A slight but statistically insignificant diminution in the apoptotic index was observed when each inhibitor was used separately; however, when both inhibitors were present at nontoxic concentrations, atRA-induced apoptosis was virtually abolished (#$P<0.05$ vs atRA). Results are representative of 2 independent studies in which at least 300 cells per dish were scored for the presence or absence of apoptotic nuclei as determined by DAPI staining.

Discussion

Three major findings emerge from this study. First, we have shown that atRA rapidly induces the expression of tTG mRNA with subsequent elevations in immunoreactive tTG protein and in vitro cross-linking activity. The activation of tTG in SMCs is a relatively specific response to retinoids, because no other reagent tested induced the expression of this multifunctional enzyme. Its rapid kinetics of expression and independence of de novo protein synthesis indicate that tTG is an immediate-early retinoid response gene. Second, using 2 independent assays (DAPI and TUNEL staining), we have shown that atRA can induce apoptosis in vascular SMCs. Finally, we provide evidence for a mechanistic link between atRA-induced tTG activity and apoptosis by showing the near complete inhibition of apoptosis in the presence of MDC (a tTG substrate inhibitor) and cystamine (a tTG active site inhibitor). These results identify tTG as a critical target of retinoid action in cultured SMCs and suggest that atRA-mediated effects on vascular SMC phenotype and vessel remodeling after injury may, in part, involve the action of this multifunctional enzyme.

tTG (EC 2.3.2.13) and other members of the transglutaminase gene family exert calcium-dependent posttranslational modifications of substrate-specific proteins through an acyl transferase reaction involving γ-carboxamide groups of glutamine residues (donor peptide) with ε-amino groups of lysine residues (acceptor peptide). The resulting covalent modification imparts an irreversible cross-link that stabilizes proteins, conferring structural integrity to intracellular and extracellular microenvironments.[42] tTG protein is present at high levels in disease-free blood vessels[28] and is restricted to adult rat aorta by Northern blotting (unpublished data, July 2000), suggesting that tTG is part of the SMC differentiation program of gene expression. In this context, levels of tTG have been shown to drop precipitously in RASMCs after enzymatic dispersion and cell passaging, indicating that the loss of tTG coincides with the phenotypic modulation of these cells in vitro.[43] The dramatic upregulation of tTG after atRA administration is consistent with this retinoid's prodifferentiation effects in cultured SMCs.[24,44]

In a previous study, atRA was shown to modestly elevate tTG mRNA in human venous SMCs but did not elevate tTG

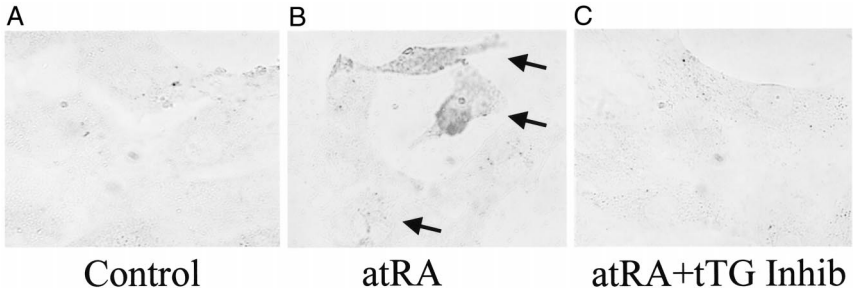

Figure 6. TUNEL staining of RASMCs treated with atRA in the presence of tTG inhibitors. RASMCs were cultured for 24 hours in 0.25% FBS before exposure to DMSO (A), 2×10^{-6} mol/L atRA (B), or the same concentration of atRA plus both tTG inhibitors (C) for 22 hours and then processed for TUNEL staining. Results are representative of 2 independent studies. Arrows in panel B indicate TUNEL positive RASMC, whereas no such cells are seen in control or atRA+both inhibitor conditions. Magnification is ×100.

activity in these cells. Human arterial SMCs showed a 3-fold increase in tTG activity with atRA stimulation, but the mRNA expression of tTG was not reported in these cells.[29] Interestingly, tTG colocalized with stress fibers in human arterial and venous SMCs and was shown to coimmunoprecipitate with myosin, suggesting that codistribution of tTG with stress fibers was attributable to tTG's interaction with myosin.[29] Although we did observe some evidence of stress fiber formation in atRA-treated SMCs, which is consistent with atRA's prodifferentiation effects in these cells, extensive confocal microscopy failed to reveal any colocalization of tTG with the stress fiber apparatus (data not shown). A likely explanation for these varying results may relate to the species differences in vascular SMCs.

Although tTG-mediated cross-linking reactions have been implicated to play a role in diverse biological processes, the precise physiological function of the enzyme remains unclear, particularly with respect to vascular biology. Previous studies have shown atRA to antagonize growth factor–stimulated SMC proliferation.[7–12] The findings reported here suggest that one component of this growth-inhibitory effect may be related to apoptosis. In support of this concept are several studies carried out in various non-SMC lines. For example, cystamine inhibited tTG activity while elevating cell growth in cultured WI-38 human lung cells.[45] atRA-stimulated human epithelial prostate cells showed elevated tTG and apoptosis, both of which correlated with reduced cell growth.[41] A similar correlation was observed in rat tracheobronchial epithelial cells.[46] Melino et al[47] showed increased cell death and a marked reduction in proliferative capacity of several tumor cell lines that were engineered to overexpress tTG. Gentile et al[48] showed comparable effects of overexpressing tTG in Balb-c 3T3 fibroblasts. Conversely, cells transfected with tTG in the antisense orientation resulted in a reduction of both spontaneous and atRA-induced cell death.[47,49] Collectively, these studies point to an important role for activated tTG in mediating cellular apoptosis and, consequently, modulating growth responses. Although the mechanisms remain to be clarified, evidence suggests that tTG-mediated cross-linking of intracellular proteins, such as the retinoblastoma protein and histones, may be critical in signaling an apoptotic program.[50] However, we cannot rule out effects of tTG on SMC apoptosis that are independent of its cross-linking activity.

Significant inhibition of atRA-induced SMC apoptosis required the simultaneous addition of both tTG inhibitors (Figures 5 and 6). Several factors may explain why, individually, each inhibitor was ineffective in significantly blocking atRA-mediated apoptosis. First, our data cannot distinguish the effects of each inhibitor on tTG activity versus other, undefined effects. Thus, it is possible that tTG-independent pathways of apoptosis are blocked in the presence of both inhibitors. Second, concentrations used for each inhibitor in this study (150 μmol/L) were comparatively lower than in previous cell culture studies (>250 μmol/L).[45,51] Another explanation for the nonsignificant effect of each inhibitor on atRA-induced apoptosis may relate to their distinct modes of inhibition; although cystamine seems to modify the active site cysteine residue (Figure 1), MDC acts as a pseudosubstrate inhibitor. Thus, there seems to be a requirement for both modes of inhibition to effect significant blockage of atRA-induced apoptosis. In this context, tTG inhibitors only partially attenuated in vitro tTG activity but, when combined, always elicited near complete inhibition of in vitro tTG activity (see text and Reference 45). Clearly, more work in this area is required, including the use of more specific reagents (eg, antisense tTG) and an analysis of apoptosis in atRA-treated SMCs derived from tTG knockout mice.

It is important to stress that although virtually all SMCs showed elevated immunoreactivity for tTG after atRA treatment, only a subpopulation (<8%) of such cells actually underwent apoptosis. This finding is consistent with in vivo immunohistochemistry studies showing substantial levels of tTG protein in blood vessels[28,30] where apoptosis of contractile SMCs is low or near absent. As calcium is a critical determinant of tTG activity,[27] we hypothesize that those tTG-positive cells undergoing apoptosis achieve a critical threshold of intracellular calcium levels that activate the cross-linking activity of the enzyme. Alternatively, the 8% apoptotic index may be explained by the heterogeneity of SMC subtypes in these cultures having variable apoptotic pathways or tTG substrate profiles. The function of tTG in cells that do not undergo apoptosis is unclear but could relate to signal transduction events involving its GTP-binding domain.[52] Future studies will be necessary to determine the rules governing tTG activity and its downstream biological effects in atRA-treated SMCs.

In summary, the data presented here are consistent with the hypothesis that tTG activation mediates SMC apoptosis after atRA stimulation. However, we emphasize that atRA likely orchestrates myriad changes in gene expression that could impinge on the apoptotic program independent of tTG expression and activity. Future studies should be directed toward a more complete understanding of atRA-induced gene expression in SMC as well as tTG signaling in SMC after atRA stimulation (eg, GTP-binding and phospholipase C–me-

diated signaling[52]). Finally, an appraisal of the proposed atRA-tTG-apoptosis axis in the setting of vascular remodeling after injury warrants investigation.

Acknowledgments

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