

Inhibition of Tissue Factor Gene Induction and Activity Using a Hairpin Ribozyme

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Background—Tissue factor (TF) is a membrane-bound glycoprotein that initiates the clotting cascade. Inhibition of the TF pathway has been shown to prevent thrombosis and restenosis after arterial injury in a variety of animal models.

Methods and Results—We describe a novel approach to inhibiting the expression of the TF protein that involves the targeted destruction of cellular TF mRNA with the use of a tetraloop hairpin ribozyme. After construction of the ribozyme and determination of its optimal length and kinetic parameters, a ribozyme expression vector that used the retroviral vector pMV12 was constructed. The ability of this expression vector to generate anti-TF ribozyme was further augmented by positioning of the anti-TF ribozyme downstream of a rat tRNA val (RNA polymerase II) promoter. The resultant construct containing the anti-TF ribozyme was then used to transfect vascular smooth muscle cells and generate a variety of clonal cell lines. Northern blot analyses performed on 3 transfected and 3 untransfected clones demonstrated markedly reduced TF mRNA levels in the transfected clones both during quiescence and after serum stimulation. Cell lysates analyzed for total TF activity by monitoring factor Xa generation similarly demonstrated a statistically significant and concordant reduction in TF activity in smooth muscle cells transfected with the ribozyme expression vector compared with both untransfected clones and clones transfected with the empty vector.

Conclusions—These results demonstrate the feasibility of an antithrombotic strategy based on ribozyme technology. (*Circulation*. 2002;105:2282-2287.)

Key Words: restenosis ■ thrombosis ■ muscle, smooth

Tissue factor (TF) is a membrane-bound glycoprotein that initiates the clotting cascade and is considered to be a major regulator of coagulation, hemostasis, and thrombosis.^{1,2} Arterial thrombosis is believed to arise from the interaction of TF in the vascular wall with platelets and coagulation factors in circulating blood. Acute thrombosis occurring spontaneously within the vessel wall can lead to arterial occlusion and result in myocardial infarction, unstable angina, and sudden death.^{3,4}

Because of its pivotal role in thrombosis, there have been numerous attempts to block either the expression or the activity of TF. Indeed, inhibition of the TF pathway has been shown to attenuate the activation of coagulation.⁵ Recently, there has been much interest in TF inhibition as an approach to the prevention of thrombosis and restenosis after arterial injury. Such attempts at an in vivo TF knockout after experimental injury have yielded encouraging results, demonstrating reduced thrombogenicity of disrupted atherosclerotic plaques⁶ and decreased neointimal thickening.^{7,8} To date, efforts at TF inhibition in this setting have concentrated on the exogenous administration of either recombinant TF pathway inhibitor (TFPI)^{6,9} or antibodies directed against TF.¹⁰ More recently, local TFPI gene transfer has also been used successfully.^{11,12} Although the idea is theoretically

sound, it is unknown whether the short-term administration of a peptide can overcome the increased TF expression seen after injury and provide long-term protection. Furthermore, the systemic doses of recombinant TFPI necessary to prevent arterial thrombosis and possibly restenosis may be associated with significant hemorrhagic risks. Although local gene transfer of TFPI appears to be promising, the long-term safety in humans of eliciting site-specific production of recombinant protein remains unknown.

A novel approach to inhibiting the expression of a protein involves the targeted destruction of cellular mRNA with the use of catalytic RNA, or ribozymes.¹³ Ribozymes are a class of RNA molecules that possess enzymatic properties.^{14,15} On binding to complementary nucleic acid strands, catalytic degradation takes place via a cleavage reaction. In contrast to traditional pharmaceutical products, ribozymes disrupt the flow of genetic information rather than inhibiting protein function.¹⁶ Ribozyme technology has emerged as a powerful extension of the antisense approach to inhibition of gene expression.¹⁷ A number of different types of ribozymes have been described, including hammerhead and hairpin varieties. Hairpin ribozymes possess a secondary structure consisting of 4 helices and 5 loop regions. Two helices, helices 1 and 2,

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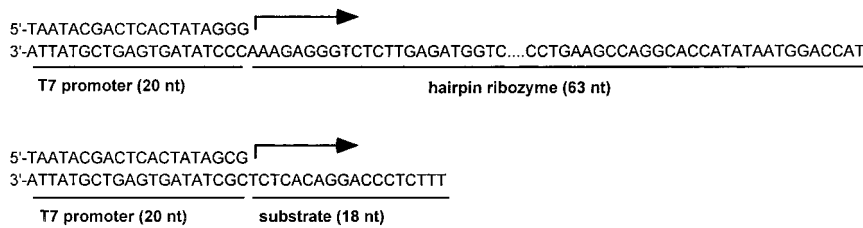


Figure 1. DNA templates for synthesis of the anti-TF hairpin ribozyme and TF mRNA target sequence. Templates for both the ribozyme and the substrate contained an upstream T7 RNA polymerase promoter. Templates were hybridized to a complementary T7 promoter primer, and [α - 32 P]CTP transcripts were synthesized by runoff transcription using T7 RNA polymerase.

form between the substrate and ribozyme, whereas helices 3 and 4 are within the ribozyme itself.¹⁸

To inhibit the accumulation of TF mRNA in vascular cells, we designed a novel tetraloop hairpin ribozyme against TF mRNA at the GUC sequence between nucleotides (nt) 808 and 811 of the rat cDNA. To produce cultured vascular smooth muscle cells (VSMCs) that stably express high levels of anti-TF ribozyme, a retroviral vector was engineered. To augment ribozyme expression beyond levels that would result from the 5'LTR (long-terminal-repeat) alone, the anti-TF ribozyme was positioned downstream of a rat tRNA val (RNA polymerase II) promoter. We then analyzed our permanently transfected cell lines and, comparing them with controls, were able to demonstrate a reduction in both TF mRNA and TF activity as determined by factor Xa generation.

Methods

Cell Culture

VSMCs were isolated by enzymatic dissociation from the thoracic aortas of 200- to 300-g male Sprague-Dawley rats as previously described.¹⁹ Cells were grown in 100-mm plates containing DMEM supplemented with 10% heat-inactivated calf serum (CS), 100 U/mL penicillin, and 100 mg/mL streptomycin and serially passaged before reaching confluence. To produce quiescence, cells were incubated in a defined medium composed of DMEM supplemented with 1 μ mol/L insulin, 5 μ g/mL transferrin, 0.2 mmol/L L-ascorbic acid, and 25 μ g/mL BSA for 48 hours. VSMCs infected with anti-TF ribozyme constructs were maintained in DMEM supplemented with 10% CS and 200 μ g/mL hygromycin B initially (Sigma Chemical Co); the hygromycin concentration was decreased to a maintenance dose of 50 μ g/mL after individual clones were isolated. All cell lines retained a stable phenotype after >20 passages in monolayer culture.

Construction of the Hairpin Ribozyme and Optimization of Length

Single-stranded DNA templates were synthesized (Midland Certified Reagent Co) for both the ribozyme and substrate RNA. The specific sequences are as shown in Figure 1. Templates for both the ribozyme and the substrate contained an upstream T7 RNA polymerase promoter. Templates were hybridized to a complementary T7 promoter primer, and [α - 32 P]CTP transcripts were synthesized by runoff transcription using T7 RNA polymerase. Cleavage reactions to determine the optimal anti-TF hairpin ribozyme helix 1 were carried out by mixing substrates of various lengths and the 63-nt anti-TF ribozyme at 37°C in 12 mmol/L MgCl₂, 2 mmol/L spermidine, 40 mmol/L Tris, pH 7.5. Reactions were performed with 25 nmol/L ribozyme and 100 nmol/L substrate concentrations for the entire study. Reaction products were analyzed on denaturing acrylamide gels, identified by autoradiography, cut out, and quantified by scintillation counting.

Determination of Michaelis-Menten Kinetics

To determine the Michaelis-Menten kinetic parameters and the specificity constant (at pH 7.0 and temperature 37°C) for the

ribozyme, time-course and reaction-velocity experiments were performed. Cleavage reactions as described above were carried out with a constant concentration of ribozyme (50 nmol/L) and increasing concentrations of substrate (from 25 to 200 nmol/L) for time points ranging from 0 to 240 minutes. Results of the time-course study were used to determine the incubation time needed to perform experiments under conditions that are consistent with the steady-state approximations of Michaelis-Menten kinetics. An optimal incubation time period was determined during which there was both (1) a linear relation between percent cleavage versus time and (2) <10% of the original substrate concentration cleaved by the ribozyme. Reaction velocity experiments were then performed to determine the kinetic parameters k_{cat} and K_M . The reaction velocity (V) was calculated for a series of cleavage reactions that were performed with constant ribozyme and varying concentrations of substrate. The initial reaction velocity (V_0) for each substrate concentration was determined by monitoring the amount of substrate cleaved and then applying the formula $V_0 = \%$ of substrate cleaved $\times$ initial concentration of substrate/incubation time.

The plot of V_0 versus substrate concentration was best fitted to a polynomial function from which V_{max} was determined. K_M was calculated by determining the substrate concentration at $0.5 V_{max}$. k_{cat} was determined by solving $V_{max}/[\text{ribozyme}]$. The specificity constant (k_{cat}/K_M) was also determined.

Construction of the Ribozyme Expression Vector

To produce cultured VSMCs that stably expressed high levels of the anti-TF ribozyme, a retroviral vector, pMV12-Rbz (Figure 2), was engineered. pMV12 contains the 5' and 3' long-terminal-repeat sequences of the Moloney murine sarcoma virus and the hygromycin phosphotransferase gene (*hph*) driven by the herpes simplex thymidine kinase promoter.^{20,21} Whereas the *hph* is independently transcribed by the herpes simplex virus thymidine kinase promoter, cDNA clones inserted into the multiple cloning site of pMV12 are under the transcriptional control of the 5'LTR. To augment ribozyme expression beyond levels that would result from the 5'LTR alone, the anti-TF ribozyme was positioned downstream of the tRNA val promoter. This was achieved by inserting an *XhoI/MluI* polymerase chain reaction (PCR) fragment encoding the hairpin ribozyme into pBtV5-111, which contains the tRNAval coding and upstream regions. The tRNAval/ribozyme sequences were then cloned into the *NsiI/NotI* site of pMV12 by use of PCR with primers containing the appropriate restriction sequences.

The resulting final construct contains an upstream nonprocessing tRNAvalCAC promoter and a tetraloop downstream of the ribozyme sequence. The tRNAvalCAC containing 4 mutations was previously demonstrated to prevent 3' processing.²² The mutant tRNA val sequence was introduced into pTV1 containing the valCAC tRNA to generate a nonprocessing tRNA. The 3' tetraloop was introduced to promote stability of the 3' end of the RNA.²³ The mutant tRNA tetraloop was generated by annealing 2 overlapping synthetic oligonucleotides (100 mers) in 1 cycle of PCR to generate full-length complementary sequences. The fragment was cloned into the *SacI/HindIII* sites of pTV1.²⁴

Isolation of Cell Lines and Stable Transfectants

Psi-2 packaging cells were transfected with 20 μ g per plate of pMV12-anti-TF ribozyme construct using calcium phosphate. *Psi-2* cells were split into selective media containing 200 μ g/mL hygromycin. After 24 hours, the viral supernatant was collected, centri-

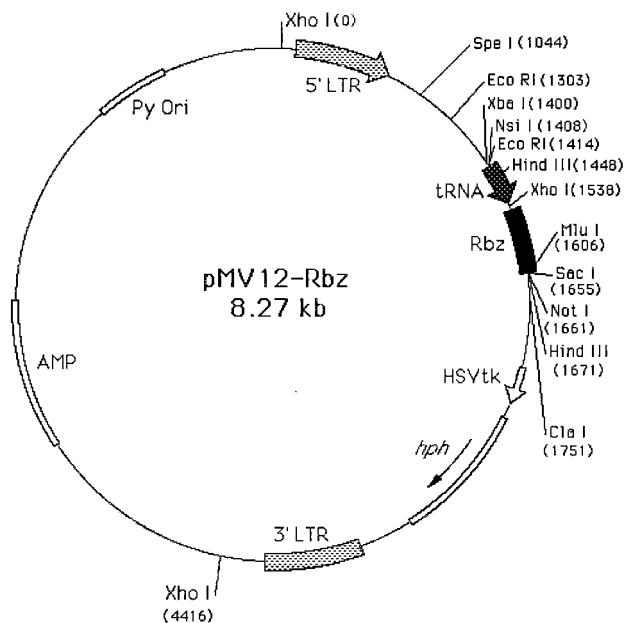


Figure 2. Construction of the ribozyme expression vector. The anti-TF ribozyme, along with its upstream tRNA val promoter, was inserted into the multiple cloning site of pMV12 with PCR by use of primers containing the appropriate restriction sequences. With the anti-TF ribozyme downstream of both the retroviral 5'LTR and the tRNA val promoter, ribozyme expression could be augmented to levels beyond that which could be obtained from the 5'LTR alone. The tRNA val/ribozyme sequence itself was obtained by insertion of a PCR fragment encoding the hairpin ribozyme into pBtV5-111, which contains the tRNA val coding and upstream regions.

fused at 1000 rpm at 4°C, filtered through a 0.2- μ m filter, and frozen at -80°C. VSMCs were exposed to 3 mL of viral supernatant with 8 μ g/mL polybrene. Forty-eight hours after infection, cells were split into hygromycin-containing medium. Clones were selected by ring cloning, expanded, and frozen at -80°C. Four clones were randomly selected and analyzed for TF expression and activity.

Measurement of TF mRNA

Total RNA was extracted from VSMCs by the guanidinium isothiocyanate/CsCl procedure.²⁵ Agarose gel electrophoresis, transfer to nitrocellulose, and hybridization to ³²P-labeled DNA were performed as described.²⁶ Prehybridization and hybridization were performed at 42°C. Final washes for all blots were in 0.1 $\times$ standard saline citrate (1 $\times$ =0.15 mol/L NaCl/0.015 mol/L sodium citrate, pH 7) and 0.1% SDS at 65°C for 1 hour. The full-length insert from mouse TF²⁷ was labeled by random oligomer priming to a specific activity of >108 cpm/ μ g and used at 2 $\times$ 10⁶ cpm/mL. As a control, filters were also hybridized with cDNA encoding the constitutively expressed smooth muscle myosin regulatory light chain.²⁸ All experiments shown in the figures represent RNA blots in which all lanes had equal loading as confirmed both by staining of 18S and 28S ribosomal RNA with

ethidium bromide and by hybridization with the regulatory light chain.

Determination of TF Activity by Monitoring Factor Xa Generation

TF activity was assessed by monitoring hydrolysis of factor X, as previously described.²⁹ Confluent monolayers of control and permanently transfected rat aortic VSMCs seeded on tissue culture plates were incubated for various times in incubation medium containing the indicated agents. Duplicate sets of chamber slides were stimulated in each single experiment to allow for simultaneous determination of total (cell lysates) and surface-associated TF. To measure factor Xa generation in cell lysates, monolayers were lysed by incubation with 15 mmol/L octyl- β -D-glycopyranoside for 15 minutes at 37°C. Factor VIIa (1 nmol/L) and factor X (150 nmol/L) were added sequentially. Aliquots of 40 μ L were taken every minute and added to 96-well plates, and the wells were filled with 100 μ L EDTA buffer (bicine buffer, pH 8.5: 25 mmol/L EDTA, 1 g/L BSA) to stop factor Xa production. Twenty-five microliters of a 15-mmol/L solution of D-isoleucine-L-proline-L-arginine-p-nitroanilide (IPR-pNA) was added to each well, and absorption at the 405-nm wavelength was measured in a kinetic plate reader (Tmax, Molecular Devices). The concentration of factor Xa was calculated from the slope of the absorption curve.

Statistics

Experiments were performed at least 3 times, and values were presented as the mean $\pm$ SD. A value of $P<0.05$ was considered significant. For TF activity experiments, statistical analysis was performed by 1-way ANOVA, and comparisons between groups were made by use of the Student-Newman-Keuls method.

Results

Specific Cleavage of TF mRNA by Hairpin Ribozyme In Vitro

The hairpin ribozyme was engineered to cleave a site in the TF mRNA at the GUC sequence between nts +808/809 relative to the transcription initiation site of the rat cDNA (Figure 3). The target sequence is AGAGU*GUCCUGG-GAGAAA, with cleavage occurring at *. The hairpin ribozyme was engineered so that it could connect by base pairs to the 2 sequences flanking the U*GUC to form helices 1 and 2. Helix 2 is fixed in length at 4 bases by the functional requirements of the hairpin ribozyme; however, the length of helix 1 could be varied. The length of helix 1 that provided optimal catalytic activity was experimentally determined to be 6 to 10 nt (Figure 4).

In vitro, this ribozyme cleaved the target substrate with high efficiency as determined by its kinetic parameters (Figures 5 and 6). Under very mild reaction conditions (37°C in 12 mmol/L MgCl₂/40 mmol/L Tris/2 mmol/L spermidine at pH 7.5), K_m was calculated as 88 nmol/L and k_{cat} as 2.6 min⁻¹. This gives an enzyme efficiency of $k_{cat}/K_m=0.029$

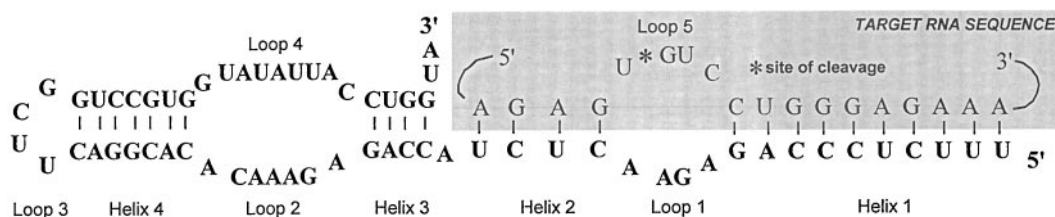


Figure 3. 2D model of the hairpin ribozyme used to cleave the TF substrate RNA (gray area). The ribozyme contains 4 helices and 4 loops. A fifth loop is formed within the substrate RNA on binding to the ribozyme, where cleavage occurs (*).

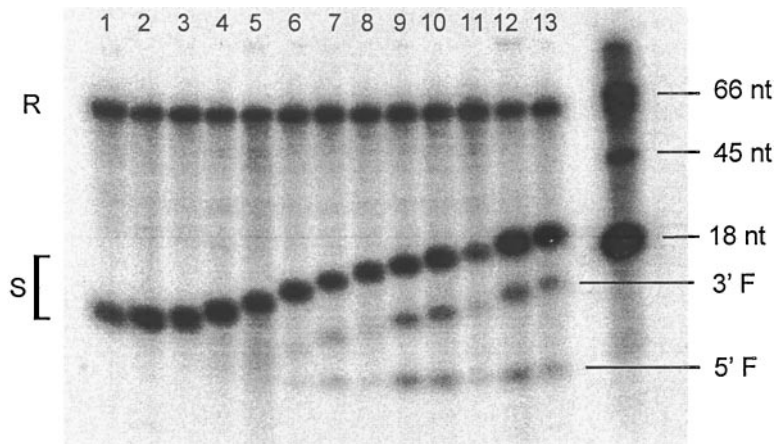


Figure 4. Anti-TF hairpin ribozyme helix 1 optimization. Substrates corresponding to varying helix-1 length were prepared by T7 transcription and then incubated with the 63-nt anti-TF ribozyme containing a full-length (10-nt) helix 1 domain. Substrates (10-, 9-, 8-, 6-, 5-, 4-, 3-, 2-, 1-, and 0-nt helix 1) are shown in lanes 13 through 3, respectively. Reactions were performed with 25 nmol/L ribozyme and 100 nmol/L substrate concentrations. Efficient cleavage occurs with a 6-bp helix 1, but not with shorter helices.

nmol/L $\cdot$ min⁻¹, compared with 0.07 for original native hairpin ribozyme. The catalytic efficiency, relative to the original tobacco ringspot ribozyme, is therefore 42%.

Effects of Ribozyme on TF mRNA Expression and TF Activity in a Permanently Transfected Cell Line

The practical application of ribozymes as therapeutic agents in vivo will depend on their ability to function in a complex cellular

environment. This requires stable expression of the ribozyme in the cell, specificity for the target RNA, accessibility of mRNA targets for cleavage, and lack of cytotoxicity of the endogenously expressed ribozyme. To address these issues, the sequences containing the anti-TF ribozyme were cloned into a retroviral expression vector under the control of the retroviral promoter.

To ascertain that TF mRNA levels were indeed diminished in VSMCs that were infected with the retroviral pMV12-Rbz, Northern blot analyses were performed using a TF cDNA insert (Figure

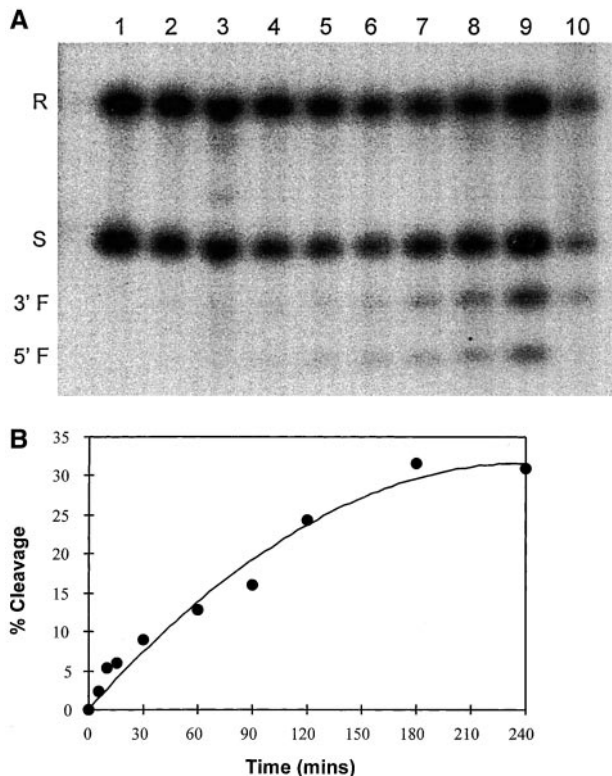


Figure 5. Time course of (n-1) ribozyme (R)-induced substrate (S) cleavage. A, Cleavage reactions were performed at 37°C with the ribozyme (R) at 50 nmol/L and the substrate (S) at 100 nmol/L for 0 minutes (lane 1), 5 minutes (lane 2), 10 minutes (lane 3), 15 minutes (lane 4), 30 minutes (lane 5), 60 minutes (lane 6), 90 minutes (lane 7), 120 minutes (lane 8), 180 minutes (lane 9), and 240 minutes (lane 10). 3' F indicates 3' cleavage fragment; 5' F, 5' cleavage fragment. B, Percent cleavage of S was plotted versus time and best-fitted to a second-order polynomial function that demonstrated linearity up to 60 minutes.

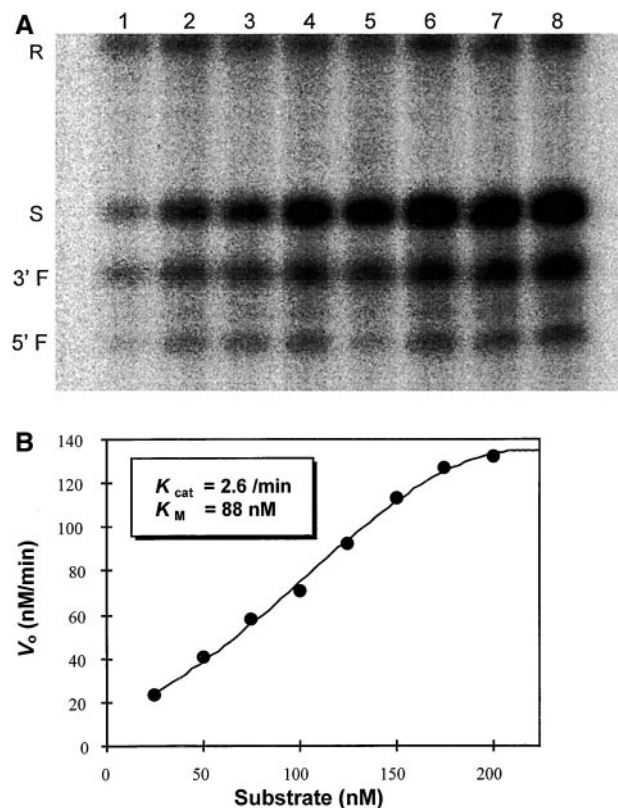


Figure 6. In vitro reaction kinetics. A, Cleavage reactions were performed at 37°C for 60 minutes with the ribozyme (R) at 50 nmol/L and the substrate (S) at increasing concentrations (25 to 200 nmol/L increments; lanes 1 to 8). 3' F indicates 3' cleavage fragment; 5' F, 5' cleavage fragment. B, The initial reaction velocity (V₀) derived from each lane was plotted versus S and best-fitted to a third-order polynomial function that yielded a V_{max} of 131 nmol/L $\cdot$ min⁻¹. From these data, K_M=88 nmol/L; k_{cat}=2.6 min⁻¹.

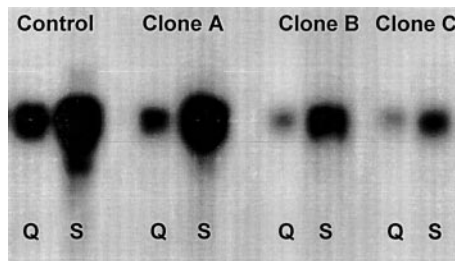


Figure 7. Northern blot analyses of control cells and cells transfected with the anti-TF ribozyme using a TF cDNA insert. Both during quiescence and after serum stimulation, the 3 clones (A, B, and C) transfected with the retroviral pMV12-Rbz demonstrated markedly reduced TF mRNA levels.

7). Expression of high levels of TF mRNA was detected in control clones that were not transfected with the retroviral pMV12-Rbz. In accord with previous reports, quiescent VSMCs expressed basal levels of TF mRNA that were substantially increased after stimulation with serum. In contrast, the 3 clones that were transfected with the retroviral pMV12-Rbz demonstrated markedly reduced TF mRNA levels both during quiescence and after serum stimulation. Cell lysates were also analyzed for total TF activity by monitoring factor Xa generation. Concordant with the mRNA data, VSMCs transfected with the retroviral pMV12-Rbz (clones A, B, and C) demonstrated a statistically significant reduction in TF activity (12.45 ± 7.34 , 8.95 ± 6.90 , and 3.85 ± 3.18 fmol/cm², respectively) compared with both untransfected control VSMCs (44.77 ± 2.05) and VSMCs transfected with the empty vector (28.78 ± 18.73) (Figure 8). Furthermore, there was a direct correlation between the extent of reduction in TF mRNA and the TF activity in each of the different cell lines.

Discussion

In normal arteries, there is very little or no TF in the intima or media, although it is abundant in the adventitia. It has therefore been proposed that the initiation of intravascular coagulation may require the "induced" expression of TF in the vessel wall.^{19,30} In animal models of balloon arterial injury, TF is rapidly induced in the smooth muscle cells

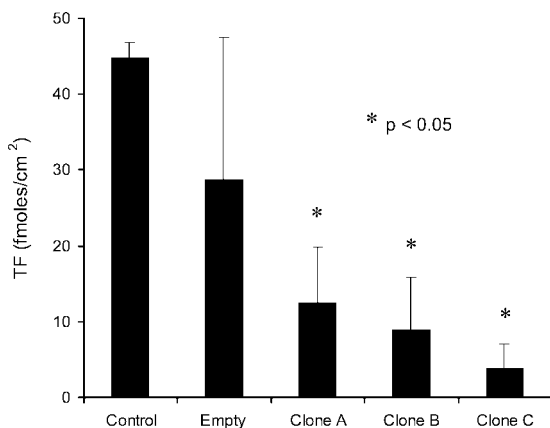


Figure 8. Analysis of cell lysates for total TF activity by monitoring factor Xa generation. Compared with lysates from untransfected control cells and cells transfected with the empty vector, lysates from cells transfected with the anti-TF ribozyme (clones A, B, and C) demonstrated a statistically significant reduction in TF activity.

(SMCs) of the media and also accumulates in the SMCs of the developing neointima.³¹ Induction of TF in SMCs appears to be primarily responsible for the prolonged procoagulant activity in rabbit balloon-injured aortas.³² In tissue culture, TF is rapidly induced in arterial SMCs by platelet-derived growth factor and thrombin. This induction appears to be caused primarily by an increase in TF transcription.³³

Because of the evidence that the initial response of normal arteries to injury is the rapid transcription of TF in medial SMCs, we thought that an approach that targeted gene expression of TF would be most effective. We chose the ribozyme technology because of the promise of this methodology based on its successful in vitro application in a variety of disease states, most notably HIV infection.^{17,34} Although conventional antisense RNAs and DNAs have been shown to impair gene expression, the stoichiometric nature of these molecules may prevent their usefulness in situations characterized by very high levels of mRNA induction, as seen with TF. Ribozymes possess the properties of antisense RNA with the additional ability of catalytic cleavage. As opposed to hammerhead ribozymes, hairpin ribozymes cleave at maximum rates at relatively low Mg²⁺ concentrations. Therefore, hairpin-based ribozymes may be particularly advantageous because the conditions required for their optimum function are nearly physiological.³⁴ In this study, we used a TF-specific hairpin ribozyme under the control of the tRNA val promoter and demonstrated a reduction in TF gene expression.

The practical application of ribozymes as therapeutic agents in vivo requires a high concentration of ribozyme so that substrate binding is not limiting. Endogenous delivery of ribozymes requires a vector containing the gene for the ribozyme behind a suitable promoter. The vector we chose was a retroviral vector, with subsequent transcription of the ribozyme achieved from a stably transfected cell line. We used the retroviral system because of its established efficacy in obtaining stable transfectants and its high transfection efficiency. To further augment the expression of the ribozyme in the cell beyond that which could be achieved by the viral 5'LTR promoter alone, an additional tRNA val promoter was placed upstream of the ribozyme gene. Although we did not actually measure ribozyme levels within the stably transfected cell lines, we did demonstrate a parallel decrease in TF mRNA by Northern blot analysis and a decrease in TF activity.

To the best of our knowledge, this is the first demonstration of an antithrombotic strategy based on ribozyme technology. With the development of local delivery catheters,³⁵ it is conceivable that a vessel wall could be pretreated with a ribozyme before injury with balloon dilatation or another percutaneous technique. Such pretreatment could conceivably reduce the thrombosis associated with vascular injury and possibly even inhibit the restenotic process. There are limitations to the applicability of this approach to animal and human models. First, the retroviral gene delivery vector we used clearly has several drawbacks. These include the low frequency of gene transfer, the relative lability of retroviral particles compared with other viruses, and the rapid in vivo inactivation of retroviral particles (presumably by the presence of complement in serum).³⁶ In addition, replication of target cells is necessary for proviral integration.³⁷ Thus, it is unknown whether stable high-level gene expression in vivo can be achieved with retrovirally transduced cells. Further-

more, the long-term safety of retroviral vectors remains unknown, because integration into chromosomal DNA could potentially result in dysregulation of cellular genes and/or insertional mutagenesis.^{36,38} In vivo experiments in humans in the context of angioplasty would probably require a nonintegrating viral vector such as adenovirus.³⁹ Adenoviruses effectively infect both dividing and nondividing mammalian cells and remain extrachromosomal.^{36,40} Such a vector would give transient expression, which would most likely suffice, given the relatively short-lived surface expression of TF after vessel injury. Another limitation of our study is our inability, with current technology, to completely eliminate the TF mRNA. Even in tissue culture, we were unable to fully inhibit TF mRNA induction. Transfection efficiency in an atherosclerotic human model may be even lower.⁴¹ It may be that this strategy would result in insufficient knockout of TF in such an in vivo model. With the ongoing advances in vector technology, however, it is anticipated that vectors and strategies will be available within the foreseeable future to give very high levels of in vivo expression.

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