Inhibition of MMP-2 gene expression with small interfering RNA in rabbit vascular smooth muscle cells

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Small interfering RNA (siRNA) specifically suppresses gene expression in mammalian cells in vitro (11). This inhibitory effect results from selective degradation of target mRNA (24). In addition, recent studies showing that siRNA can be used for inhibition of gene expression in vivo set the path for therapeutic applications, either locally (7) or systemically (36). Molecular therapies have been tested in animal models of human diseases and in clinical trials for more than a decade. Successful modification of arterial biology can be achieved using overexpression of therapeutic genes (38) or antisense oligonucleotides (3). However, clinical application of these approaches is limited by the low efficiency of vascular cell transfection in vivo, which can be overcome only by using potentially hazardous viral vectors (38), and by concerns about the stability and specificity of antisense technologies (3). Theoretically, the small size (21–23 bp) of siRNA molecules, together with the absence of a requirement for nuclear translocation of siRNA, suggests that transfection efficiency of siRNA in vascular cells is likely to be superior to that of antisense oligonucleotides (3) or plasmid DNA or viral vectors (4).

Matrix metalloproteinase (MMP)-2 is an interesting target for siRNA-based arterial therapy, since MMP-2 is constitutively expressed in vascular smooth muscle cells (VSMCs), and increased MMP-2 expression and activity have been associated with progression and rupture of atherosclerotic plaques, restenosis (17), and aneurysm progression (12). MMP-2 is secreted as an inactive zymogen (pro-MMP-2), which, once activated, is able to hydrolyze numerous extracellular matrix components and, thus, facilitate VSMC migration and fibrous cap degradation (18).

The aim of the present study was to investigate the effects of MMP-2 siRNA on rabbit VSMCs in vitro and ex vivo. We first tested the ability of MMP-2 siRNA to inhibit MMP-2 gene expression, MMP-2 activity, and VSMC migration in vitro. We then explored the feasibility of ex vivo endoluminal application of MMP-2 siRNA in hypercholesterolemic rabbit balloon-injured carotid arteries. We provide evidence that the internalization of MMP-2 siRNA in intimal cells leads to inhibition of MMP-2 activity. Our results indicate that local delivery of siRNA in the arterial wall may be a new approach for the study and treatment of arterial diseases.

MATERIALS AND METHODS

Cell culture. A rabbit VSMC line (Rb-1) (25) was cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin; GIBCO-BRL). At 24 h before transfection, VSMCs were plated in 24-well plates at a density of 5 × 10^4 cells per well and cultured at 37°C in 95% air-5% CO_2.

siRNA preparation. Rabbit MMP-2 siRNA and scramble siRNA sequences were determined with the Ambion algorithm (http://www.ambion.com/techlib/misc/siRNA_finder.html) and blasted against the GenBank databases (accession no. D63579). MMP-2 siRNA [5'-UCU-UCG-UAG-UUG-GUU-G99-3' (sense) and 3'-99A-GUA-GCA-GCA-UCA-ACC-AAC-5' (antisense)] and scramble siRNA [5'-AAG-CAA-CCU-AAG-UAG-GUC-GAA-GAA-3' (sense) and 3'-99U-GUG-GUG-GAU-GCC-GUA-5' (antisense)] were synthesized and tagged with tetramethylrhodamine (TAMRA) or FITC by Eurogentec. siRNA stock solutions were stored at −20°C.

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Transfection of VSMCs in vitro. VSMCs (50–70% confluence) were washed with PBS; then 50 nmol/l TAMRA-tagged MMP-2 siRNA and 1 μl of cationic lipid jet SI-ENDO (Eurogentec) in 600 μl of culture medium were added for 24 h at 37°C according to the manufacturer’s protocol. A second group of VSMCs were transfected with 50 nmol/l TAMRA-tagged scramble siRNA according to the same protocol. Nontransfected VSMCs (no siRNA and no jet SI-ENDO) were used as control. For each study of flow cytometry, confocal microscopy, RT-PCR and gelatin zymography, VSMC migration, and VSMC viability, three transfection experiments were performed, and each transfection condition was repeated in six wells.

Assessment of siRNA internalization using flow cytometry and confocal microscopy. At 24 h before transfection, 5 x 10^4 VSMCs were seeded in 24-well plates for fluorescence-activated cell sorting (FACS; Epics XL-MCL, Beckman Coulter), and 2 x 10^5 VSMCs were seeded in Lab-Tek cover glass four-chamber slides for confocal laser scanning microscopy (model LSM 510, Carl Zeiss). MMP-2 siRNA and scramble siRNA transfections were performed as described above. TAMRA- and FITC-tagged siRNA were used in confocal and flow cytometry studies, respectively. FACS data were analyzed using Cell Quest software (Beckton Dickinson). For confocal microscopy analysis, siRNA-transfected VSMCs were fixed in 4% paraformaldehyde-PBS solution for 15 min and incubated with the fluorescent DNA-binding dye YOYO-1 iodide (Invitrogen) following the manufacturer’s protocol. Images were analyzed with LSM 5 Image Browser software.

Real-time RT-PCR. Transfection of VSMCs was performed as described above. Total RNA was extracted from VSMCs 48 h after transfection using TRIzol (Invitrogen) (14). RNA was reverse transcribed with the Thermoscript RT-PCR system (Invitrogen) and amplified by PCR. Real-time RT-PCR assays were performed using a Light-Cycler with the FastStart DNA MasterPLUS SYBR Green I kit (Roche). All samples were run in parallel during the same PCR, allowing for head-to-head comparisons between MMP-2 siRNA, scramble siRNA, and no siRNA groups. MMP-2, tissue inhibitor of metalloproteinase (TIMP)-1, and TIMP-2 mRNA levels were expressed in arbitrary units after normalization to 18S rRNA levels (15).

The following primers were used: 5'-CATGTCATATTGGGGGGAAC-3' (forward) and 3'-GGGTAAATGTGGATGTGCTTCTT-5' (reverse) for MMP-2, 5'-CCACCAAGATGTTCAAAG-3' (forward) and 3'-GTAACGAAACCTGTCTG-5' (reverse) for TIMP-1, 5'-GCGGAGAAGGAGGTGGACT-3' (forward) and 3'-TAGGGGACGATGTAGAGGA-5' (reverse) for TIMP-2, and 5'-TTTGCACGCAACCATCTCA-3' (forward) and 3'-GTCGGTGCCCTCTA-ACTCTTAT-5' (reverse) for 18S.

Gelatin zymography and ELISA. VSMCs were transfected as described above. After 24 h of transfection, VSMCs were incubated in serum-free medium at 37°C for 24, 48, and 72 h. VSMC-conditioned media were collected and stored at −20°C. Gelatinolytic activities were measured in the VSMC-conditioned medium samples. Denstometric analysis of scanned gelatinolytic bands was performed with Scion Image and NIH (release Beta 3b) software (National Institutes of Health).
of Health). ELISA (Amersham) of TIMP-1 and TIMP-2 levels was performed in the VSMC-conditioned medium samples.

VSMC wound migration assay. The in vitro migratory activity of VSMCs was measured using a wound migration assay (13). An injury line was created with a single scratch at the center of a VSMC monolayer (50–70% confluence) using a sterile 1.15-mm-diameter pipette tip. Then VSMCs were transfected as described above. VSMCs were photographed with phase contrast microscopy (model CK40, Olympus; ×10 objective) immediately thereafter and at 24, 48 and 72 h after transfection (15 images at each time point). Distance between cells at both sides of the wound was measured for five pairs of cells per image. Cell migration was expressed as a percentage of initial wound width.

Assessment of cell adherence and viability. Viable adherent VSMCs were quantified after transfection with MMP-2 siRNA, scramble siRNA, and no siRNA using a (3,4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazolium bromide (MTT) test (Sigma), as previously described (37). Enzymatic activity of lactate dehydrogenase (14) was quantified in the VSMC-conditioned medium using a colorimetric assay (Promega). The apoptosis of VSMCs was evaluated by TdT-mediated dUTP nick end labeling (Sigma) on MMP-2 siRNA-transfected, scramble siRNA-transfected, and nontransfected VSMCs. Five different fields per experimental condition were chosen to quantify the percentage of apoptotic cells.

Transfection of rabbit injured carotid arteries ex vivo. The animal protocol was approved by the Bichat University Institutional Animal Care and Use Committee. Male New Zealand White rabbits (n = 15, 3.5–4.0 kg body wt) were fed a 0.3% cholesterol diet for 30 days. Before angioplasty, animals were premedicated with intramuscular 1% acepromazine and then anesthetized with intravenous 0.1% pentobarbital sodium. Right carotid artery angioplasty was performed with a 3.5-mm-diameter 30-mm-long balloon-catheter introduced percutaneously via a 5-Fr femoral sheath and advanced into the right carotid artery under fluoroscopic guidance (BV Endura, Philips) (6). The balloon was inflated three times at 10 atm, and then the skin wound was repaired. The animals were euthanized by intravenous pentobarbital sodium overdose 2 wk after angioplasty. The carotid arteries were harvested, flushed with saline, and cleaned of adipose tissue. Transfections were performed with a final concentration of 5 μmol/l TAMRA-tagged MMP-2 siRNA (n = 5 arteries), scramble siRNA (n = 5 arteries), or no siRNA (n = 5 arteries) in the presence of 66 μl of jet SI-ENDO in a total culture medium volume of 340 μl. Transfection solutions were injected ex vivo through an indwelling catheter in 30-mm-long arterial segments sealed at both ends by sutures. After 24 h of incubation at 37°C, arterial segments were rinsed with PBS and cut into 4-mm-long rings. Five rings per treatment group were frozen in liquid nitrogen-cooled isopentane, cut into 7-μm-thick cross sections with a cryostat (model CM 1900, Leica), and incubated with 1 mg/ml 4,6-diamidino-2-phenylindole (DAPI) solution for 15 min (Sigma). Stained arteries were visualized using fluorescence microscopy. Five rings per treatment group were incubated for 72 h in 200 μl of serum-free medium at 37°C in 95% air-5% CO₂. The conditioned media were collected each 24 h and stored at −20°C until gelatinolytic activities were measured (see above). Results are expressed in densitometric units (DU)/μl of conditioned medium. *P < 0.0005 vs. scramble siRNA and no siRNA.

Fig. 2. Quantification of MMP-2 mRNA levels in siRNA-transfected VSMCs using real-time RT-PCR. VSMCs were transfected with MMP-2 siRNA or scramble siRNA or not transfected. Note significant reduction of MMP-2, but not tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, mRNA levels 48 h after transfection with MMP-2 siRNA. Values (means ± SD) are presented as ratio of MMP-2, TIMP-1, and TIMP-2 mRNA to 18S rRNA. *P < 0.005 vs. scramble siRNA and no siRNA.

Fig. 3. Gelatinolytic activities in conditioned media of siRNA-transfected VSMCs. VSMCs were transfected with MMP-2 siRNA or scramble siRNA or not transfected. A: representative gel showing reduced pro-MMP-2 activity (70-kDa band) 24, 48, and 72 h after transfection with MMP-2 siRNA (vs. scramble siRNA-transfected and nontransfected VSMCs). B: pro-MMP-2 activity in each treatment group over time. Values (means ± SD) are expressed in densitometric units (DU)/μl of conditioned medium. *P < 0.0005 vs. scramble siRNA and no siRNA.
in 4% paraformaldehyde and embedded in paraffin for histology and immunohistochemistry studies.

**Histology and immunohistochemistry.** Adjacent 7-μm-thick arterial cross sections were cut and stained with hematoxylin-phloxin-safran, orcein, or Masson’s trichrome. Additional cross sections were immunostained with monoclonal antibodies against Ki-67, a nuclear proliferation marker (1:50 dilution; Immunotech), RAM-11, a marker of rabbit macrophage cytoplasm (1:50 dilution; Dako, Trappes, France), and smooth muscle α-actin (1:50 dilution; Dako), as described elsewhere (9).

**Statistical analysis.** Values are means ± SD. A two-way ANOVA was used to compare the overall effects of treatment and time followed by post hoc Fisher’s test for intergroup comparisons when appropriate (StatView 4.5). Differences were considered statistically significant at *P* < 0.05.

**RESULTS**

**Cellular uptake of siRNA in vitro.** At 24 h after transfection with fluorescent siRNA, FACS analysis indicated that 77 ± 9% and 84 ± 4% of VSMCs contained fluorescent MMP-2 siRNA and scramble siRNA, respectively (Fig. 1). Similarly, confocal microscopy studies performed 24 h after transfection with TAMRA-tagged siRNA indicated significant internalization of siRNA in ~80% of VSMCs with a cytoplasmic localization of siRNA (Fig. 1). When transfections were performed without jet SI-ENDO, <2% of VSMCs showed siRNA internalization (data not shown).

Cell adhesion by MTT (0.33 ± 0.03 vs. 0.32 ± 0.05 vs. 0.33 ± 0.06, *P* = 0.45) and viability by lactate dehydrogenase (0.23 ± 0.06 vs. 0.23 ± 0.09 vs. 0.27 ± 0.09, *P* = 0.11) measurements were not different in MMP-2 siRNA-transfected vs. scramble siRNA-transfected and nontransfected VSMCs up to 72 h.

TdT-mediated dUTP nick end labeling showed no significant difference in cell apoptosis between MMP-2 siRNA-transfected (10 ± 4%) vs. scramble siRNA-transfected (11 ± 9%) and nontransfected VSMCs (7 ± 4%) at 48 h (*P* = 0.61) (data not shown).

**MMP2-siRNA inhibits MMP2 expression and activity in VSMCs in vitro.** At 2 days after in vitro siRNA transfection, real-time RT-PCR analysis (Fig. 2) demonstrated a significant reduction of MMP-2 mRNA levels in MMP-2 siRNA-transfected vs. scramble siRNA-transfected and nontransfected VSMCs.

![Fig. 4. Effect of siRNA transfection on VSMC migration.](#) A monolayer of VSMCs at 70–80% confluence was scraped with a sterile pipette tip to create an injury line. VSMCs were transfected with MMP-2 siRNA or scramble siRNA or not transfected. A: representative phase contrast images before transfection and 24, 48, and 72 h after transfection. At each time point after transfection, distance between VSMCs on both sides of the wound (white lines) is greater in MMP-2 siRNA-transfected than in scramble siRNA-transfected and nontransfected VSMCs. B: VSMC migration in each treatment group over time. Values are means ± SD. *P* < 0.0005 vs. scramble siRNA and no siRNA.
VSMCs ($P < 0.005$), corresponding to a $44 \pm 19\%$ reduction in MMP-2 mRNA levels in MMP-2 siRNA-transfected vs. scramble siRNA-transfected VSMCs ($P = 0.003$). There were no significant differences in TIMP-1 and TIMP-2 mRNA levels between MMP-2 siRNA-transfected vs. scramble siRNA-transfected and nontransfected VSMCs at 48 h ($P = 0.69$ and $P = 0.77$, respectively; Fig. 2).

Gelatin zymography of VSMC-conditioned media (Fig. 3) showed a single 70-kDa band corresponding to rabbit pro-MMP-2 (10). Pro-MMP-2 activity was markedly reduced in MMP-2 siRNA-transfected vs. scramble siRNA-transfected and nontransfected VSMCs $[P < 0.0005, P < 0.0001$ (time effect), $P < 0.005$ (interaction)]. Specifically, there was a $26 \pm 12\%$, $43 \pm 14\%$, and $51 \pm 7\%$ reduction in pro-MMP-2 activity in MMP-2 siRNA- vs. scramble siRNA-transfected VSMCs at 24, 48, and 72 h, respectively (Fig. 3).

ELISA of VSMC-conditioned media at 48 h showed no significant difference in TIMP-2 concentration between MMP-
2-siRNA-transfected (127 ± 22 ng/ml) vs. scramble siRNA-transfected (137 ± 59 ng/ml) and nontransfected (186 ± 44 ng/ml) VSMCs (P = 0.46).

**MMP2-siRNA inhibits VSMC migration in vitro.** VSMC migration via a wound migration assay was significantly reduced in MMP-2 siRNA-transfected vs. scramble siRNA-transfected and nontransfected VSMCs [P < 0.0001, P < 0.0001 (time effect), P < 0.0001 (interaction); Fig. 4]. There was a 57 ± 10%, 36 ± 14%, and 18 ± 8% reduction in VSMC migration in MMP-2 siRNA-transfected vs. scramble siRNA-transfected VSMCs at 24, 48, and 72 h, respectively.

**Histology and immunohistochemistry of ex vivo siRNA-transfected rabbit carotid arteries.** A concentric neointima (Fig. 5, D–F) developed in balloon-injured arteries in all hypercholesterolemic animals. At this early time point after arterial injury, no endothelial cell was visible. The neointima consisted essentially of VSMCs, identified by positive α-actin staining (Fig. 5G). Proliferative activity, indicated by Ki-67 nuclear stain, predominated in the superficial layers of the neointima and was only occasionally observed in the media (Fig. 5H). Few RAM-11-positive macrophages were present in the superficial layers of the media (Fig. 5I). Analysis of an adjacent cross section suggests that transfected cells in the superficial layers of the neointima (Fig. 5, A–C) are of smooth muscle cell origin.

Fluorescence microscopy analysis of arterial cross sections was performed 24 h after ex vivo transfection (Fig. 6). Red fluorescence was observed in the superficial layers of the neointima (Fig. 6, I and N; Fig. 5, A and C), as well as in rare adventitial cells, in all (10 of 10) arteries transfected with TAMRA-tagged MMP-2 siRNA but in none (0 of 5) of the nontransfected arteries. Analysis of merged fields (DAPI and TAMRA) also suggested a cytoplasmic localization of TAMRA-tagged siRNA (Fig. 6, J and O).

**Decreased MMP2 activity after MMP2-siRNA transfection ex vivo.** Gelatin zymography of the conditioned media of balloon-injured and ex vivo transfected arteries showed 70-, 60-, and 98-kDa gelatinolytic bands corresponding to pro-MMP-2, MMP-2, and pro-MMP-9 (10), respectively (Fig. 7A). There was a marked decrease of pro-MMP-2 activity in MMP-2 siRNA-transfected vs. scramble siRNA-transfected and nontransfected arteries [P < 0.0001, P < 0.0001 (time effect), P = 0.56 (interaction)]. Specifically, there was a 32 ± 9%, 27 ± 7%, and 50 ± 32% reduction in pro-MMP-2 activity in MMP-2 siRNA-transfected vs. scramble siRNA-transfected arteries at 24, 48, and 72 h, respectively (Fig. 7B). Similarly, there was a significant reduction of MMP-2 activity in MMP-2 siRNA-transfected vs. scramble siRNA-transfected and nontransfected arteries [P < 0.0005, P < 0.0001 (time effect), P < 0.05 (interaction)], corresponding to 50 ± 19%, 34 ± 14%, and 42 ± 15% reduction in MMP-2 activity in MMP-2 siRNA-transfected vs. scramble siRNA-transfected arteries at 24, 48, and 72 h, respectively (Fig. 7C). In contrast, there was no significant difference in pro-MMP-9 activity in MMP-2 siRNA-transfected vs. scramble siRNA-transfected and nontransfected arteries up to 72 h of incubation (P = 0.91; Fig. 7D).

**Fig. 6.** Ex vivo transfection of rabbit carotid arteries with siRNA. Balloon-injured carotid arteries of hypercholesterolemic rabbits were transfected ex vivo with MMP-2 siRNA or scramble siRNA or not transfected. Cross sections were obtained 24 h after transfection from nontransfected (A–E) or TAMRA-tagged MMP-2 siRNA-transfected (F–O) carotid arteries. The same sections were observed using phase microscopy (A, F, and K) or fluorescence microscopy (B, G, and L (green fluorescence of elastic tissue), C, H, and M (blue fluorescence of DAPI nuclear staining), D, I, and N (red fluorescence of TAMRA-tagged siRNAs), E, J, and O (merge of fluorochromes)]. Magnification ×20 (A–J) and ×40 (K–O). Note TAMRA-tagged siRNA in superficial layers of neointimal cells (white arrow), as well as in some adventitial cells, in I (white arrowhead) and N.
DISCUSSION

Our data demonstrate that VSMCs can be efficiently transfected with synthetic siRNA and that siRNA targeted at MMP-2 markedly inhibits MMP-2 gene expression, MMP-2 activity, and VSMC migration in vitro, with no apparent toxic (i.e., apoptotic) effect. In addition, we showed that siRNA can be delivered ex vivo in the arterial wall and inhibit MMP-2 activity.

RNA interference is a phenomenon by which double-stranded RNA molecules are cut into siRNA duplexes, which recognize and induce the selective degradation of complementary mRNA, eventually leading to inhibition of endogenous gene expression. Even though RNA interference is thought to occur spontaneously in plants, nematodes, and mammals, a considerable amount of evidence suggests that it can be replicated in cultured cells by transfection of synthetic siRNA or siRNA-expressing vectors (20), thus allowing the use of RNA interference in gene expression studies (22, 29). The prospect of using siRNA therapeutically stems from recent studies demonstrating that intravenous injections of naked siRNA in mice result in gene silencing, which can be assessed systemically (36). Alternatively, other studies focusing on local delivery of siRNA into solid tumors showed convincing therapeutic effects in vivo (27, 28). Interestingly, in a recent study, intravenous injection of siRNA lipoplexes (31) in mice resulted in significant endothelial uptake of formulated siRNA and potent inhibition of angiogenesis and tumor growth (32). These results suggest that siRNA may be used to study endothelial gene functions in animal models of atherosclerosis. However, the therapeutic value of knocking down systemically endogenous genes in endothelial cells is doubtful, given the protective effects of the endothelium against atherosclerosis (30). In addition, the intravenous application of siRNA may lead to undesirable accumulation of siRNA in nontarget organs (33).

In contrast, local strategies aimed at inhibiting VSMC activation successfully prevented in-stent restenosis, vascular graft occlusion, and transplant vasculopathy in experimental models (8), and inhibition of VSMC proliferation and migration by drug-eluting stents has become the gold standard for prevention of restenosis after percutaneous coronary interventions in patients (34). Thus VSMCs are potential targets for newly designed siRNA-based therapeutic strategies against vascular diseases. Several studies convincingly demonstrated siRNA-mediated inhibition of gene expression in cultured VSMCs (16). However, in vivo delivery of siRNA in the arterial wall remains a technical challenge. Banno et al. (2) reported some medial transport of periadventitiously delivered siRNA in a murine model of jugular vein-to-carotid artery interposition vein grafts, but this approach requires low-flow conditions and surgical exposure of the target artery. More recently, Corteling et al. (5) successfully transfected the endoluminal part of deendothelialized rat cerebral arteries with anti-RhoA siRNA, which resulted in significant inhibition of gene expression. In these ex vivo studies, however, reverse permeabilization of the

Fig. 7. MMP-2 activities in conditioned media of siRNA-transfected rabbit carotid arteries. Balloon-injured carotid arteries of hypercholesterolemic rabbits were transfected ex vivo with MMP-2 siRNA or scramble siRNA or not transfected. A: representative gel showing reduced pro-MMP-2 (70 kDa), MMP-2 (60 kDa), but not pro-MMP-9 (98 kDa), activity 24, 48, and 72 h after transfection with MMP-2 siRNA (vs. scramble siRNA and no siRNA) in conditioned media of explanted arteries. B–D: pro-MMP-2, MMP-2, and pro-MMP-9 activities in each treatment group over time. Values are means ± SD. *P < 0.01 vs. scramble siRNA and no siRNA.
arterial wall was used to facilitate siRNA uptake by vascular cells, an approach that may not be used in vivo.

In the present study, we took advantage of the constitutive expression of MMP-2 by VSMCs to study the ability of synthetic siRNA to transfect and inhibit gene expression in VSMCs in vitro and ex vivo. Our in vitro data demonstrate that transfection efficiencies in the range of 80% can be achieved when siRNAs are mixed with cationic lipid vectors, whereas virtually no transfection was detected with naked siRNA. VSMCs transfected with MMP-2 siRNA showed reduced MMP-2 mRNA levels and pro-MMP-2 activity, resulting in ~50% inhibition of VSMC migration 24 h after transfection, without apparent cell toxicity. In contrast, MMP-9, TIMP-1, and TIMP-2 expression did not differ between MMP-2 siRNA-transfected, scramble siRNA-transfected, and nontransfected VSMCs, suggesting that the inhibitory effect of MMP-2 siRNA was gene specific.

To assess the feasibility of siRNA-mediated transfection of VSMCs in the whole arterial wall, we used an ex vivo model in which the siRNA-jet SI-ENDO lipoplex is incubated in contact with the luminal surface of rabbit carotid arteries. Since preliminary experiments indicated that siRNAs do not cross the internal elastic laminae (data not shown), a highly cellular lesion was induced by balloon angioplasty and a high-cholesterol diet prior to transfection, so that target cells were directly exposed to siRNA. No attempt to chemically permeabilize the arterial wall was made. In all arteries exposed to the TAMRA-tagged siRNAs, a circumferential red fluorescence indicated that an siRNA deposit had formed at the arterial wall-lumen interface. We found that arterial siRNA uptake was strictly limited to neointimal cells (no medial transfection), as well as to rare adventitial cells (probably through the vasa vasorum). The perinuclear TAMRA fluorescence suggested an intracellular localization of siRNA. Immunostaining studies revealed the presence of VSMCs, but not macrophages, in the TAMRA-positive superficial layers of the neointima. These observations favor local MMP-2 siRNA transfection of neointimal VSMCs. Moreover, our observation that pro-MMP-2 and MMP-2 activities are significantly reduced after MMP-2 siRNA transfection, scramble siRNA-transfected, and nontransfected VSMCs, suggesting that the inhibitory effect of MMP-2 siRNA was gene specific.

Several issues remain to be addressed before therapeutic application of siRNA technology in the field of cardiovascular diseases can be considered. 1) It is unclear whether the high transfection efficiencies achieved in our ex vivo model can be replicated in vivo. In vivo applications will require significant progress in bioengineering and vectorology, so that high transfection efficiencies can be achieved in the arterial wall using a percutaneous approach without flow interruption. Newly designed bioactive stents have been used for arterial delivery of genetic materials (21, 26) and represent an attractive option for slow release of siRNA in vascular cells in vivo. Whether the cationic lipid vector used in the present study will lend itself to such an approach is unknown. Alternatively, siRNA-expressing viral vectors that may improve transfection efficiency in vivo have been designed (23). 2) Therapeutic use of RNA interference may rely not only on the use of synthetic 21- to 23-bp siRNAs, but also on potentially more efficient modified siRNAs (36), longer double-stranded RNA molecules (19), or short hairpin RNAs (35), which have not been tested in models of local arterial transfection. 3) Even though we demonstrated that ex vivo effects of siRNA transfection lasted ≥72 h, it is not possible to extrapolate these ex vivo data to an in vivo setting, in which several factors, including blood flow and the presence of inflammatory cells, may reduce the window of efficacy of siRNAs. 4) The issue of gene target screening must be further explored. Genes involved in VSMC proliferation and migration, leukocyte and platelet recruitment, inflammation and oxidative stress, apoptosis, extracellular matrix degradation, or thrombus formation are all, separately or in association, potential targets for siRNA-based local treatments of atherosclerotic plaques. Our study was not designed to test the therapeutic effects of siRNA-mediated inhibition of MMP-2. However, the known involvement of MMP-2 in various complications of atherosclerosis, including plaque destabilization, restenosis, and aneurysm progression and rupture (13), suggests that MMP-2 inhibition may be of therapeutic value.

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