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# Antisense oligonucleotides to stromelysin mRNA inhibit injury-induced proliferation of arterial smooth muscle cells

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**Summary.** Smooth muscle cell migration and proliferation are important events in the formation of intimal lesions associated with atherosclerosis and restenosis following balloon angioplasty. To make this possible, the smooth muscle cell has to change from a contractile to an activated repair cell with capacity to synthesize DNA and extracellular matrix components. There is now considerable evidence that the extracellular matrix has important functions in modulating the phenotypic properties of smooth muscle cells, but less is known about the role of the matrix metalloproteinases. The present study investigates the role of stromelysin in the modulation of rat aortic smooth muscle cell morphology and function following mechanical injury in vitro and in vivo. Antisense mRNA oligonucleotides were used to investigate the role of stromelysin expression in injury-induced phenotypic modulation and the subsequent migration and proliferation of vascular smooth muscle cells. Cultured rat aortic smooth muscle cells and balloon-injured rat carotid arteries were used as experimental models. Light- and electron microscopy were used to follow changes in smooth muscle cell phenotype and lesion formation and incorporation of $^{3}$H-thymidine to detect DNA synthesis. Injury-induced DNA synthesis and migration in vitro were inhibited by 72% and 36%, respectively, by adding stromelysin antisense oligonucleotides to the medium prior to injury. In primary cultures, 67% of the smooth muscle cells treated with stromelysin antisense were retained in a contractile phenotype as judged by analysis of cell fine structure, compared to 15% untreated cells and 40% in cells treated with mismatched oligonucleotides. Examination of the carotid arteries one week after balloon injury likewise demonstrated a larger fraction of contractile cells in the inner parts of the media in vessels treated with antisense oligonucleotides compared to those treated with mismatched oligonucleotides. The neointima was also distinctly thinner in antisense-treated than in mismatched-treated and control arteries at this time. These findings indicate that stromelysin mRNA antisense oligonucleotides inhibited phenotypic modulation of rat arterial smooth muscle cells and so caused a decrease in migration and proliferation and neointima formation in response to vessel wall injury.

**Key words:** Balloon-injury, Stromelysin, Phenotypic modulation, Restenosis, Rat, Smooth muscle cells

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## Introduction

Vascular smooth muscle cells (SMC) are a major component of the intimal lesions associated with atherosclerosis and restenosis following balloon angioplasty (McBride et al., 1988; Liu et al., 1989; Ross, 1993). The subcellular reorganization of SMC in response to mechanical injury has been studied in a rat model of balloon injury to the carotid artery (Thyberg, 1998). Before injury all SMC in the media are in a contractile phenotype with a cytoplasm dominated by myofilaments. After injury the SMC in the inner layer of the media change into a synthetic phenotype by loss of myofilaments and formation of a large endoplasmic reticulum and Golgi complex. Some of these cells then migrate to the intima where they proliferate and secrete extracellular matrix components.

In the media, the SMC are surrounded by a basement membrane and embedded in an interstitial matrix. The basement membrane consists of laminin, collagen type IV, entactin and proteoglycans while the interstitial matrix is composed of collagen type I and III, elastin, proteoglycans, fibronectin, and thrombospondin (Wight, 1996). In response to an injury of the vessel wall these barriers must be reorganized in order to allow migration of SMC from the media to the intima. The mechanisms responsible for activation of phenotypic modulation, migration and proliferation of SMC remain to be fully

understood but growth factors and cytokines released by injured cells are believed to play important roles in these events (Ross, 1993; Libby and Ross, 1996). Furthermore, there is now considerable evidence that the extracellular matrix has essential functions in modulating various aspects of SMC behaviour (Carey, 1991; Assoian and Marcantonio, 1996; Thyberg, 1996).

Activation of matrix metalloproteinases (MMPs) represents an aspect of the SMC response to injury. The MMPs fall into three subclasses: 1) the collagenases which specifically degrade connective tissue collagens, 2) the gelatinases which degrade gelatin, basement membrane collagen and elastin, and 3) the stromelysins which have a broad substrate specificity, degrading proteoglycans, laminin, fibronectin, gelatin and basement membrane collagen (Birkedal-Hansen, 1995). Collagenase and stromelysin are activated by plasmin which is generated from plasminogen (He et al., 1989). There is increasing evidence that SMC produce MMP in response to injury of the vessel wall (Clowes et al., 1990; Jackson and Michael, 1993; Reidy et al., 1996; Aoyagi et al., 1998) and treatment with synthetic MMP inhibitors affects the wound-healing response in the injured vessel (Bendeck et al., 1996; Zempo et al., 1996). Stromelysin mRNA have been demonstrated in human atherosclerotic plaques and in rabbit carotid arteries after balloon denudation (Henney et al., 1991; Galis et al., 1995; Aoyagi et al., 1998). The expression is associated with SMC in the intima but not in the media, which may reflect local tissue rearrangement to allow growth and formation of the lesion. The production, secretion and activation of the metalloproteinases have also been studied extensively in cultured SMC. The results from these studies indicate that collagenase and stromelysin are synthesized by human SMC in response to TNF-α or IL-1 (Galis et al., 1994) and tissue collagenase in response to PDGF (Yanagi et al., 1991). Mechanical injury of cultured rabbit smooth muscle-derived Rb-1 cells is associated with induction of mRNAs for collagenase and stromelysin (James et al., 1993), indicating that mechanical factors may regulate these genes. We have recently demonstrated a tenfold increase in stromelysin mRNA transcripts two days after seeding rat aortic SMC in primary culture (Hultgårdh-Nilsson et al., 1997). In addition, Webb and coworkers demonstrated detectable levels of stromelysin as early as two hours after injury of the rat carotid artery (Webb et al., 1997). These observations suggest that stromelysin may be involved in regulating the phenotypic state of the SMC, for example by controlling the organization of the basement membrane. In favour of this hypothesis, it has been demonstrated that migration of cultured SMC through a basement membrane barrier is collagenase-dependent and is inhibited by cellular differentiation (Pauly et al., 1994).

The objective of this study was to investigate the role of stromelysin in the modification of SMC morphology and function following mechanical injury in an in vitro model (Calara et al., 1996) and in vivo after de-endothelialization. Primarily the study is focused on changes in phenotype and the initiation of migration and proliferation, which normally take place during the first few days after injury. Accordingly, the in vivo experiments presented here stretch over only a seven-day period. In order to inhibit stromelysin gene expression, the injured cultures and arteries were treated with stromelysin antisense oligonuleotides. As controls, mismatched oligonucleotides, lipofectin and saline were used.

## Materials and methods

### *Materials*

Ham's medium F12, newborn calf serum (NCS) and collagenase were obtained from Life Technologies (Paisley, Scotland), bovine serum albumin (BSA) from Sigma (St. Louis, Mo., USA), trypsin from Difco (Detroit, Mich., USA) and cell culture plastics from Nunc (Roskilde, Denmark). The medium was supplemented with 10 mM Hepes/10 mM Tes (pH 7.3), 50 μg/ml L-ascorbic acid and 50 μg/ml gentamycin sulfate. Fibronectin was isolated from human plasma by affinity chromatography on gelatin-Sepharose (Hedin and Thyberg, 1987). To prepare cell culture substrate, fibronectin was diluted in phosphate-buffered saline (PBS, pH 7.3) to a concentration of 20 μg/ml. After 20 hours at 20 °C, the dishes were rinsed twice with PBS and incubated with medium F12/0.1%BSA for 30 minutes to block unspecific binding. Phosphorothioate-modified oligonucleotides were purchased from Scandinavian Gene Synthesis AB (Köping, Sweden). Lipofectin was from Life Technologies (Paisley, Scotland).

### *Cell culture*

SMC were isolated from the aortic media of 350-400 g male Sprague-Dawley rats by digestion with collagenase in medium F12/0.1%BSA as previously described (Thyberg et al., 1990). After rinsing, the cells were seeded in primary culture in 60-mm plastic petri dishes (35.000 cells/cm$^2$) and in 24-well multidishes (13.000 cells/cm$^2$) in medium F12/10% NCS or in 60-mm dishes (50.000 cells/cm$^2$) on a substrate of fibronectin in medium F12/0.1% BSA. For analysis of injury-induced mRNA expression, DNA synthesis and migration, cells were trypsinized once and seeded in 100-mm dishes (11.000 cells/cm$^2$) and on glass slides in six-well plates (21.000 cells/cm$^2$) in F12 medium containing 10% NCS. For electron microscopy, the freshly isolated cells were first allowed to attach to a fibronectin substrate for twelve hours in the absence of the experimental reagents. The medium was then changed to F12 without BSA and the oligonucleotides (final concentration 15 μM) or the corresponding volumes of saline or lipofectin were added (medium and reagents changed again after two days). On day three,

F12 medium with 0.1% BSA but without reagents was added to all cultures. After a total of 6 days in culture, the cells were fixed in 3% cacodylate-buffered glutaraldehyde, photographed in an inverted microscope, scraped off the dishes with a plastic spatula, and processed for electron microscopy.

### *In vitro injury*

Secondary cultures on glass slides or in 100-mm dishes were allowed to grow to confluence in the presence of 10% NCS. The cultures were subsequently growth-arrested for 48 hours in serum-free F12 medium containing 0.1% endotoxin-free BSA. Prior to injury the cells were given fresh serum-free medium with or without 0.1% BSA. The injury was made by gentle pressure with a 3-mm-wide soft plastic tube to the bottom of the culture dish for five seconds.

### *Antisense oligonucleotides*

The antisense oligonucleotide was a phosphorothioate-modified single-stranded DNA containing 24 nucleotides corresponding to the translation initiation site of rat stromelysin (MMP-3), including 2 codons upstream from the translation initiation codon (5' GAC TGG GAG CCC TTT CAT TTC CAC 3'). A mismatched sequence with 6 altered bases (5' GAG TCG GAC GCC TTT CAT TTG GAC 3'; altered bases underlined) was used as control. To enhance cellular uptake of DNA, the oligonucleotides were diluted in F12 medium together with lipofectin following the manufacturer's protocol.

### *Analysis of injury-induced DNA synthesis and migration*

Fresh medium F12, with or without addition of stromelysin oligonucleotides, was added to confluent serum-starved SMC cultures 1.5 hours before injury. After 7 hours fresh medium F12/0.1% BSA was added and [$^3$H]thymidine (final concentration, 1 $\mu$Ci/ml) was added after 24 hours. The cells were incubated in this medium for another 24 hours before fixation in 3% cacodylate-buffered glutaraldehyde. The specimens were then dehydrated in ethanol and mounted on glass slides. The slides were dipped in Kodak NTB2 emulsion, air-dried, exposed at 4 °C for 3 days, developed in Kodak D-19 and stained with methylene blue. DNA synthesis was determined by counting the total number of labeled nuclei in the wounded area. Migration was quantified by counting the total number of cells that had translocated from the borders into the injured zone of the cultures.

### *RNA isolation*

RNA from secondary confluent cultures was extracted essentially according to Chirgwin et al., (1979). SMCs were washed twice with PBS at 4 °C, lysed in 4M guanidine isothiocyanate, 0.03M sodium acetate (pH 6.0) and 1% ß-mercaptoethanol, and scraped off the dishes using a disposable cell scraper. The lysates were loaded onto 4 ml of 5.7M cesium chloride and centrifuged at 33,000 rpm for 20 hours at 20 °C using a SW40Ti rotor. The RNA pellet was resuspended in Tris-EDTA buffer (pH 8.0). The solubilized RNA was precipitated overnight in 0.3M sodium acetate and 2.5 volumes of 99% ethanol at -70 °C. The isolated RNA was pelleted by centrifugation at 15,000 rpm for 30 minutes at 4 °C, washed with 70% ethanol, dried and resuspended in sterile water. Quantity and purity was determined by spectrophotometry at 260 and 280 nm.

### *Northern blot analysis*

The RNA was electrophoretically separated (15 $\mu$g/lane) on 1.4% agarose gels containing 2.2 mM formaldehyde (Lehrach et al., 1977) and transferred to Hybond-N membranes (Amersham). Hybridizations were performed in 50% formamide, 5xSSC (43.8 g/l sodium chloride and 22 g/l sodium citrate), 5 x Denhardt's solution (1 g/l polyvinylpyrrolidone, 1 g/l BSA, 1 g/l Ficoll 400), 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml salmon sperm DNA and 10% dextran sulfate for 20 hours at 42 °C with cDNA probes labeled with [$^{32}$P]dCTP using the random priming technique (Stratagene, La Jolla, Calif., USA) or the nick translation technique (Amersham). After hybridization the filters were washed for 60 minutes at 55 °C in 0.1xSSC with 0.5% SDS and exposed to Fuji RX-L film for 1-24 hours. Filters used for several hybridizations were boiled in 0.1xSSC, 0.1% SDS.

### *Preparation for light- and electron microscopy*

For light microscopy the specimens were dehydrated in graded ethanol (70-100%), cleared in xylene and embedded in paraffin. Sections of 7 $\mu$m thickness were cut on a standard microtome, routinely stained with hematoxylin-eosin and photographed in a Nikon Labophot microscope. Generally, the ratio of neointima area/media area is preferred to estimate neointimal thickening. However, this was not feasible at the early time point studied here, since an even and solid neointima had not yet formed after seven days. Instead, each vessel section was divided into 12 segments and the width of the neointima in the midst of each segment was measured with values given in $\mu$m. Segments lacking a neointima were registered as 0 $\mu$m.

For electron microscopy, the specimens were postfixed for 2 hours at 4 °C in 2% osmium tetroxide in 0.1M cacodylate buffer (pH 7.3) containing 0.5% potassium ferrocyanate, dehydrated in graded ethanol (70-100%), stained with 2% uranyl acetate in ethanol and embedded in Spurr low viscosity epoxy resin. Thin sections were cut with a diamond knife on an LKB Ultrotome IV, picked up on carbon-coated Formvar films, stained with alkaline lead citrate, and examined in a JEOL 100CX electron microscope at 60 kV.

### *Balloon injury of the carotid artery and administration of oligonucleotides*

Adult male Sprague-Dawley rats (400-500 g) were anesthetized with sodium pentobarbital (20 mg/kg i.p.). The left carotid artery was exposed and an F2 embolectomy catheter advanced through the external carotid artery to the aortic arch. The catheter with inflated balloon was withdrawn three times. The main carotid artery was clamped with hemoclips for local administration of 15 $\mu$M stromelysin antisense and mismatched oligonucleotides with lipofectin (1 $\mu$g/ml) diluted in saline, lipofectin in saline, or saline alone. After 15 minutes the external carotid was tied off and the hemoclips released. The rats were euthanized 7 days after injury and the carotid arteries were perfusion fixed under physiological pressure with 3% cacodylate-buffered glutaraldehyde, removed and cleaned from surrounding tissues, and immersed in fresh fixative for subsequent processing (see above).

### *Statistical methods*

Data are expressed as mean±SD and were evaluated by unpaired Student's t test. A probability value of less than 0.05 was taken as significant.

### *Ethical considerations*

The investigation was performed with permission from the local ethical committee and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

## Results

### *Effects of stromelysin antisense oligonucleotides on the fine structural reorganization of rat aortic SMC in primary culture*

During the first week in primary culture, rat arterial SMC go through a fine structural reorganization strongly resembling that seen in vivo after vascular injury (Thyberg, 1996, 1998). This modification is basically the same if the cells are grown in plastic dishes in a serum-containing medium (Thyberg et al., 1983) or on a substrate of fibronectin in a serum-free medium (Hedin

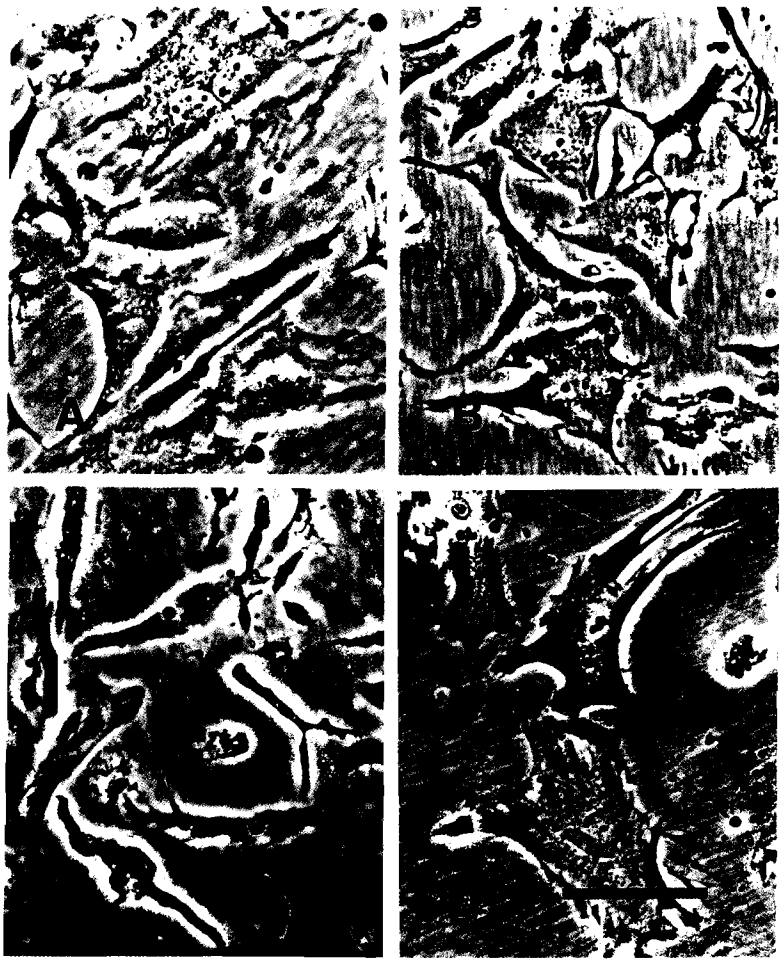

**Fig. 1.** Phase contrast micrographs of SMC grown in primary culture in a serum-free medium for six days. Experimental treatments: **A,** saline; **B,** lipofectin; **C,** stromelysin antisense oligonucleotides, 15 $\mu$M; **D,** mismatched oligonucleotides, 15 $\mu$M. Bar: 50 $\mu$m.

et al., 1988). Here, the latter condition was used to study the effect of stromelysin antisense oligonucleotides on the shift in phenotype of rat aortic SMC in primary culture. Like in the in vivo experiments described below, saline, lipofectin, and mismatched oligonucleotides served as controls.

Inspection of the cultures in the inverted microscope revealed that the cells in the saline and lipofectin groups spread out on the substrate and that the majority of them showed a highly flattened shape after six days (Fig. 1A, B). In contrast, a sizable fraction of the cells treated with the stromelysin antisense oligonucleotides retained a spindle-like shape throughout the culture period (Fig. 1C), whereas no such effect was evident in the cultures incubated with the mismatched oligonucleotides (Fig. 1D). The electron microscopic analysis further disclosed that the main part of the cells in the saline (85%) and lipofectin (77%) groups had adopted a synthetic phenotype after six days of culture with a large endoplasmic reticulum and Golgi complex and a myofilament system of markedly decreased size (compare Fig. 7). Following exposure to the stromelysin antisense oligonucleotides, this reorganization was inhibited (33% cells in a synthetic phenotype after 6 days of culture) and at the end of the experiment a large number of cells showed a morphology indistinguishable from that of freshly isolated cells in a contractile phenotype (compare Fig. 7). With the mismatched oligonucleotides, only a small effect on the change in phenotype was noted (60% cells in a synthetic phenotype after six days of culture).

*Injury-induced stromelysin gene expression in vitro*

To determine the expression pattern of stromelysin mRNA in the in vitro injury-model confluent and serum-starved SMC cultures were injured as described. SMC were harvested at different time-points and RNA extracted. Northern blotting of RNA from 3 independent experiments demonstrated that stromelysin mRNA was expressed at low levels in uninjured cultures as well as in cultures harvested 0.5 and 2 hours post injury (Fig. 2, lane 1, 2, and 3). A marked increase in accumulation of stromelysin mRNA was detected 6 hours post injury (Fig. 2, lane 4), and returned to the basal level 16 hours after injury (Fig. 2, lane 5). Notably, the rise in stromelysin mRNA expression was preceded by a marked increase in mRNA levels for the transcription factor Ets-1 (Fig. 2). Unfortunately, the lack of antibodies reacting with rat stromelysin have prevented Western analysis to prove a blocking effect of the antisense oligonucleotides on stromelysin production.

*Effects of stromelysin antisense oligonucleotides on injury-induced DNA synthesis and migration in vitro*

It has recently been shown that the in vitro injury model used in this study induces activation of DNA synthesis and cell migration (Calara et al., 1996). To investigate the possibility that stromelysin gene expression takes part in the injury-induced activation of the SMC, antisense oligonucleotides hybridizing with a 24-bp sequence surrounding the translation initiation

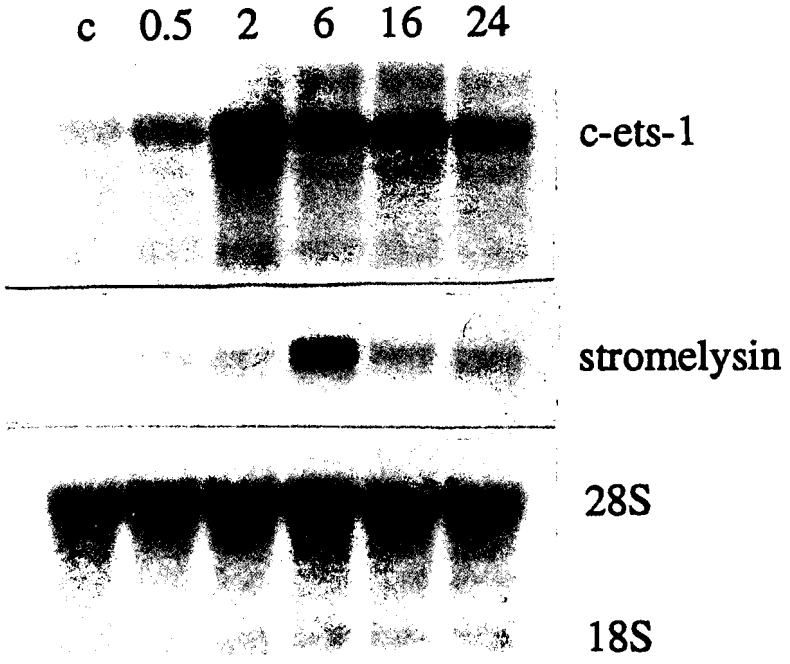


**Fig. 2.** Northern blot showing c-ets-1 (5.3 kb band) and stromelysin (1.9 kb band) mRNA-expression in secondary cultures of SMC at different time points (hours) after in vitro-injury. A clear increase in c-ets-1 mRNA is seen two hours after injury and in stromelysin mRNA six hours after injury. The control is not injured. Ribosomal RNA (18S and 28S) was visualized as a check of the equality in loading between the different lanes.

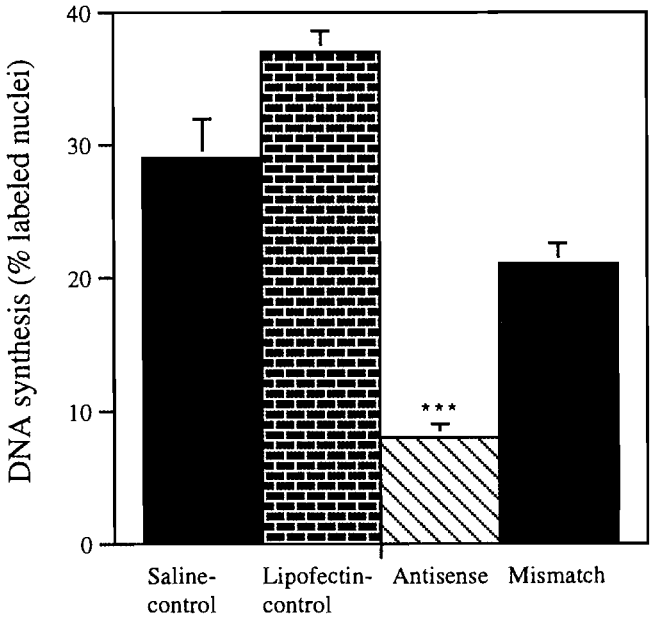


**Fig. 3.** Bar graph showing a dose-related inhibition of SMC DNA synthesis by stromelysin antisense oligonucleotides after in vitro injury as assessed by analysis of [$^3$H]-thymidine incorporation. The results are presented as means of nine cultures with the SD indicated as a vertical bar. A significant difference between the antisense-treated (striped bars) and mismatch-treated (grey bars) cultures is observed at 15 $\mu$m (***: P=0.000; S: saline control; L: lipofectin control).

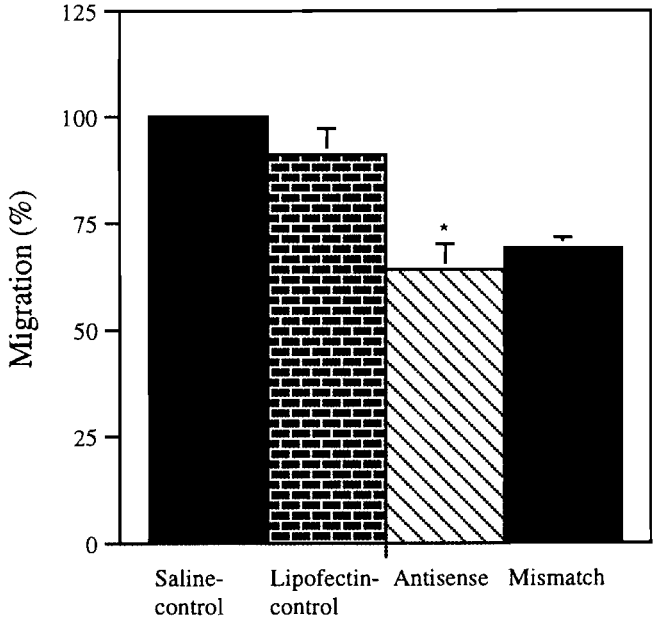


**Fig. 4.** Bar graph showing a dose-dependent inhibition of SMC migration after in vitro-injury in cultures treated with stromelysin antisense oligonucleotides (striped bars) and a non-specific, dose-independent and less striking effect with mismatched oligonucleotides (grey bars). The results are presented as means of nine cultures with the SD indicated as a vertical bar. A significant difference between the antisense and mismatched oligonucleotides is obtained at 15 $\mu$m (*: P=0.062).

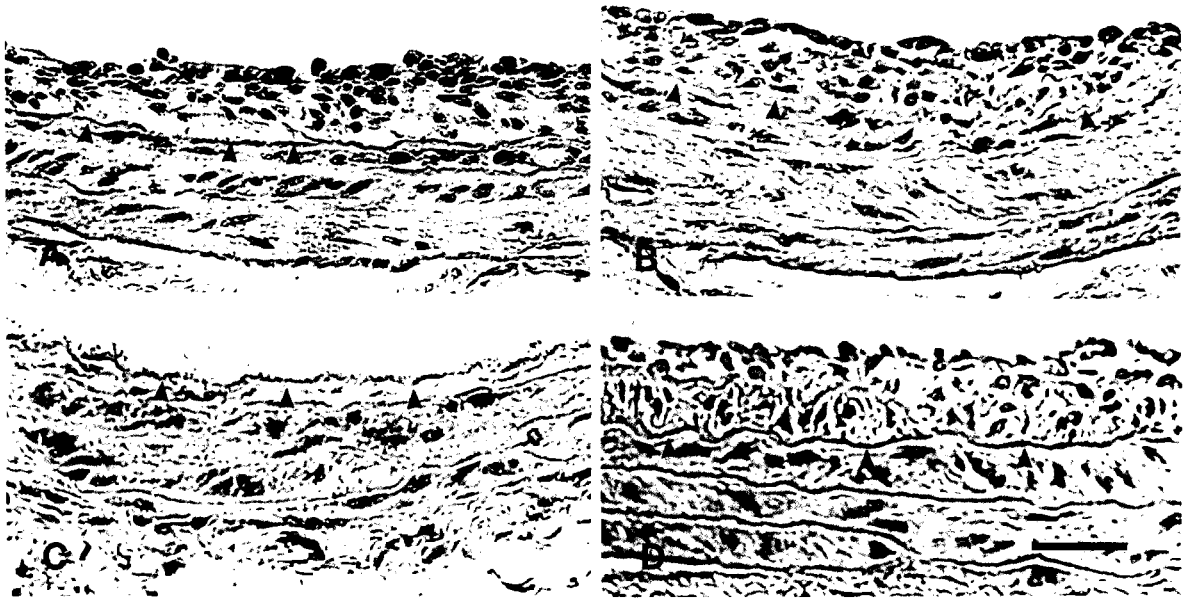


**Fig. 5.** Light micrographs of rat carotid arteries fixed seven days after balloon injury. Experimental treatments: A, saline; B, lipofectin; C, stromelysin antisense oligonucleotides; D, mismatched oligonucleotides. A multilayered neointima is observed inside the internal elastic lamina (arrowheads) in the vessels treated with saline **(A)**, lipofectin **(B)**, and the mismatched oligonucleotides **(D)**. On the other hand, no neointima has formed in the vessel treated with the stromelysin antisense oligonucleotides **(C)**. Bar: 50 $\mu$m.

codon of stromelysin mRNA were used. At 15 $\mu$M concentration these oligonucleotides significantly inhibited injury-induced DNA synthesis with a reduction in labelling index from 29% in the control group to 8% in the antisense-treated group (Fig. 3). Cell migration was reduced with 36% as compared to the control value of 100% migrating cells (Fig. 4). Addition of the mismatched sequence at the same concentration also decreased the level of replicating cells (Fig. 3) and the total number of migrating cells (Fig. 4). Incubation of the cells with 0.3 $\mu$M and 3 $\mu$M antisense and mismatch oligonucleotides did not significantly reduce injury-induced DNA synthesis and migration (data not shown).

### *Effects of stromelysin antisense oligonucleotides on the early stages of neointimal thickening after vascular injury in the rat*

To look into the role of stromelysin in phenotypic modulation of SMC and the formation of neointimal thickenings after vascular injury, we examined the effect of stromelysin antisense oligonucleotides on this process. Saline, lipofectin, and mismatched oligonucleotides served as controls. The reagents were applied locally for 12-15 minutes after balloon catheterization of the left carotid artery of adult rats. Due to the apparent risk for degradation of the oligonucleotides and a subsequent "catch up" in the repair process by antisense-treated vessels, the animals were killed already seven days after the injury and the vessels processed for light and electron microscopy. In the saline- and lipofectin-treated arteries, the neointima had a mean thickness of 7.5 and 8.3 $\mu$m, respectively (Fig. 5A,B). However, large local variations were seen at this early time of intimal thickening. Administration of the antisense oligonucleotides reduced the neointima to 3.1±2.1 $\mu$m, whereas no effect was obtained with the mismatched oligonucleotides, which gave a neointima of 11.6±4.8 $\mu$m (Fig. 5C,D). Statistical analysis revealed that the difference in effect between the antisense and mismatched oligonucleotides was significant at the level P=0.015 and between antisense- and saline-treated at P=0.016 (Fig. 6A). Study of the light microscopic sections also showed that the fraction of the vessel circumference covered by a neointima varied markedly between the groups. In the saline- and lipofectin-treated arteries this value was high, 70% and 64%, respectively. On the other hand, only 37% of the vessel circumference was covered by a neointima in the arteries exposed to the antisense oligonucleotides, whereas the corresponding value was 68% in the arteries exposed to the mismatched oligonucleotides (Fig. 6B).

Electron microscopic examination of the injured arteries added further detail to the aforementioned observations. In all experimental groups, the majority of the SMC in the media were in a contractile phenotype as defined by the predominance of myofilaments in the cytoplasm (Fig. 7A). Nevertheless, a large fraction of the cells in the innermost layer of the media (i.e. directly underneath the internal elastic lamina) had been modified into a synthetic phenotype with few myofilaments and a large secretory apparatus. This

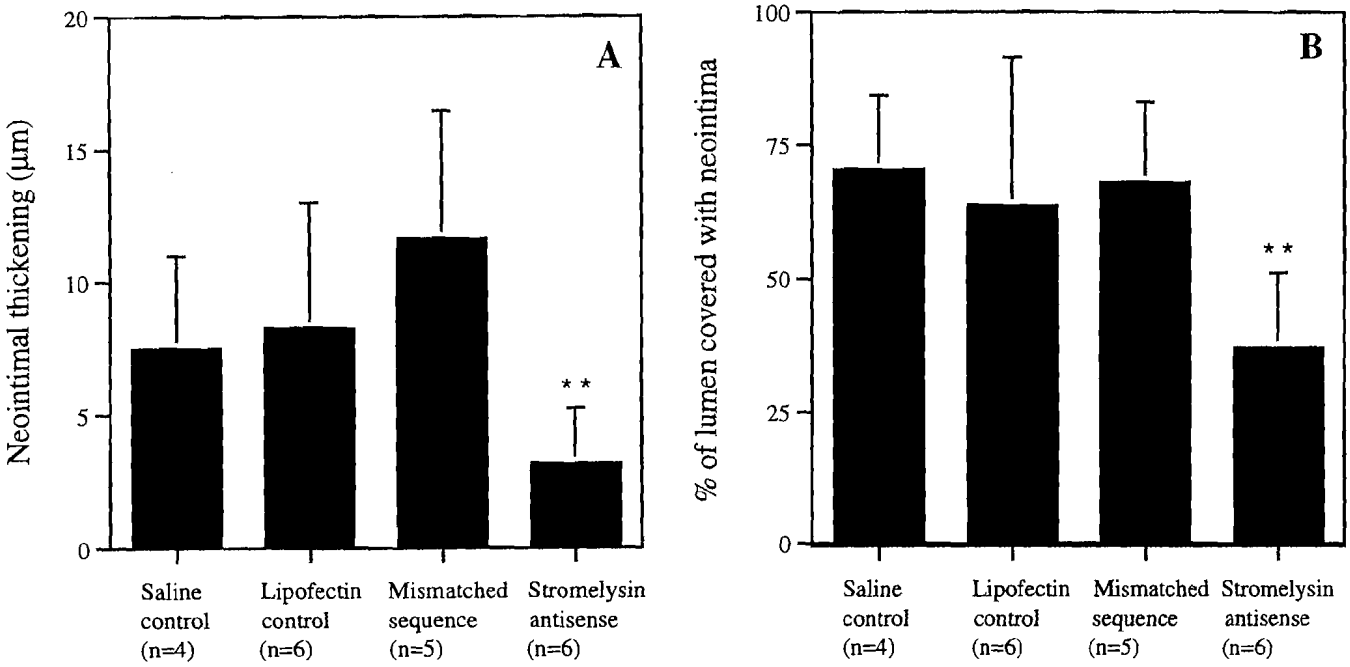


**Fig. 6. A.** Bar graph showing neointimal thickening seven days after balloon injury and administration of oligonucleotides. Average thickness of the neointima calculated on the whole circumference of the aortic section (12 measuring points/section). **: P=0.015. **B.** Bar graph showing the percentage of the vessel wall circumference covered by a clearly visible neointima.

pattern has been described earlier (Thyberg et al., 1997; Thyberg, 1998) and was especially evident in vessels treated with saline, lipofectin, and the mismatched oligonucleotides (Fig. 7B), whereas a larger number of contractile cells remained in the inner part of the media in vessels treated with the antisense oligonucleotides (Fig. 7C). However, the change in SMC morphology was not completely blocked in the last-mentioned group.

Once in the intima, the SMC showed a further increase in size of the secretory apparatus (i.e. the endoplasmic reticulum and the Golgi complex). In addition, many mitotic cells were noted here (Thyberg, 1998). Accordingly, a multilayered neointima consisting of synthetic SMC surrounded by a loosely arranged extracellular matrix could already be found seven days after endothelial denudation in vessels treated with saline, lipofectin, and the mismatched oligonucleotides (Fig. 8A,B). As indicated above, the neointima was distinctly thinner in vessels treated with the antisense oligonucleotides and the exposed internal elastic lamina was covered either by a layer of degranulated platelets or just a thin layer of SMC (Fig. 8C). In any case, the intimal SMC were in a synthetic phenotype and mitoses were noted also in this group.

On the basis of these results, it is concluded that treatment of injured rat carotid arteries with stromelysin antisense oligonucleotides inhibited the early steps in the formation of a neointimal thickening. This may be due to an effect either on the initial shift in phenotype of the SMC or their subsequent migration from the media to the intima, or both.

## Discussion

Injury to the arterial media induces a wound-healing response in which the SMC start to migrate, proliferate and secrete extracellular matrix components. Previous findings using in vitro as well as in vivo injury models indicate that in order to activate the repair functions, the SMC first have to modulate from a contractile to a synthetic phenotype (Thyberg et al., 1995; Thyberg 1996, 1998). The present study demonstrates that antisense oligonucleotides to stromelysin mRNA inhibit the injury-induced formation of neointimal thickenings in the rat carotid artery. To study if these effects may be due to effects on the phenotypic state of the cells, two in vitro models were used. (1) primary cultures, where it is possible to study the change in phenotype and (2) an in vitro injury model in which cell migration and proliferation can be analyzed. In conclusion, our data suggest a role for injury-induced activation of the stromelysin gene in both migration and DNA synthesis

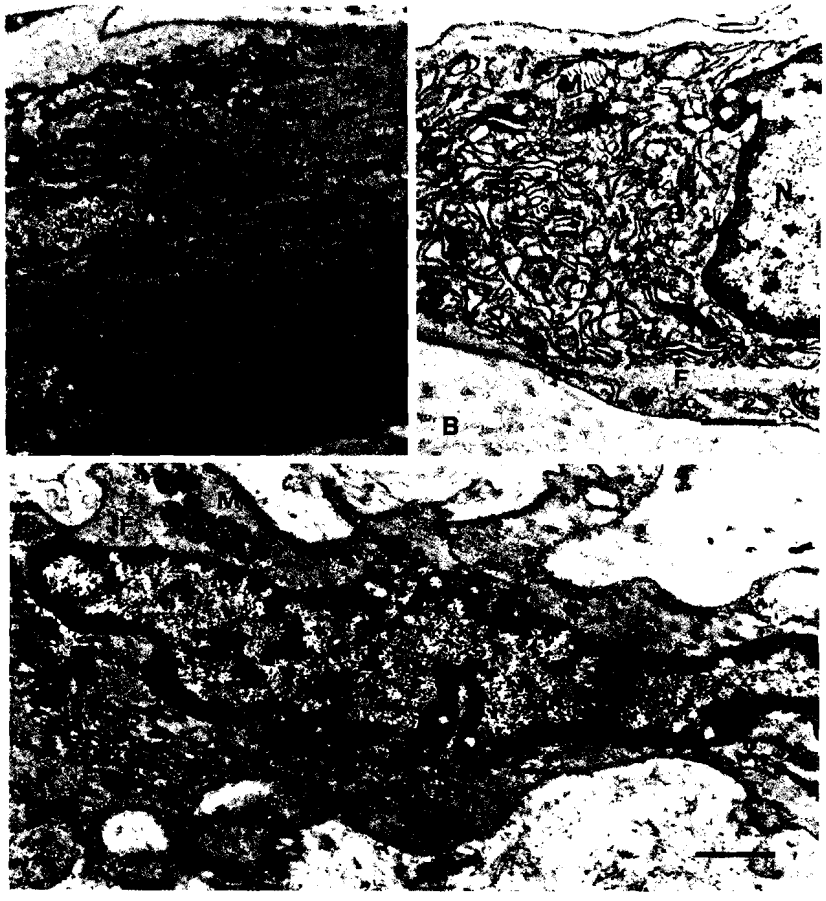


**Fig. 7.** Electron micrographs of SMC in the media of carotid arteries fixed seven days after balloon injury. Experimental treatments: **A and B**, mismatched oligonucleotides; **C**, stromelysin antisense oligonucleotides. The cell in A is from the third layer of the media and the cells in B and C are from the first layer of the media (directly underneath the internal elastic lamina). The cells in A and C are in a contractile phenotype with a cytoplasm dominated by myofilaments (F) and numerous mitochondria (M). The cell in B is instead in a synthetic phenotype with a large endoplasmic reticulum (ER) and Golgi complex (G) and only few myofilaments (F). N marks the nucleus. Bars: 1 $\mu$m.

of SMC at the borders of the injured cell area. Our findings further suggest that the inhibitory effects of the antisense oligonucleotides in part may be coupled to a decreased capacity of the cells to change from a normal contractile to an activated repair cell.

Over the last few years an increasing number of publications have demonstrated the use of antisense oligonucleotides as tools for identifying mechanisms involved in SMC migration and proliferation (Bennett et al., 1994; Fox and Shanley, 1996; Gunn et al., 1997; Sirois et al., 1997). The adoption of the antisense technology has raised questions concerning the specificity of the obtained effects. Previous findings demonstrate a nonantisense antiproliferative effect both in SMC and epithelial cells of phosphorothioate oligonucleotides with a 4-guanosine (4-G) sequence (Yaswen et al., 1992; Villa et al., 1995). Nevertheless, using a stromelysin antisense sequence without 4-G motifs, we found a significant reduction in DNA synthesis and migration in vitro as well as intimal thickening after balloon injury in vivo, as compared with a mismatched sequence. The significant differences in effects on migration and DNA synthesis between the antisense and the mismatched sequence indicate that the effects of the antisense oligonucleotides at least in part are specific. Other nonspecific mechanisms by which the oligonucleotides may influence the cells include expression of polyanionic properties mimicking heparin by binding growth factors (Stein and Krieg, 1994) and induction of γ-interferon, a cytokine inhibiting SMC proliferation by formation of double-stranded RNA (Hansson et al., 1989; Hovanessian, 1989; Meurs et al., 1990). These nonantisense mechanisms are unlikely to explain the inhibitory effects on proliferation and migration observed in the in vitro injury-model. By using the medium deprived of serum before, during and after treatment with antisense oligonucleotides and injury, the amounts of growth factors and cytokines in the medium are minute.

In the rat carotid artery injury model, the stromelysin antisense oligonucleotides significantly reduced neointimal thickening. Electron microscopic analysis of all experimental groups revealed that the majority of the SMC in the media were in a contractile state excluding the innermost layer where a large fraction of the cells had modified to a synthetic phenotype after the balloon

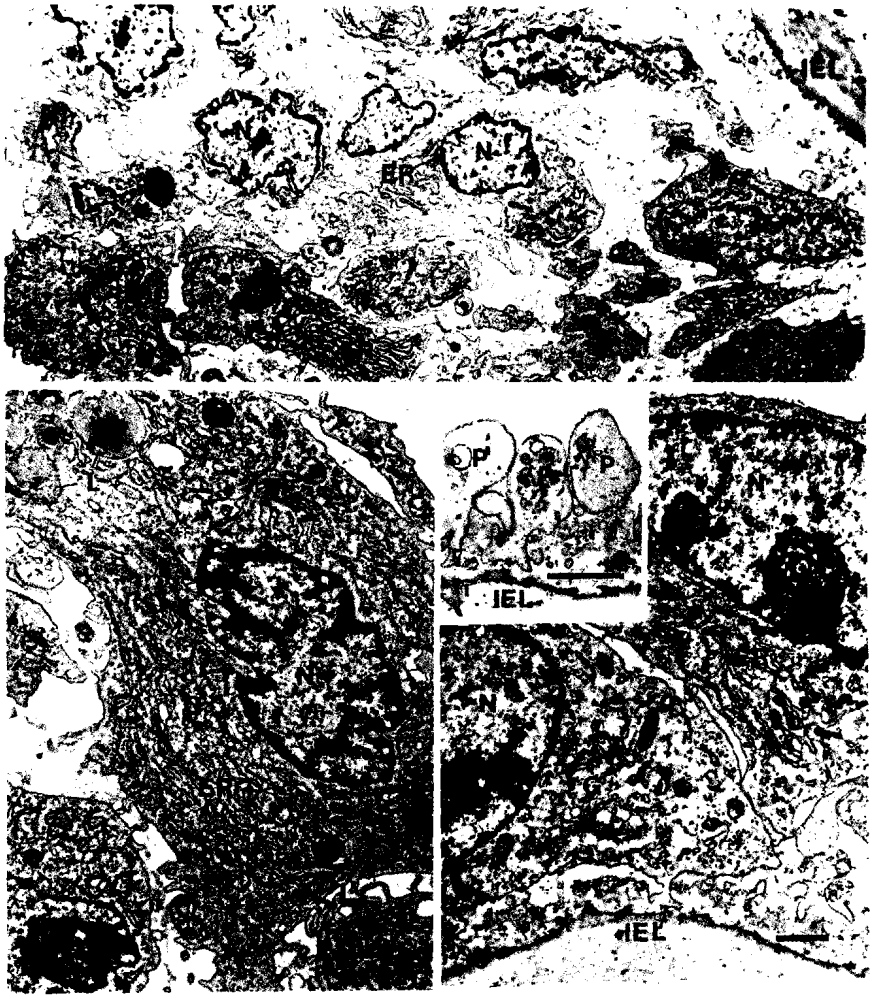


**Fig. 8.** Electron micrographs of SMC in the neointima of carotid arteries fixed seven days after balloon injury. Experimental treatments: **A and B**, mismatched oligonucleotides; **C**, stromelysin antisense oligonucleotides. At this time, the cells in the neointima are all in a synthetic phenotype with a large endoplasmic reticulum (ER) and Golgi complex (G). The inset in C shows platelets (P) adhering to the exposed internal elastic lamina (IEL). IEL: internal elastic lamina; L: lysosomes with inclusions of lipid-like material; N: nucleus. Bars: 1 μm.

injury. However, the change in SMC morphology in this part of the media was incomplete in the vessels treated with antisense oligonucleotides. A more pronounced effect of stromelysin antisense oligonucleotides on phenotypic modulation was obtained in primary cultures. After six days, only one third of the cells treated with antisense oligonucleotides were in a synthetic state, whereas the majority of the SMC in the controls had lost most of their myofilaments and had adopted a synthetic morphology. The differences between the effects in vivo and in vitro may reflect the differences in time of exposure to the stromelysin antisense oligonucleotides. Thus, the oligonucleotides were present for three days altogether in the cell culture dish, while the in vivo injury model allowed only 12 minutes of uptake from the vessel lumen. Another possible explanation could be that the oligonucleotides in the in vivo situation had to penetrate through the matrix before reaching the target cells. To obtain a more efficient effect on the phenotypic modulation in vivo, the oligonucleotides may have to be present for a longer period of time. Use of a matrix release device could possibly overcome this problem. This was done to suppress intimal thickening by antisense oligonucleotides to the PDGF-β receptor subunit (Sirois et al., 1997). However, so far no data have been presented to demonstrate the uptake of oligonucleotides from such a device into the adventitia and through the media.

The requirement of matrix metalloproteinases for migration of SMC in vitro as well as in animal models of balloon injury has been demonstrated (Pauly et al., 1994; Bendeck et al., 1996; Zempo et al., 1996; Webb et al., 1997). A role for these enzymes in proliferation is less clear, but some investigators have presented data suggesting that metalloproteinases are involved in the initiation of SMC proliferation (Southgate et al., 1992; Zempo et al., 1996). There is also evidence that gelatinase, collagenase and stromelysin are produced by cytokine-stimulated human SMC (Galis et al., 1994).

To our knowledge, this is the first study dealing with the role of stromelysin gene expression in the structural reorganization of SMC prior to migration and proliferation. We have recently demonstrated that the stromelysin gene is strongly upregulated on day 2 in rat SMC primary cultures (Hultgårdh-Nilsson et al., 1997). At this time the cells start to modulate from a contractile to a synthetic state, a prerequsite for migration and proliferation. In conclusion, our experiments with the stromelysin antisense oligonucleotides support the hypothesis that metalloproteinases do have a role in the modulation of SMC phenotype. The mechanism by which stromelysin can regulate the state of SMC may be by cleavage of matrix proteins. We have preliminary data demonstrating that BB-94, a drug which blocks metalloproteinase activity, inhibits signaling pathways and gene expression induced by injury. One hypothesis would be that the injury actvivates stromelysin, leading to cleavage of matrix proteins. This may give rise to protein fragments with (1) new functions, (2) exposure of binding sites to the cell surface, and (3) possible release of bioactive factors/substances from the matrix. These events may act independently or in concert to alter the phenotypic state of the SMC. The basement membrane surrounding the SMC is another target for stromelysin. A degradation of the basement membrane, which supports the differentiated state of SMC, may initiate events leading to an altered gene expression and, subsequently, a modification of cell structure and function.

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