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European Journal of Pharmacology 309 (1996) 95–105

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Oligodeoxynucleotides directed to early growth response gene-1 mRNA inhibit DNA synthesis in the smooth muscle cell

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Received 30 October 1995; revised 17 April 1996; accepted 23 April 1996

Abstract

Vascular smooth muscle cell proliferation plays a central role in the pathophysiology of cardiovascular diseases. The induction of the early growth response gene-1 (egr-1) mRNA is associated with different cellular processes such as cell proliferation. Antisense oligodeoxynucleotides seem to provide a promising new pharmaceutical tool for effective modification of the expression of specific genes. Hence, in the present study, the effect of 15-mer antisense oligodeoxynucleotides (targeted to the initial codon region of the egr-1 mRNA) on the angiotensin II- and platelet-derived growth factor-BB-induced growth promoting effects of aortic smooth muscle cells was evaluated. Angiotensin II- and platelet-derived growth factor-BB induced egr-1 mRNA (3.4 kb) and Egr-1 protein (80 kDa) in a time- and concentration-dependent fashion. No effects of the sense and antisense oligodeoxynucleotides on the agonist-induced elevation of the egr-1 mRNA and on the Egr-1 protein could be demonstrated. However, they effectively inhibited the angiotensin II- and the platelet-derived growth factor-BB-induced DNA synthesis. Our findings provide evidence that the oligodeoxynucleotides inhibit vascular smooth muscle cell growth via nonantisense mechanism(s).

Keywords: Oligodeoxynucleotide; Immediate-early gene; Growth factor

1. Introduction

Smooth muscle cell proliferation participates in the pathophysiology of both hypertension and atherosclerosis (Schwarz and Reidy, 1987; Bevan et al., 1976; Folkow et al., 1973). Local generation and autocrine or paracrine actions of angiotensin II has been shown to occur in the vascular system and the heart (Campbell, 1986; Schelling et al., 1991). Thus, it has been proposed that angiotensin II participates in the mechanisms of structural vascular changes by enhancing cell growth in vascular smooth muscle cells. The platelet-derived growth factor-BB dimeric isoform is consisted of two polypeptide chains B and is a potent mitogen in vitro for different cell types including vascular smooth muscle cells (Sachinidis et al, 1990). Platelet-derived growth factor may play an important role in the pathogenesis of atherosclerosis (Ross et al., 1986).

The early growth response gene-1 (egr-1), also known as krox-24, zif-268, NGFI-A, has been identified as a transcription factor belonging to a class of immediate-early genes (Milbrandt, 1987; Sukhatme et al., 1988; Sukhatme, 1990). Like other immediate-early growth response genes such as c-fos, c-myc and c-jun (Murakami et al., 1991; Sukhatme, 1990), induction of egr-1 mRNA in different cell types by several growth factors is associated with different cellular processes such as cellular differentiation, cell development, cell hypertrophy and cell proliferation (Milbrandt, 1987; Sukhatme et al., 1988; Sukhatme, 1990). The Egr-1 protein with a molecular weight of 80 kDa has been identified (Cao et al., 1990). The c-fos protein (Verma and Sassone-Corsi, 1987) is often coregulated with the Egr-1 protein. Both proteins are located in the nucleus and play an important role as transcription factors (Sukhatme et al., 1988; Sukhatme, 1990).

Antisense oligodeoxynucleotides have been used to inhibit specific gene expression. Although the underlying mechanisms of action of antisense oligodeoxynucleotides are not well understood, it is assumed that antisense oligodeoxynucleotides directed to a specific segment of a selected mRNA block mRNA translation upon hybridization by base pairing and consequently interrupt the synthe-

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PII S0014-2999(96)00323-8

sis of a particular protein (Stein et al., 1988; Toulme and Helene, 1988). These physical interactions between antisense oligodeoxynucleotides and the target gene sequence are proposed at either the pre-mRNA or at the mRNA level resulting in an inhibition of RNA processing or RNA translation (Leonetti et al., 1993). Alternatively, DNA-RNA hybrids may occur which may be hydrolysed by the RNase H resulting in degradation of mRNA. In addition, it has been proposed that oligodeoxynucleotides may inhibit the binding of translation initiation factors or directly inhibit the translation of the mRNA by ribosomes (Leonetti et al., 1993).

The possibility to inhibit the expression of genes which are involved in the regulation of vascular smooth muscle cell growth might provide a new simple pharmaceutical tool for 'gene therapy' of cardiovascular diseases (for review see Epstein et al., 1993). Thus, we applied the 'antisense' approach by investigating the effect of non-modified antisense oligodeoxynucleotides directed against the egr-1 gene on the platelet-derived growth factor-BB- and angiotensin II-induced DNA synthesis in vascular smooth muscle cells. Control experiments were performed using sense and scrambled oligodeoxynucleotides. In order to examine whether the inhibitory mechanisms of the oligodeoxynucleotides can be attributed to putative antisense mechanisms we investigated the effects of sense and antisense oligodeoxynucleotides on the agonist-induced increase in egr-1 mRNA and protein.

2. Materials and methods

2.1. Materials

Fluoresceinisothiocyanate-conjugated monoclonal anti-α-smooth muscle actin and angiotensin II were obtained from Sigma (Deisenhofen, Germany). Dulbecco's modified Eagles medium (DMEM), Ham's F-10 and Dulbecco's phosphate-buffered saline (PBS) and other culture media were obtained from Gibco BRL (Eggenstein, Germany). Cell culture flasks were purchased from Falcon (Heidelberg, Germany). The 2.1 kb Fragment (OC68 insert) of the egr-1 gene and the rabbit antiserum R5232-T to the Egr-1 protein were kindly provided from Vikas Sukhatme, Harvard Medical School, Beth Israel Hospital, Boston/MA 02215, USA. A 0.77 kb cDNA for β-actin (Dianova/Oncor Science, Hamburg, Germany) was used as DNA probe for β-actin. Hybond N^+ membranes, [^{32}P]deoxycytidine triphosphate ([^{32}P]dCTP), [methyl-^{3}H]thymidine, [^{125}I]-platelet-derived growth factor-BB, 3[^{125}I](iodotyrosyl) angiotensin II and horse radish peroxydase-labelled donkey anti-rabbit immunoglobulin G were obtained from Amersham Buchler (Braunschweig, Germany). Kodak X-OMAT films (8 × 10 inch) were obtained from Kodak (Rochester, USA). Fetal calf serum, platelet-derived growth factor-BB, bacteriophage T4 polynucleotide kinase and molecular weight standard RNA were obtained from Boehringer Mannheim (Mannheim, Germany). Polyvinylidenedifluoride membranes were obtained from Millipore, Bedford, USA. Bio-Gel P-60 was obtained from Bio-Rad (Munich, Germany). Hybond N^+ membranes were obtained from Amersham (Little Chalfont, England). 2-*n*-Butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)-biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (EXP 3174) was a gift from MSD research laboratories, West Point, USA. Benzonase (250 units/μl) was obtained from (Merck (Darmstadt, Germany).

2.2. Methods

Vascular smooth muscle cells were isolated from medial segments from thoracic rat aorta of female Wistar-Kyoto rats (6–8 weeks old) (Charles River Wiga GmbH, Sulzfeld, Germany) and cultured over several passages as previously described (Ross, 1971). The culture medium was DMEM supplemented with 10% fetal calf serum, nonessential amino acids, penicillin 100 IU/ml and streptomycin 100 μg/ml. Cells (3×10^6) in 75 cm^2 flasks were allowed to grow in 5% CO_2, 95% air at 37°C and became confluent after 4–5 days. Vascular smooth muscle cells in culture showed the characteristic 'hill and valley' growth pattern. The purity of vascular smooth muscle cells was confirmed by immunocytochemical localization of smooth muscle specific α-smooth muscle actin using fluoresceinisothiocyanate-conjugated monoclonal anti-α-smooth muscle actin. Experiments were performed with three different cell lines at passage 5–20.

The ATG codon of murine Egr-1 (also known as krox-24, zif-268, NGFI-A) sequence (position 259) as suggested by Sukhatme et al. (1988) was taken as the starting codon. Milbrandt (1987) had proposed an ATG 84 nucleotides downstream from this site. The sequence of the nerve growth factor (NGF) inducible cDNA (NGFI-A) from the rat pheochromocytoma PC12 line isolated from Milbrandt (1987) is homolog to murine egr-1 sequence (Sukhatme et al., 1988). The putative initiation ATG of the NGFI-A cDNA chosen by Milbrandt (1987) (cDNA position 353) corresponds to position 343 in the murine egr-1 cDNA Sukhatme et al. (1988).

Oligodeoxynucleotides (15-mer) were synthesized on a Gene Assembler Plus (Pharmacia LKB, Munich Germany) using standard protocols. The oligodeoxynucleotides were dried down, resuspended in PBS and quantified by spectrophotometry and gel electrophoresis.

The following oligonucleotide (15-mer) sequences were used:

(1) oligodeoxynucleotides which are either antisense or sense to the translation initiation codon region (base 259–273) of the egr-1 gene
- antisense [3′-TACCGTCGCCGGTTC-5′](AS1)
- sense [5′-ATGGCAGCGGCCAAG-3′] (S1)

(2) oligodeoxynucleotides directed to bases 278–292

- antisense [3′-TCTACGTTAACTACA-5′] (AS2)
- sense [5′-AGATGCAATTGATGT-3′] (S2)
- scrambled [3′-ATACTAGCTATACTC-5′(SCR-AS2)

(3) polyadenylate

- 3′-AAAAAAAAAAAAAAA-5′(PolyA)

For measurement of the cellular uptake AS1 oligodeoxynucleotide was 5′-endlabelled by a bacteriophage T4 polynucleotide kinase with γ-[^{32}P]ATP] as described previously (Sambrook et al., 1989). Ten picomoles of the oligonucleotide were incubated with 8 units of bacteriophage T4 polynucleotide kinase and 10 pmol of γ-[^{32}P]ATP at 37°C for 45 min followed by an incubation at 70°C for 10 min to inactivate the enzyme. The efficiency of transfer of [^{32}P]ATP to the oligodeoxynucleotide and the specific activity was determined chromatographically using a polyethyleneimine-cellulose column. The purification of the radiolabelled oligodeoxynucleotide was performed by chromatography through a Bio-Gel P-60. The column was prepared in a sterile pasteur pipette. When radioactivity first started to eluate from the column, five-drop fractions were collected, analyzed by another chromatography and the appropriate fractions pooled. The radiolabelled oligonucleotide was added to the confluent cells (10^5 cpm/well) for different time periods. After three washes with PBS cells were lysed in 0.5 M NaOH and counted. Protein was measured according to Bradford (1976).

For the the determination of the [^{125}I]platelet-derived growth factor-BB or [^{125}I]angiotensin II binding in vitro, confluent cells (24-well culture plates) were preincubated in serum-free medium (0.5 ml) for 24 h. After cells were incubated with the S1 or AS1 oligodeoxynucleotides for 2 h [^{125}I]platelet-derived growth factor-BB (25 000 cpm/well, 5 ng/ml) or [^{125}I]angiotensin II (145 000 cpm, 0.4 nM) was added to the medium for 10 or 45 min at 37°C, respectively, chilled on ice, and then washed 3 times with 0.5 ml PBS containing 0.25% bovine serum albumin. Subsequently, cells were solubilized with 0.5 M NaOH and γ-radiation was quantified with a Gamma-C12 γ-counter (Hermann Biermann, Bad Nauheim, Germany). Nonspecific binding was determined using a 50-fold excess of unlabelled platelet-derived growth factor-BB or angiotensin II.

The effect of the platelet-derived growth factor-BB and angiotensin II on DNA synthesis was measured by a slight modification of a method as described previously (Nemecek et al., 1986). Vascular smooth muscle cells were seeded in 24-well culture plates and cultivated until confluence. Then the initial medium was replaced by serum-free medium consisting of a mixture of DMEM and Ham's medium (1 : 1). Following another 22–24 h cultivation in serum-free medium, cells were incubated with the oligodeoxynucleotides for 2 h before stimulation with platelet-derived growth factor-BB or angiotensin II. Cultures were exposed to platelet-derived growth factor-BB or angiotensin II for 20 h before 3 μCi/ml [methyl-^{3}H]thymidine were added to the serum-free medium. Four hours later experiments were terminated by aspirating the medium and subjecting the cultures to sequential washes with Dulbecco's phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% trichloroacetic acid and ethanol/ether (2 : 1, v/v). Phase-contrast microscopy was used to inspect the dishes for evidence of cell detachment or changes in cell morphology. Acid-insoluble [^{3}H]thymidine was extracted into 250 μl/dish 0.5 M NaOH and 100 μl of this solution was mixed with 5 ml scintillator and quantified by using a liquid scintillation counter Beckman LS 3801 (Düsseldorf, Germany). Then 50 μl of the residual solution was prepared for the determination of protein using the Bio-Rad protein assay according to the method of Bradford (1976).

The expression of egr-1 mRNA was studied after preincubation of the cells for 24 h in serum-free medium in 75 cm^2 culture flasks. Cells were then preincubated with sense and antisense oligonucleotide for 2 h and stimulated with angiotensin II (100 nM) or platelet-derived growth factor-BB (50 ng/ml) for 30 min. Total RNA was extracted from cells by the guanidinium isothiocyanate/CsCl procedure (Chirgwin et al., 1979). Ten micrograms of total RNA were separated by electrophoresis in a 6% formaldehyde/1.2% agarose gel, blotted on Hybond N^+ membranes washed at room temperature in 75 mM sodium citrate buffer containing 0.75 M sodium chloride for 5 min, and fixed by UV irradiation. After fixing, the blots were washed at 60°C in 1.5 mM sodium citrate buffer containing 15 mM sodium chloride and 0.1% sodium dodecylsulfate for 5 min. Prehybridization and hybridisation were performed overnight at 60°C in a buffer consisting of 75 mM sodium citrate, 0.75 M sodium chloride, 0.2% sodium dodecyl sulfate, 50 mM sodium phosphate and 10 × Denhardt's solution 200 μg/ml salmon sperm (ss) DNA. The DNA probes were labelled with [^{32}P]deoxycytidine triphosphate ([^{32}P]dCTP) by random oligonucleotide priming to a specific activity of $2–4 \times 10^9$ dpm/μg DNA. The stringency of the final wash was 0.2 × SSC containing 0.1% sodium dodecyl sulfate) at 65°C for 2 × 45 min. A 2.1 kb Fragment (OC68 insert) of Egr-1 including three zinc-finger domains (Sukhatme et al., 1988), a 1.0 kb fragment of v-fos and a 1.56 kb fragment of c-myc were used as probes. Blots were exposed to Kodak films for 3–7 days at −70°C. Blots were standardised using a 0.77 kb cDNA probe for β-actin. The size in kilobases (kb) of the detected mRNA was calculated by comparison with the 18S (1.8 kb) and 28S (4.6 kb) ribosomal RNA migration from the gel wells. Control experiments were performed using the angiotensin II AT1 receptor antagonist 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (EXP3174).

The Egr-1 protein analysis was performed by a modification of the enhanced chemiluminescence western blot-

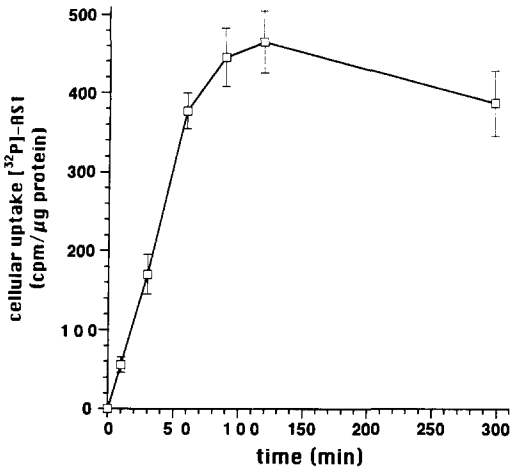


Fig. 1. Uptake of the AS1 oligonucleotides into vascular smooth muscle cells. The AS1 oligonucleotides directed to the egr-1 mRNA were 5′-endlabelled with γ-[^{32}P]ATP. Confluent cells were preincubated in serum-free medium for 24 h. Then radiolabelled antisense oligonucleotides (10^5 cpm) were added to the confluent cells for different time periods. After 3 washes with PBS cells were lysed in 0.5 M NaOH and counted. The data represent the mean ± S.D. from one representative experiment performed 6-fold.

ting method as previously described by Simm et al. (1992).

Cells were seeded in 24-well culture plates (4×10^5 cells/well, well diameter 12 mm) and cultivated in culture medium until confluent. The medium was then replaced by serum-free medium consisting of a mixture of DMEM and Ham's F-10 medium (1 : 1). Following another 24 h cultivation in serum-free medium, cells were stimulated for different time periods. The medium was removed and then cells were lysed with a buffer containing 50 mM Tris, 2% sodium dodecyl sulfate, 2% mercaptoethanol, 1 mM sodiumorthovanadate, pH 6.7. The samples were vigorously shaking for 5 min and then benzonase (2.5 μl; 250 units/μl) was added. After 5 min of vigorous shaking aliquots were used for protein determination using the Bio-Rad protein assay according to Bradford (1976). Sample solutions containing 30 μg protein were transferred into microtest tubes and bromphenol blue (6 μl) in 50% glycerol was added. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 7.5% acrylamide gel using a mini gel system (Bio-Rad, Munich, Germany). Proteins were transferred to a Polyvinylidenedifluoride membranes over night by 100 mA with a buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3. The protein transfer was checked using Ponseau S dye. The membranes were then washed using PBS containing 0.1% Tween-20 and 1%

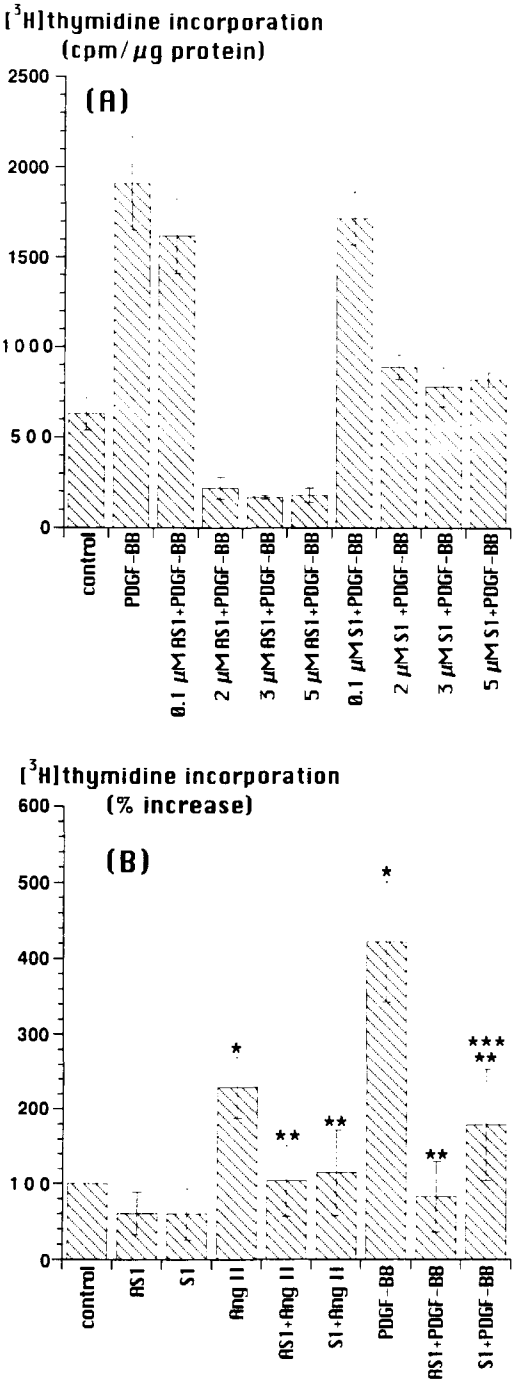


Fig. 2. Effects of the S1 or AS1 oligodeoxynucleotides on platelet-derived growth factor-BB- and angiotensin II-induced [^{3}H]thymidine incorporation into cell DNA. (A) Representative experiment performed in triplicates wells. Confluent cells (24-well plates) were precultured for 22 h in the serum-free medium. Oligodeoxynucleotides have been added at different concentrations to serum-free medium 2 h before stimulation with platelet-derived growth factor-BB. Data represent the means ± S.D. of a triplicate determination. (B) Data from individual experiments each performed with triplicate wells were standardised by calculating the means ± S.D. of the individual experiments and expressed as percentage of the value from unstimulated cells (control = 100%). * $P < 0.05$ for angiotensin II or platelet-derived growth factor effect versus control. ** $P < 0.05$ for AS1 + angiotensin II or S1 + angiotensin II effect versus angiotensin II effect. *** $P < 0.05$ for AS1 + platelet-derived growth factor-BB or S1 + platelet-derived growth factor-BB effect versus platelet-derived growth factor-BB effect. **** $P > 0.05$ for S1 + platelet-derived growth factor-BB effect versus AS1 + platelet-derived growth factor-BB effect.

nonfat dry milk (standard incubation buffer). Nonspecific binding blockade was performed in PBS containing 0.1% Tween-20 and 5% nonfat dry milk for 2 h. Following a quick rinse with standard incubation buffer, membranes were incubated for 1 h in a standard solution containing anti-Egr-1 antisera R5232-T (1 : 4000 dilution) and subsequently washed three times for 5 min using standard incubation buffer. Then membranes were incubated for 1 h in standard incubation buffer containing horse radish peroxydase-labelled donkey anti-rabbit immunoglobulin G (1 : 5000 dilution). Membranes were washed three times for 5 min with the standard incubation buffer. The detection of the Egr-1 protein was performed using the chemiluminescence method with luminol. Briefly, sheets were soaked in 3 ml chemiluminescence buffer containing 100 mM Tris, 2.5 mM luminol, 400 μM *p*-coumaric acid, pH 8.5, to which 3 ml hydrogen peroxide buffer was added (5.4 mM H_2O_2, 100 mM Tris, pH 8.5). After 1 min the sheets were dried with filter papers and wrapped in transparent foil. Light emission was detected with Kodak X-OMAT films.

3. Results

3.1. Cellular uptake of the [^{32}P]AS1 oligonucleotide

Cellular uptake of the [^{32}P]labelled [^{32}P]AS1 oligonucleotides is shown in Fig. 1. Maximal cellular uptake of oligodeoxynucleotides occured at 1 h of incubation and uptake remained unchanged during 5 h. The maximal cellular uptake of the radiolabelled antisense oligonucleotide was approximately 7%. Percentage uptake was calculated with the formula: % uptake = (cpm after cell lysis/10^5) × 100.

3.2. Inhibition of vascular smooth muscle cell DNA synthesis by AS1 and S1 oligodeoxynucleotides

AS1 and S1 oligodeoxynucleotides at a concentration of 0.1 μM lacked significant effects on the platelet-derived growth factor-BB-induced DNA synthesis (Fig. 2A, one representative experiment performed in triplicate wells). AS1 or S1 oligodeoxynucleotides at concentrations of 2, 3 and 5 μM completely inhibited the effect of platelet-derived growth factor-BB effect on DNA synthesis below or to control values, respectively. Data from four individual experiments each performed with triplicate wells were normalised by calculating the means ± S.D. of the individual experiments and expressed as percentage of the value from unstimulated cells (control = 100%) (Fig. 2B). AS1 or S1 oligodeoxynucleotides at a concentration of 3 μM reduced the effect of angiotensin II on DNA synthesis to control values. The difference in the inhibitory efficacy of the AS1 and S1 oligodeoxynucleotides on the platelet-derived growth factor-BB-induced DNA synthesis was statistically not signifficant ($P > 0.05$). Statistical analysis of the data was performed using the Mann-Whitney U-test (StatView 512^{+TM}, version 1.0, Apple Computer).

3.3. The AS1 or the S1 oligodeoxynucleotides do not inhibit the expression of egr-1 mRNA

The expression of egr-1 mRNA was analyzed by Northern blot analysis following incubation of the cells with platelet-derived growth factor-BB and angiotensin II. An-

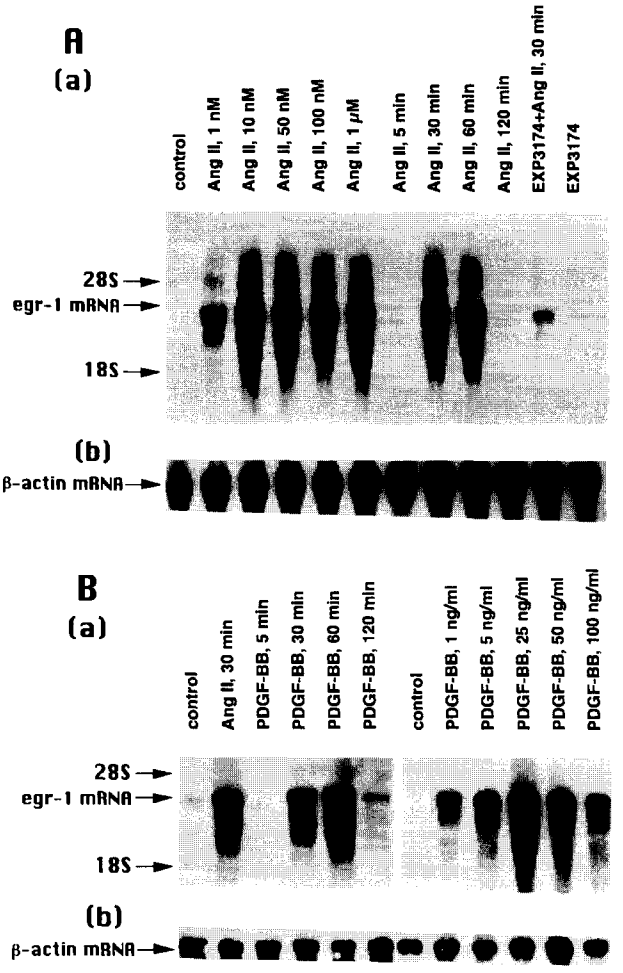
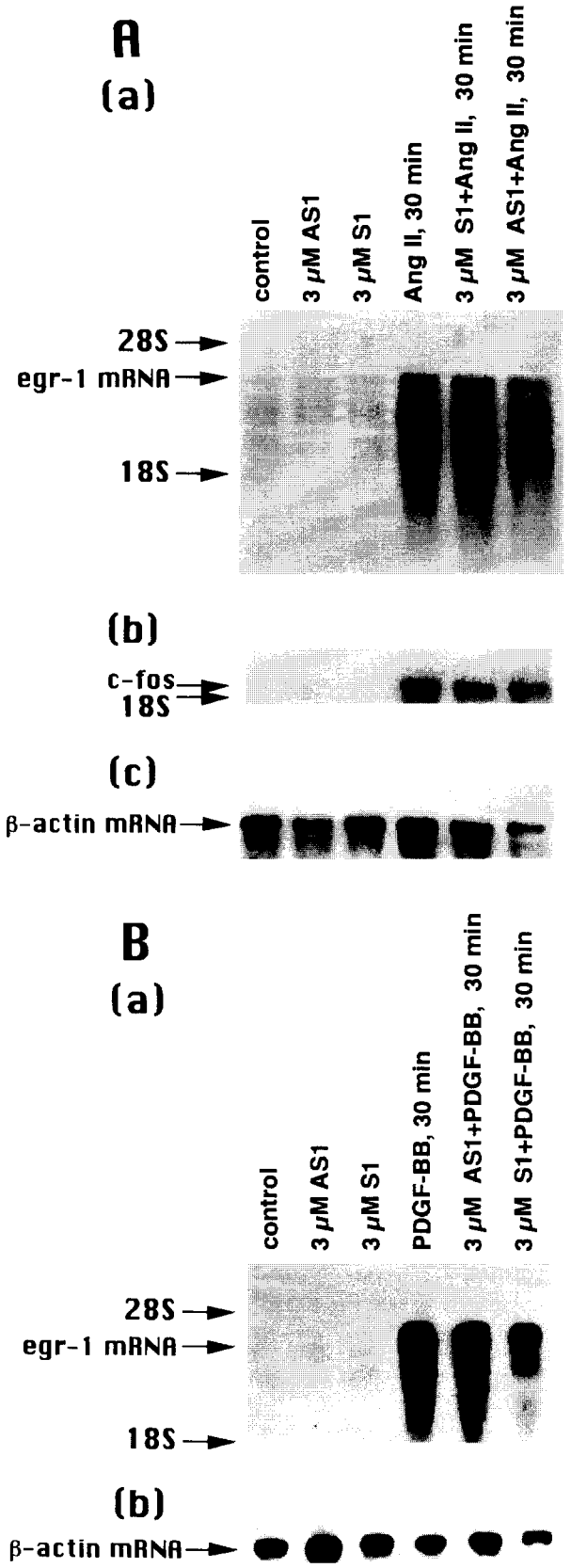


Fig. 3. Effects of platelet-derived growth factor-BB and angiotensin II on the egr-1 mRNA expression. Confluent cells were precultured for 24 h in the serum-free medium. (A) Then cells were stimulated with angiotensin II (100 nM) for different time periods or with different concentrations angiotensin II for 30 min. Control experiments were performed after preincubation of the cells with EXP3174 (100 nM) for 10 min and then cells were stimulated with 100 nM angiotensin II for 30 min. (B) Cells were stimulated with platelet-derived growth factor-BB (50 ng/ml) for different time periods or with different concentrations of platelet-derived growth factor-BB for 30 min. Ten micrograms of total RNA were separated on a formaldehyde-agarose gel, blotted onto Hybond N^+ membranes and probed with a ^{32}P-labelled 2.1 kb fragment of egr-1 which hybridised to the 3.4 kb egr-1 mRNA. (B) The same membrane was rehybridized with a 0.77 kb cDNA probe for β-actin. Arrows indicate the 28S (4.6 kb), the 18S ribosomal RNA (1.8 kb), the egr-1 mRNA (3.4 kb) and the β-actin mRNA (2.0 kb).

giotensin II induced a rapid accumulation of 3.4 kb egr-1 mRNA with a maximum between 30 min and 60 min (Fig. 3A). The egr-1 mRNA declined to basal levels after 2 h.



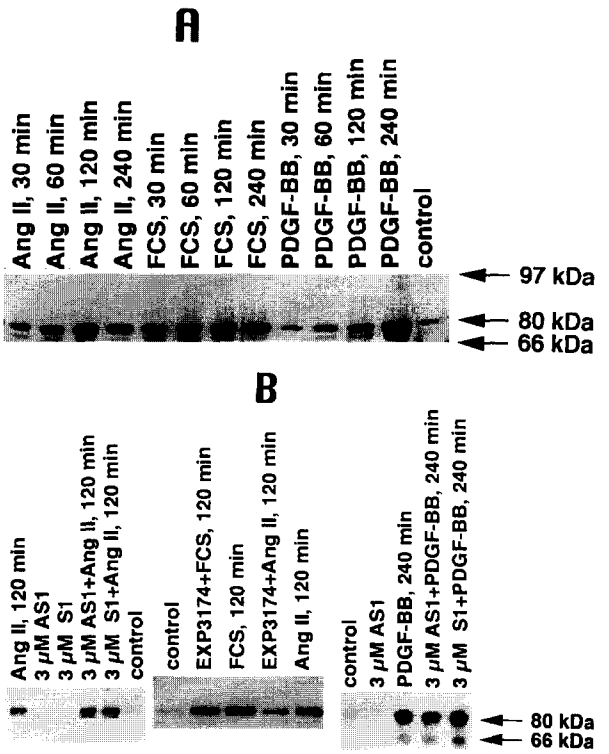


Fig. 5. Effects of angiotensin II, platelet-derived growth factor-BB and fetal calf serum on the Egr-1 protein formation; effects of the S1 and AS1 oligodeoxynucleotides on the angiotensin II- and platelet-derived growth factor-BB-induced increase in Egr-1 protein. (A) Cells were incubated for 24 h in serum-free medium. Then cells were stimulated with 50 ng/ml platelet-derived growth factor-BB, 100 nM angiotensin II and 10% fetal calf serum for different time periods. Cells were harvested and lysed as described in Methods. After sodium dodecyl sulfate-polyacrylamide gel elektrophoresis in a 7.5% polyacrylamide gel, Western blot analysis was performed with the R5232-T antiserum. (B) Effect of the S1 and AS1 on the angiotensin II- and platelet-derived growth factor-BB-inducede Egr-1 protein level at times indicated. Cells were preincubated with 3 μM for 2 h before stimulation with platelet-derived growth factor-BB or angiotensin II. Control experiment showing the effect of EXP3174 on the angiotensin II-induced induction of the Egr-1 protein. Cells were precultured with 100 nM EXP3174 for 10 min before stimulation with 100 nM angiotensin II.

Fig. 4. Effects of the S1 and AS1 oligodeoxynucleotides on angiotensin II- and platelet-derived growth factor-BB-induced increase of egr-1 mRNA. Confluent cells were precultured for 24 h in the serum-free medium. Oligodeoxynucleotides (3 μM) have been added to quiescent cells 2 h before stimulation with 100 nM angiotensin II (A) or platelet-derived growth factor-BB (50 ng/ml) (B). Ten micrograms of total RNA were separated on a formaldehyde-agarose gel, blotted onto Hybond N^+ membranes and probed with a [^{32}P]labelled 2.1 kb fragment of egr-1 which hybridised to the 3.4 kb egr-1 mRNA. A(b) and (c): the same blot previously hybridised with the 2.1 kb fragment of egr-1 was rehybridised with a [^{32}P]labelled 1.0 kb v-fos cDNA probe and then again rehybridised with a [^{32}P]labelled 0.77 kb cDNA probe for β-actin mRNA. B(b): the same blot previously hybridised with the 2.1 kb fragment of egr-1 was rehybridised with a [^{32}P]labelled 0.77 kb cDNA probe for β-actin mRNA. Arrows indicate the 28S (4.6 kb), the 18S ribosomal RNA (1.8 kb), the egr-1 mRNA (3.4 kb), the mRNA of c-fos (2.2 kb) and the β-actin mRNA (2.0 kb).

Furthermore, angiotensin II induced a dose-dependent increase in egr-1 mRNA level with a maximum at 10 nM. Control experiments with the AT1 receptor antagonist EXP3174 showed that EXP3174 blocked the expression of egr-1 mRNA almost to basal levels (Fig. 3A). As shown in Fig. 3B, platelet-derived growth factor-BB also induced a rapid accumulation of 3.4 kb egr-1 mRNA with a maximum at 60 min and declined to basal levels after 2 h. The angiotensin II-induced increase of the egr-1 mRNA at 30 min was approximately equivalent to that induced by platelet-derived growth factor-BB at 60 min. Platelet-derived growth factor-BB caused a dose-dependent increase of the egr-1 mRNA with a maximum at a concentration between 25 and 50 ng/ml.

As shown in Fig. 4A and Fig. 4B, both the sense or antisense oligonucleotides lacked effects on the angiotensin II- and platelet-derived growth factor-BB-induced expression of the maximal egr-1 mRNA formation at 30 min (one representative experiment from three separate experiments). The possibility that the used oligodeoxynucleotides could unspecifically influence the c-fos levels causing a suppression of the agonist-induced cell proliferation is excluded since c-fos levels also remained unchanged in the presence of the oligodeoxynucleotides [Fig. 4A(b)]. As demonstrated in Fig. 3A, EXP3174 blocked the expression of egr-1 mRNA to basal levels. Densitometric analysis from three different independent experiments revealed that in contrast to EXP3174 (100 nM), which inhibited the angiotensin II-induced egr-1 mRNA expression by 80 ± 8% (mean ± S.D.), both oligodeoxynucleotides lacked significant effects on egr-1 mRNA levels.

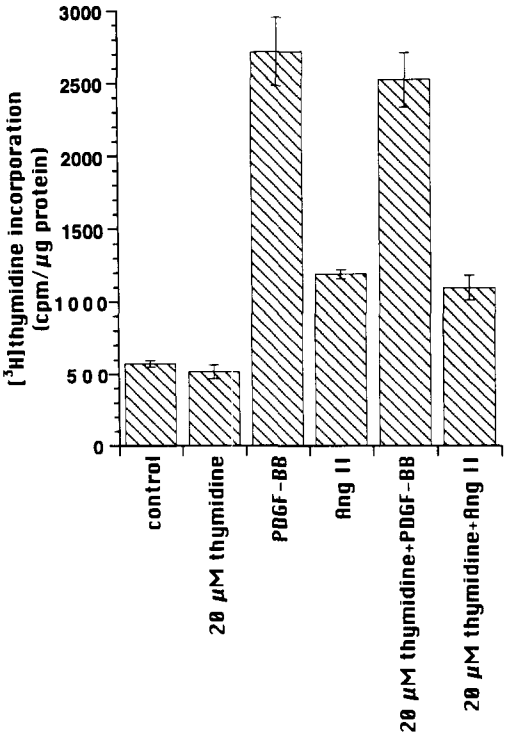


Fig. 6. Effects of α-thymidine on the angiotensin II- and platelet-derived growth factor-BB induced increase in DNA synthesis. Confluent cells were cultured in serum-free medium for 22 h. Then 20 μM α-thymidine has been added to the cells 2 h before stimulation with platelet-derived growth factor-BB (50 ng/ml) or angiotensin II (100 nM). Following another 20 h incubation, cells were exposed to 3 μCi/ml [³H]thymidine. The reaction was terminated 4 h later and [³H]thymidine incorporation into the cell DNA was quantified (mean ± S.D., one representative experiment performed in triplicate).

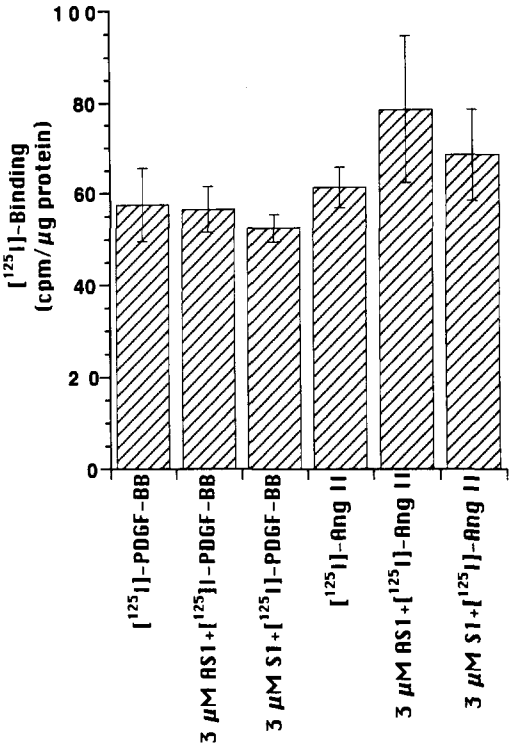


Fig. 7. Effects of the S1 and AS1 oligodeoxynucleotides on the [¹²⁵I]angiotensin II and [¹²⁵I]platelet-derived growth factor-BB binding on vascular smooth muscle cells: confluent cells in 24-well culture plates were preincubated in serum-free medium for 22 h. Then cells were preincubated with oiligodeoxynucleotides for 2 h before [¹²⁵I]platelet-derived growth factor-BB (25000 cpm/well) or [¹²⁵I]angiotensin II (145000 cpm/well) were added to the medium for 10 or 45 min at 37°C, respectively. Data represent the mean ± S.D. of a triplicate determination.

3.4. The AS1 or the S1 oligodeoxynucleotides do not inhibit the Egr-1 protein translation

Stimulation of vascular smooth muscle cells with angiotensin II, fetal calf serum and platelet-derived growth factor-BB increased the sythesis of 80 kDa Egr-1 protein which reached a maximum between 120 and 240 min (Fig. 5A). Fig. 5B shows the effect of the oligodeoxynucleotides on the angiotensin II- and platelet-derived growth factor-BB-induced production of the Egr-1 protein. Densitometric analysis from three different independent experiments revealed that in contrast to EXP3174 which caused an 70 ± 13% inhibition of the Egr-1 protein (mean ± S.D.),

both the antisense and sense oligodeoxynucleotides did not influence the Egr-1 protein formation induced by angiotensin II and platelet-derived growth factor-BB. As expected, the fetal calf serum-induced increase of the Egr-1 protein was not influenced by EXP3174 indicating the high specificity of EXP3174 to angiotensin II.

3.5. Unlabelled thymidine had no effects on the [^{3}H]thymidine incorporation

Oligodeoxynucleotides might undergo degradation in the cell culture medium releasing thymidine bases which might compete with exogenous [^{3}H]thymidine, and thereby resulting in a nonspecific suppression of [^{3}H]thymidine incorporation. To exclude this hypothesis, control experiments were performed using different concentrations of nonlabelled thymidine. As demonstrated in Fig. 6 even 20 μM of α-thymidine did not influence the [^{3}H]thymidine incorporation into cell DNA.

3.6. The AS1 or the S1 oligodeoxynucleotides do not interact with the angiotensin II or platelet-derived growth factor-BB receptors

To rule out a possible interaction of the oligodeoxynucleotides with the angiotensin II- or platelet-derived growth

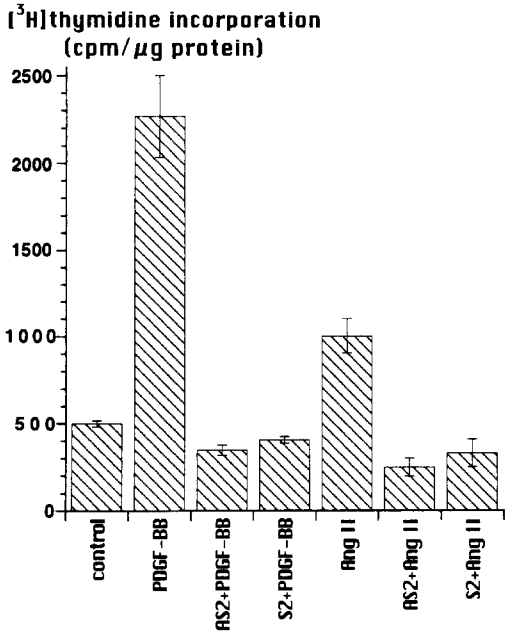


Fig. 8. Effects of the S2 and AS2 oligodeoxynucleotides on the platelet-derived growth factor-BB- and angiotensin II-induced cell DNA synthesis in vascular smooth muscle cells. (A) Confluent cells (24-well plates) were precultured for 22 h in the serum-free medium. Oligodeoxynucleotides have been added at a concentration of 3 μM to serum-free medium 2 h before stimulation with platelet-derived growth factor-BB. Following another 20 h incubation, cells were exposed to 3 μCi/ml [^{3}H]thymidine. Four h later the reaction was terminated and cell protein and [^{3}H]thymidine incorporation into cell DNA was quantified. Data represent the means ± S.D. of a triplicate determination.

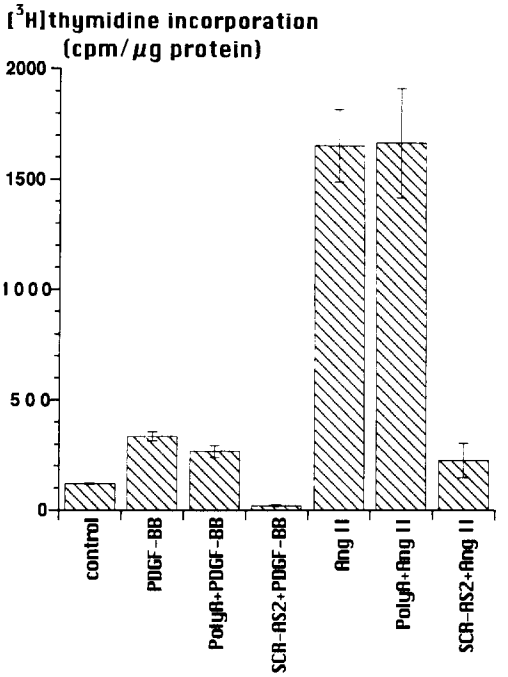


Fig. 9. Effects of PolyA and SCR-AS2 oligonucleotides on the cell DNA synthesis. Confluent cells (24-well plates) were precultured for 22 h in the serum-free medium. Oligodeoxynucleotides have been added at a concentration of 3 μM to serum-free medium 2 h before stimulation with platelet-derived growth factor-BB. Following another 20 h incubation, cells were exposed to 3 μCi/ml [^{3}H]thymidine. Four hours later the reaction was terminated and cell protein and [^{3}H]thymidine incorporation into cell DNA was quantified. Data represent the means ± S.D. of a triplicate determination.

factor receptors, the effect of the oligodeoxynucleotides on the [^{125}I]angiotensin II- and [^{125}I]platelet-derived growth factor-BB binding to vascular smooth muscle cells was investigated. As indicated in Fig. 7, both oligodeoxynucleotides lacked significant effects on the binding [^{125}I]angiotensin II- and [^{125}I]platelet-derived growth factor-BB.

3.7. The AS2, the S2 and the SCR-AS2 oigodeoxynucleotides inhibit the cell DNA synthesis

As shown in Fig. 8, platelet-derived growth factor-BB and angiotensin II caused a 4.5-fold and 2-fold increase in [^{3}H]thymidine incorporation into cell DNA, respectively (mean ± S.D., one experiment performed in triplicate). In the presence of 3 μM AS2 or 3 μM S2 oligonucleotides, the effect of both agonists was completely inhibited.

To ascertain that the inhibitory effects of the oligodeoxynucleotides on cell growth are not exerted by toxic contaminants during the isolation procedure of the oligodeoxynucleotide control experiments were performed using PolyA (15-mer) and SCR-AS2 oligodeoxynucleotides. Oligodeoxynucleotides were synthesized successively. As shown in Fig. 9, PolyA lacked significant effects on the platelet-derived growth factor-BB and an-

giotensin II-induced DNA synthesis. In contrast, SCR-AS2 suppressed the effect of both agonists below basal values.

4. Discussion

Our results provide evidence that oligodeoxynucleotides inhibit platelet-derived growth factor-BB- and angiotensin II-induced DNA synthesis in vascular smooth muscle cells. However, agonist-induced increase of egr-1 mRNA and Egr-1 protein was not suppressed by antisense and sense oligodeoxynucleotides. These findings suggest that oligodeoxynucleotides inhibited DNA synthesis by mechanisms which cannot be attributed to antisense mechanisms.

The induction of egr-1 by angiotensin II, platelet-derived growth factor-BB and fetal calf serum occurred rapidly and transiently with kinetics similar to those of several other early response transcription factor genes (Murakami et al., 1991; Sukhatme, 1990). Egr-1 binding sites occur in the 5′ domains of a wide range of genes. These include egr-1 itself (Darland et al., 1991), c-fos, platelet-derived growth factor-A, c-ras and junD (Christy and Nathans, 1989) whose products could affect the activities of other genes and therefore lead to a growth response.

We have demonstrated that approximately 7% of [^{32}P]labelled AS1 oligodeoxynucleotides were taken up after 1 h and uptake remained unchanged within 5 h. The mechanism of cellular uptake of the oligodeoxynucleotides is controversially discussed. Loke et al. (1989) suggested a receptor-mediated uptake at least in cell lines, which can be inhibited by competitor DNA. In general, it is believed that oligodeoxynucleotides are taken up by pinocytosis and/or recpetor-mediated endocytosis after binding to cell surface proteins. Such candidate receptor proteins have been described (for review see Leonetti et al., 1993).

The present results show that aside from PolyA all of the antisense and sense oligodeoxynucleotides used, significantly inhibited DNA synthesis induced by two potent growth factors. Cellular toxicity of the oligodeoxynucleotides was excluded by the trypan blue method (data not shown). Furthermore, to demonstrate that the inhibitory effects of the oligodeoxynucleotides on cell growth are not exerted by any toxic contaminants during the isolation of the oligodeoxynucleotides, control experiments were performed with PolyA (15-mer). Since in contrast to oligodeoxynucleotides used in the present study, PolyA did not influence cell DNA synthesis, toxic effects of contaminants on vascular smooth muscle cells may be excluded. Also AS1 or S1 oligodeoxynucleotides purified by high pressure liquid chromatography (HPLC) from a commercial supplier (MWG-Biotech, Ebersberg, Germany) suppressed the agonist-induced increase in cell DNA synthesis without inhibition egr-1 mRNA exression or the Egr-1 protein levels (data not shown). However, the mechanisms of action remain unclear, because Northern as well as Western analysis did not reveal suppression of both egr-1 mRNA and Egr-1 protein. Our control experiments using EXP3174, an AT1 angiotensin II receptor antagonist, indicated that both methods are sensitive for detection of a suppression egr-1 mRNA and protein.

Several reports describe that oligodeoxynucleotides targeted to proto-oncogenes like c-fos (Nishikura and Murray, 1987) or c-myc (Biro et al., 1993) as well as to genes encoding nonmuscle myosin heavy chain or c-myb (Simon and Rosenberg, 1992) specifically inhibit vascular smooth muscle cell proliferation both in vitro and in vivo. Shi et al. (1993) reported that antisense oligodeoxynucleotides directed to c-myc mRNA specifically inhibited human vascular smooth muscle cell proliferation and c-myc mRNA induction. The same group reported that c-myc antisense oligodeoxynucleotides reduced the formation of neointima in denuded porcine coronary arteries whereas c-myc sense oligodeoxynucleotides were ineffective (Shi et al., 1994). Bennett et al. (1994) reported that antisense oligodeoxynucleotides directed to c-myc reduced rat vascular smooth muscle cell proliferation in vitro and significantly reduced neointima formation in carotid artery after balloon injury. Sense oligodeoxynucleotides were ineffective (Bennett et al., 1994). As recently discussed by Wagner (1994), each of these studies demonstrated an inhibition of biological effects of the antisense oligodeoxynucleotides. However rigorous inhibition of the target RNA or protein has not been demonstrated. In this context, specificity has been inferred from the biological effect of the antisense as compared to control sense oligodeoxynucleotide. However, many biological effects of the oligodeoxynucleotides are not specific and cannot be attributed to the antisense mechanism alone (for review see Epstein et al., 1993; Wagner, 1994).

In contrast to the reports indicating apparent specific effects of antisense oligonucleotides, Burgess et al. (1995) demonstrated that the antiproliferative activity of c-myc and c-myb antisense oligodeoxynucleotides in smooth muscle cells is caused by nonantisense mechanism. Our findings are supported from the results of Epstein et al. (1993), Milligan et al. (1993) and Burgess et al. (1995) who demonstrated that inhibition of the vascular smooth muscle cell proliferation by oligodeoxynucleotides in vitro is mediated through nonantisense mechanisms. These authors reported that oligodeoxynucleotides (e.g. scrambled and sense oligodeoxynucleotides) inhibited vascular smooth muscle cell proliferation similarly to antisense oligodeoxynucleotides.

The antiproliferative effect of egr-1 oligodeoxynucleotides may be mediated via several nonantisense mechanisms which are extensively discussed by Epstein et al. (1993) and Wagner (1994). Authors described that oligodeoxynucleotides are able to bind to small molecules and proteins in a nonspecific manner and thereby they may directly interact with protein components, i.e. components of the cell cycle, resulting in a suppression of cell DNA synthesis. DNA-protein interactions frequently occur at physiological temperatures. Oligodeoxynucleotides in cell

culture may be degraded both intra- and extracellularly to nucleosides or nucleotides. The degradation products of the oligodeoxynucleotides can influence cell proliferation, especially at the high concentrations used in most of the cell culture experiments. In addition, it seems to be possible that oligodeoxynucleotides may directly influence ribosomal mRNA translation in a nonspecific manner. It is also conceivable that antisense and control oligodeoxynucleotides may decrease the stability of several mRNA due to conformational changes caused by their hybridization. Antisense and control oligodeoxynucleotides (sense, and scrambled oligodeoxynucleotides) may potentially be hybridized to other cellular mRNA sequences in a nonspecific manner resulting in an inhibition of cell DNA synthesis. Cross-hybridazation of oligodeoxynucleotides or their degradation products with several mRNAs in the cytosol at the cytosolic temperature of 37°C is likely. It is possible that oligodeoxynucleotides which contain the nucleotides G and/or C may cross-hybridize with several mRNAs at 37°C and thus may influence cell DNA synthesis. Since PolyA does not contain the nucleotides G and/or C, cross-hybridization of PolyA with other mRNAs at 37°C may not be strong enough to influence DNA synthesis. The possibility that the used oligodeoxynucleotides could unspecifically influence the c-fos and c-myc mRNA levels leading to a suppression of the agonist-induced cell proliferation can be excluded since oligodeoxynucleotides lacked any effects on the c-fos and c-myc mRNA levels (c-myc data not shown).

The [^{3}H]thymidine incorporation assay is a common assay used to examine antisense efficacy for growth inhibition by measuring the incorporation of [^{3}H]thymidine into cell DNA. Objections against the use of [^{3}H]thymidine incorporation technique to assess the influence of antisense oligodeoxynucleotides on cell growth have been raised (Matson and Krieg, 1992). It has been argued that oligodeoxynucleotides might undergo degradation in the cell culture resulting in a release of thymidine which might compete with exogenous [^{3}H]thymidine, thereby resulting in an apparent suppression of [^{3}H]thymidine incorporation. This mechanism could be ruled out as we have demonstrated that high concentrations of nonradioactive exogenous thymidine had no effect on the agonist-induced [^{3}H]thymidine incorporation into cell DNA.

Oligodeoxynucleotides might also interact with the components of the cell surface such as growth factor receptors and hence disturb the binding of the growth factor to its receptor. Such interactions of labelled oligodeoxynucleotides with surface receptor proteins have previously been described in other cell types (Storey et al., 1991; Yabukov et al., 1989). In the present study interactions between oligodeoxynucleotides and the angiotensin II or platelet-derived growth factor receptor could be ruled out as no effects of oligodeoxynucleotides on the binding of [^{125}I]angiotensin II and [^{125}I]platelet-derived growth factor-BB on vascular smooth muscle cells was observed.

In conclusion, the results from the present study provide evidence that antisense and sense oligodeoxynucleotides directed to the egr-1 gene inhibit platelet-derived growth factor-BB and angiotensin II-induced cell DNA synthesis. Furthermore, these effects were not due to blunted translation with impaired synthesis of the Egr-1 protein as oligodeoxynucleotides did neither inhibit egr-1 mRNA nor Egr-1 protein synthesis. Thus, the mechanism(s) by which the egr-1 antisense oligodeoxynucleotides mediated their inhibitory effects on vascular smooth muscle cell growth cannot be attributed to antisense mechanisms. From a more general perspective, our results illustrate the need for a careful interpretation of results obtained with antisense oligodeoxynucleotides regarding their specificity.

Acknowledgements

This work was supported by a grant of the DFG (Sa 568/1-1 and 568/1-2). We are particularly indebted to Vikas P. Sukhatme, Howard Hughes Medical Institute, Department of Medicine, Molecular Genetics and Cell Biology, University of Chicago, IL 60637, USA for providing the 2.1 kb Egr-1 cDNA probe and the R5232-T antiserum. The excellent technical assistence of Marianne Appenheimer and Petra Epping was greatly appreciated.

References

Bennett, M.R., S. Anglin, J.R. McEwwan, R. Jagoe, A. Newbly and G. Evan, 1994, Inhibition of vascular smooth muscle cell proliferation in vitro and in vivo by c-myc antisense oligonucleotides, J. Clin. Invest. 93, 820.

Bevan, R.D., E. Van Marthens and J.A. Bevan, 1976, Hyperplasia of vascular smooth muscle in experimantal hypertension in the rabbit, Circ. Res. 38 (Suppl. 2), 58.

Biro, S., Y. Fu, Z. Yu and S.E. Epstein, 1993, Inhibitory effects of antisense oligodeoxynucleotides targeting c-myc mRNA on smooth muscle cell proliferation and migration, Proc. Natl. Sci. USA 90, 654.

Bradford, M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248.

Burgess, T.L., E.F. Fisher, S.L. Ross, J.V. Bready, Y.X. Qian, L.A. Bayewitch, A.M. Cohen, C.J. Herrera, S.S. Hu and T.B. Kramer, 1995, The antiproliferative activity of c-myb and c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism, Proc. Natl. Acad. Sci. USA 92, 4051.

Campbell, D.J., 1986, Circulating and tissue angiotensin system, J. Clin. Invest. 79, 1.

Cao, X., R.A. Koshi, A. Gashler, M. McKiernan, C.F. Morris, R. Gaffney, R.V. Hay and V.P. Sukhatme, 1990, Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals, Mol. Cell Biol. 10, 1931.

Chirgwin, J.J., R.J. Przybyla, R.J. MacDonald and W.J. Rutter, 1979, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease, Biochemistry 18, 5294.

Christy, B.A. and D. Nathans, 1989, DNA-binding site of the growth factor-inducible protein Zif268, Proc. Natl. Acad. Sci. USA 86, 8737.

Darland, T., M. Samuels, S.A. Edwards, V.P. Sukhatme VP and E.D. Adamson, 1991, Regulation of Egr-1 (zfp-6) and c-fos expression in differentiating embryonal carcinoma cells, Oncogene 6, 1367.

Epstein, S.E., E. Speir and T. Finkel, 1993, Do antisense approaches to the problem of restenosis make sense, Circulation 88, 1351.

Folkow, B., M. Hallback, Y. Lundgren, R. Sivertsson and L. Weiss, 1973, Importance of adaptive changes in vascular design for establishment of primary hypertension, studied in man and spontaneously hypertensive rats, Circ. Res. 32/33 (Suppl. 1), 2.

Leonetti, J.P, G. Degols, J.P. Clarenc, N. Mechti and B. Lebleu, 1993, Cell Delivery and Mechanisms of Action of Antisense Oligonucleotides, Progr. Nucl. Acid. Res. Mol. Biol. 44, 143.

Loke, S.L., C.A. Stein, X.H. Zhang, K. Mori, M. Nakanishi, C. Subasinghe, J.S. Cohen and L.M. Neckers, 1989, Characterization of oligonucleotide transport into living cells, Proc. Natl. Acad. Sci. USA 86, 3474.

Matson, S. and A.M. Krieg, 1992, Nonspecific suppression of [^{3}H]thymidine incorporation by 'control' oligonucleotides, Antisense Res. Dev. 2, 325.

Milbrandt, J., 1987, A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor, Science 238, 797.

Milligan, J.F., M.D. Matteucci and J.C. Martin, 1993, Current concepts in antisense drug design, J. Med. Chem. 36, 1923.

Murakami, Y., M. Satake, Y. Yamguchi-Iwai, M. Sakai, M. Murametsu and Y. Ito, 1991, The nuclear protooncogenes c-jun and c-fos as regulators of DNA replication, Proc. Natl. Acad. Sci. USA 88, 3947.

Nemecek, G.M., S.R. Coughlin, D.H. Handley and M. Moskowitz, 1986, Stimulation of aortic smooth muscle cell mitogenesis by serotonin, Proc. Natl. Acad. Sci. USA 83, 674.

Nishikura, K. and J. Murray, 1987, Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells, Mol. Cell. Biol. 7, 639.

Ross, R.J., 1971, The smooth muscle cell II. Growth of smooth muscle in culture and formation of elastic fiber, J. Cell Biol. 50, 172.

Ross, R., E.W. Raines and D.F. Bowen-Pope, 1986, The biology of platelet-derived growth factor, Cell 46, 155.

Sachinidis, A., R. Locher, W. Vetter, D. Tatje and J. Hoppe, 1990, Different effects of PDGF-isoforms on rat vascular smooth muscle cells, J. Biol. Chem. 265, 10238.

Sambrook, J., E.F. Fritsch and T. Maniatis, 1989, Molecular Cloning. A Laboratory Manual (Cold Spring Harbor Laboratory Press, Second Edition, New York).

Schelling, P., H. Fischer and D. Ganten, 1991, Angiotensin and cell growth: a link to cardiovascular hypertrophy, J. Hypertens. 9, 3.

Schwarz, S.M. and M. Reidy, 1987, Common mechanisms of proliferation of smooth muscle in atherosclerosis and hypertension, Hum. Pathol. 18, 240.

Shi, Y., H.G. Hutchinson, D.J. Hall and A. Zalewski, 1993, Downregulation of c-myc expression by antisense oligonucleotides inhibits proliferation of human smooth muscle cells, Circulation 88, 1190.

Shi, Y., A. Fard, A. Galeo, H.G. Hutchinson, P. Vermani, G.R. Dodge, D.J. Hall, F. Sheen and A. Zalewski, 1994, Transcatheter Delivery of c-myc antisense oligomers reduces neointimal formation in a porcine model of coronary artery balloon injury, Circulation 90, 944.

Simm, A., V. Hoppe, D. Tatje, A. Schenzinger and J. Hoppe, 1992, PDGF-AA effectively stimulates early events but has no mitogenic activity in AKR-2B mouse fibroblasts, Exp. Cell Res. 201, 192.

Simon, M. and R.D. Rosenberg, 1992, Antisense nonmuscle myosin heavy chain and c-myb oligonucleotides suppress smooth muscle cell proliferation in vitro, Circ. Res. 70, 835.

Stein, C.A., C. Subasinghe, K. Shinozuka and S. Cohen, 1988, Nucl. Acids Res. 16, 3209.

Storey, A., D. Oates, L. Banks, L. Crawford and T. Crook, 1991, Antisense phosphorothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16, Nucl. Acids Res. 19, 4109.

Sukhatme, V.P., 1990, Early transcriptional events in cell growth: the Egr family, J. Am. Soc. Nephrol. 1, 859.

Sukhatme, V.P., X. Cao, L.C. Chang, C.H. Tsai-Morris, D. Stamenkovich, P.C.P. Ferreira, D.R. Cohen, S.A. Edwards, T.B. Shows, T. Curran, M.M. LeBeau and E.D. Adamson, 1988, A zinc finger-encoding gene coregulated with c-fos during growth and differeniation, and after cellular depolarization, Cell 53, 37.

Toulme, J.J, and C. Helene, 1988, Antimessenger oligodeoxyribonucleotides: an alternative to antisense RNA for artifical regulation of gene expression: a review, Gene 72, 51.

Verma, I.M and P. Sassone-Corsi, 1987, Proto-oncogene fos: complex but versatile regulation, Cell 51, 513.

Wagner, R.W., 1994, Gene inhibition using antisense oligonucleotides, Nature 372, 333.

Yabukov, L.A., E.A. Deeva, V.F. Zarytova, E.M. Ivanova, L.V. Yurchenko and V.V. Vlassov, 1989, Mechanisms of oligonucleotide uptake by cells: involvement of specific receptors?, Proc. Natl. Acad. Sci. USA 86, 6454.