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European Journal of Pharmacology 392 (2000) 129–132

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Short communication

Effect of methylene methylimino linkage of antisense oligonucleotide to the platelet-derived growth factor A-chain on growth of vascular smooth muscle cells from spontaneously hypertensive rats

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Received 21 October 1999; received in revised form 17 January 2000; accepted 21 January 2000

Abstract

Spontaneously hypertensive rats (SHR)-derived vascular smooth muscle cells show exaggerated growth and increased expression of platelet-derived growth factor (PDGF) A-chain mRNA. We examined the effect of methylene methylimino linkage of antisense oligodeoxynucleotide, a novel modification of antisense oligodeoxynucleotide designed to increase nuclease resistance, to PDGF A-chain on the exaggerated growth of vascular smooth muscle cells from SHR. Methylene methylimino-linked oligodeoxynucleotide provided complete resistance against S1 nuclease. Methylene methylimino linkage of antisense oligodeoxynucleotide to PDGF A-chain resulted in a rapid inhibition of basal DNA synthesis of vascular smooth muscle cells from SHR. This inhibition was much greater than that produced by phosphorothioate linkage of antisense oligodeoxynucleotide to PDGF A-chain. The methylene methylimino linkage of antisense oligodeoxynucleotide to PDGF A-chain may prove useful in the treatment of arterial proliferative diseases including hypertension.

Keywords: Methylene methylimino linkage; Antisense; Platelet-derived growth factor A-chain; Spontaneously hypertensive rat (SHR); Smooth muscle cell, vascular

1. Introduction

The exaggerated growth of cardiovascular organs that is observed in patients with essential hypertension as well as in spontaneously hypertensive rats (SHR) results in severe complications such as stroke, ischemic heart disease and nephrosclerosis. We have previously demonstrated an increase in the expression of platelet-derived growth factor (PDGF) A-chain mRNA in vascular smooth muscle cells from SHR (Fukuda et al., 1997a) and noted that an antisense oligodeoxynucleotide complementary to the PDGF A-chain mRNA inhibits the exaggerated growth of cardiovascular organs in SHR in vitro and in vivo (Fukuda et al., 1997b).

In order to be able to use PDGF A-chain antisense oligodeoxynucleotide as a therapeutic agent for arterial proliferative diseases, structural modifications of the antisense oligodeoxynucleotide are required to enhance its resistance to degradation by cellular nucleases. Recently the methylene methylimino linkage, a novel modification of antisense oligonucleotide, has been reported to be resistant to cellular nuclease and to be able to hybridize effectively to its complementary RNA with a high level of base pair specificity (Perbost et al., 1995; Sanghvi et al., 1995). In this study, we examined the effect of methylene methylimino linkage of antisense oligodeoxynucleotide to the PDGF A-chain on the exaggerated growth of vascular smooth muscle cells from SHR.

2. Materials and Methods

2.1. Synthetic oligodeoxynucleotides

Wild-type and phosphorothioate-linked antisense oligodeoxynucleotide complementary to the rat PDGF A-

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00068-6

chain mRNA initiation codon region (5′-AGGTCCT-CATCGCGT-3′) were synthesized by an Applied Biosystems DNA synthesizer (model 394: Foster City, CA, USA). Phosphorothioate linkages were introduced by oxidizing the phosphate linkages with 3*H*-1, 2-benzodithio-3-one1, 1-dioxide instead of the standard iodine reagent. Methylene methylimino-linked antisense oligodeoxynucleotide and a control methylene methylimino-linked reverse oligodeoxynucleotide (5′-TGCGCTACTCCTGGA-3′) were synthesized using a convergent approach in which commercial 2′-deoxynucleotides were appropriately functionalized with a 5′-*O*-amino and a 3′-*C*-formyl group via a series of reactions (Sanghvi et al., 1995).

2.2. Cell culture

Vascular smooth muscle cells were obtained by an explant method from aortas of 10-week-old male SHR/Izumo (SHR, Funabashi, Chiba, Japan) as described previously (Hadrava et al., 1989).. They were seeded and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (Gibco Laboratories, Tokyo, Japan), 100 U/ml of penicillin and 100 mg/ml of streptomycin. When the cells reached confluency (7–10 days), they exhibited a hill-and-valley pattern, which is typical of smooth muscle cells in culture. They were passaged by trypsinization with 0.05% trypsin (Gibco) in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline and incubated in 80-cm^2 tissue culture flasks at a density of 10^5 cells/ml. Experiments were performed on the vascular smooth muscle cells after 10–18 passages. Trypsinized cells were plated in DMEM in 24-well culture dishes. They were allowed to grow in DMEM containing 10% calf serum for 24 h, and the culture medium was then changed to DMEM with 0.2% calf serum. The cells were incubated in this medium for 48–72 h to establish quiescence.

2.3. Stability of oligodeoxynucleotide against S1 nuclease

One microgram of phosphorothioate or methylene methylimino-linked oligodeoxynucleotide was incubated without or with 10 U of S1 nuclease (Takara Biochemicals, Otsu, Siga, Japan) in S1 nuclease buffer (30 mM sodium acetate, 280 mM NaCl and 1 mM $ZnSO_4$) at 23°C for 5, 10 or 30 min, respectively. The oligodeoxynucleotide content was determined by acid precipitation with 0.5 N perchloric acid and hydrolysis by heating for 20 min at 90°C, after which the oligodeoxynucleotide content was estimated using a spectrophotometer (Berk et al., 1978).

2.4. Determination of DNA synthesis

[^{3}H]Thymidine incorporation into newly synthesized DNA was measured as described previously (Frank et al., 1984). First, 24-well cluster dishes holding quiescent vascular smooth muscle cells were incubated without or with 1, 10 or 100 nM oligodeoxynucleotides for 2, 12 or 24 h, respectively. The medium was then changed to DMEM containing 0.5 mCi/ml of [^{3}H]thymidine (New England Nuclear, DE, USA). After 2 h, [^{3}H]thymidine incorporation was terminated by removal of the labeled medium. Each well was washed with 1 ml iso-osmotic solution (150 mM NaCl) to eliminate excess [^{3}H]thymidine, and the cells were fixed in 1 ml ethanol and acetic acid (3:1) solution for 10 min. The fixation solution was then discarded, and the cells were washed with 1 ml of H_2O. Acid-insoluble material was precipitated with 1.0 ml cold perchloric acid, and DNA was extracted into 1.5 ml perchloric acid by heating at 90°C for 20 min. The perchloric acid containing solubilized DNA was transferred to scintillation vials, and the radioactivity was measured using a liquid scintillation spectrometer.

3. Results

Fig. 1 shows the stability of the methylene methylimino linkage of oligodeoxynucleotide to S1 nuclease. Methylene methylimino-linked oligodeoxynucleotide showed complete resistance against S1 nuclease over 30 min. Wild-type and phosphorothioate-linked antisense oligodeoxynucleotide were rapidly degraded by S1 nuclease. Fig. 2 shows the effects of antisense oligodeoxynucleotide linked to PDGF A-chain via a methylene methylimino linkage on the growth of vascular smooth muscle cells compared to that of phosphorothioate-linked antisense oligodeoxynu-

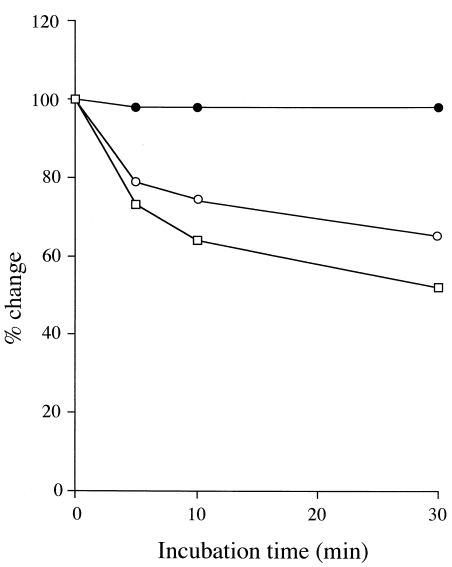

Fig. 1. Stability of oligodeoxynucleotides to S1 nuclease. Wild-type oligodeoxynucleotide (□), phosphorothioate-linked oligodeoxynucleotide (○) and methylene methylimino-linked oligodeoxynucleotide (●) were incubated without or with S1 nuclease for 5, 10 and 30 min. Oligodeoxynucleotide contents were determined by acid precipitation with 0.5 N perchloric acid and hydrolysis by heating for 20 min at 90°C, followed by estimation of oligodeoxynucleotide content by spectrophotometer.

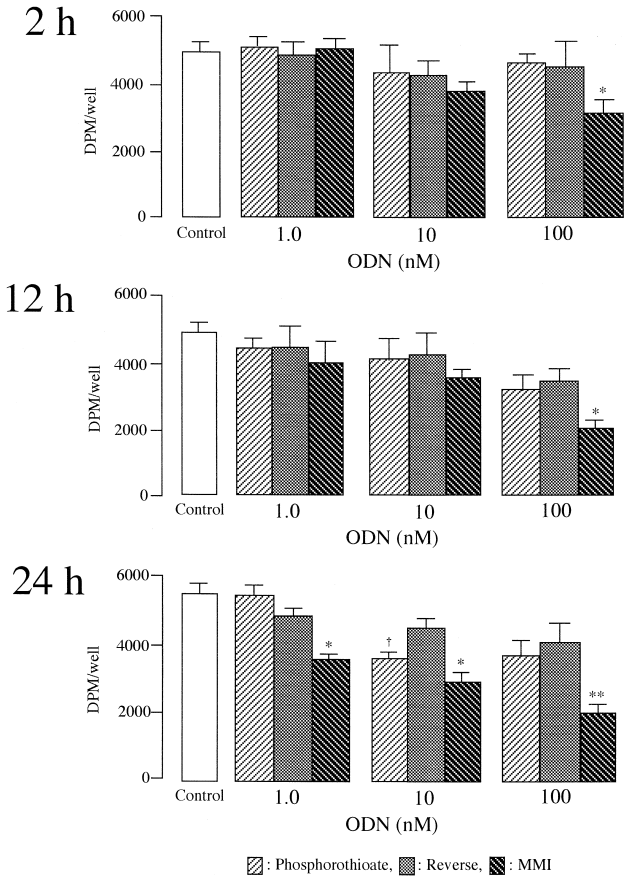

Fig. 2. Effect of antisense oligodeoxynucleotide (ODN) linked to PDGF A-chain via methylene methylimino linkage on basal DNA synthesis in vascular smooth muscle cells from spontaneously hypertensive rats. Quiescent vascular smooth muscle cells were treated without (Control) or with 1.0, 10 or 100 nM of phosphorothioate-linked antisense ODN, methylene methylimino-linked reverse ODN (Reverse) and methylene methylimino-linked antisense ODN (MMI) for 2 , 12 and 24 h. Values are expressed as means ± SEM ($n = 4$). †$P < 0.05$ vs. control, *$P < 0.05$, **$P < 0.01$ vs. phosphorothioate-linked antisense ODN. DPM, disintegrations per minute.

cleotide. At 2 and 12 h, 100 nM methylene methylimino-linked antisense oligodeoxynucleotide significantly ($P < 0.05$) inhibited basal DNA synthesis in vascular smooth muscle cells compared to phosphorothioate-linked antisense oligodeoxynucleotide. At 24 h, 1–100 nM methylene methylimino-linked antisense oligodeoxynucleotide significantly ($P < 0.05$) inhibited the basal DNA synthesis of vascular smooth muscle cells compared to the effect of phosphorothioate-linked antisense oligodeoxynucleotide. Methylene methylimino-linked PDGF A-chain reverse oligodeoxynucleotide did not affect the basal DNA synthesis of vascular smooth muscle cells.

4. Discussion

PDGF is thought to mediate the proliferation of vascular smooth muscle cells in injured arteries and to be involved in the pathogenesis of atherosclerosis (Ross et al., 1986). Nilsson et al. (1985) demonstrated that normal, growth-arrested smooth muscle cells do not express PDGF A-chain mRNA, whereas cultured vascular smooth muscle cells or vascular smooth muscle cells in atherosclerotic plaques express PDGF A-chain mRNA and secrete PDGF-AA protein. We demonstrated that inhibition of the final responsible growth factor, PDGF A-chain, by antisense oligodeoxynucleotide can suppress arterial proliferation in SHR without altering blood pressure in vivo (Fukuda et al., 1997b). In addition, two isoforms of PDGF A-chain mRNA that either include (long form) or exclude (short form) exon 6 are produced in rat vascular smooth muscle cells as a result of alternative splicing (Hu et al., 1997). We observed that SHR-derived vascular smooth muscle cells increasingly express either the short or long form of PDGF A-chain mRNA and that the long form of PDGF A-chain is important for the exaggerated growth of vascular smooth muscle cells from SHR (Fukuda et al., 1997a).

In the field of cardiovasology, several lines of research have suggested that gene therapy may be applicable for nearly every form of cardiovascular diseases, including arterial proliferative diseases such as atherosclerosis, arterial restenosis after balloon angioplasty and hypertension. One goal of gene therapy for cardiovascular diseases is to inhibit a pathologic process such as vascular smooth muscle cell hyperproliferation. For application of the drug delivery system of antisense oligodeoxynucleotide as gene therapy, several chemically modified forms of oligodeoxynucleotide such as meythylphosphorothioate, phosphorothioate or phosphoramide types have been developed to promote resistance to degradation by cellular nucleases and enhancement of cellular uptake (Karen et al., 1993). In the present study, we found that the methylene methylimino linkage of antisense oligodeoxynucleotide to PDGF A-chain resulted in complete resistance to degradation by S1 nuclease, and that this antisense oligodeoxynucleotide rapidly and potentialy inhibited the exaggerated growth of vascular smooth muscle cells from SHR.

These findings suggest that antisense oligodeoxynucleotide linked to PDGF A-chain via methylene methylimino will be a possible treatment for arterial proliferative diseases, including hypertension.

Acknowledgements

This work was supported in part by a grant-in-aid for the High-Tech Research Center from the Japanese Ministry of Education, Science, Sports and Culture to Nihon University, and by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (0967076). We thank Dr. T. Hoshiko for his helpful support to this study.

References

Berk, A.J., Sharp, P.A., 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. USA 75, 1274–1278.

Frank, D.J., Plamondon, J., Hamet, P., 1984. An increase in adenylate cyclase activity precedes DNA synthesis in cultured vascular smooth muscle cells. J. Cell. Physiol. 119, 41–45.

Fukuda, N., Kishioka, H., Satoh, C., Nakayama, T., Watanabe, Y., Soma, M., Izumi, Y., Kanmatsuse, K., 1997a. Role of long-form PDGF A-chain in the growth of vascular smooth muscle cells from spontaneously hypertensive rats. Am. J. Hypertens. 10, 1117–1124.

Fukuda, N., Kubo, A., Watanabe, Y., Nakayama, T., Soma, M., Izumi, Y., Kanmatsuse, K., 1997b. Antisense oligodeoxynucleotide complementary to PDGF A-chain mRNA inhibits the arterial proliferation in spontaneously hypertensive rats without alteration of blood pressure. J. Hypertens. 15, 1123–1136.

Hadrava, V., Tremblay, J., Hamet, P., 1989. Abnormalities in growth characteristics of aortic smooth muscle cells in spontaneously hypertensive rats. Hypertension 13, 589–597.

Hu, W.Y., Nakayama, T., Fukuda, N., Kishioka, H., Soma, M., Izumi, Y., Kanmatsuse, K., 1997. Sequences of exon 6 and intron–exon boundaries in rat platelet-derived growth factor A-chain gene: implications for alternative splicing. Biochem. Genet. 35, 395–405.

Karen, M., Nagel, B.S., Sheldon, G., Holstad, D., Keits, E., 1993. Oligonucleotide pharmacotherapy: an antigene strategy. Pharmacotherapy 13, 177–188.

Nilsson, J., Sjölund, M., Palmberg, L., Thyberg, J., Heldin, C., 1985. Arterial smooth muscle cells in primary culture produce a platelet-derived growth factor-like protein. Proc. Natl. Acad. Sci. USA 82 4418–4422.

Perbost, M., Hoshiko, T., Morvan, F., Swayze, E., Griffey, R.H., Sanghvi, Y.S., 1995. Synthesis of 5′-*O*- amino-2′-deoxypyrimidine and purine nucleotides: building-blocks for antisense oligonucleotides. J. Org. Chem. 60, 5150–5156.

Ross, R., Raines, E.W., Bowen-Pope, D.F., 1986. The biology of platelet-derived growth factor. Cell 46, 155–169.

Sanghvi, Y.S., Bellon, L., Morvan, F., Hoshiko, T., Swayze, E., Cummins, L., Freier, S., Dean, N., Monia, B., Griffey, R.H., Cook, P.D., 1995. Synthesis, biophysical and biological evaluations of novel antisense oligonucleotides containing dephosphono-internucleosidic linkages. Nucleosides Nucleotides. 14, 1087–1090.