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Effect of Ion Pairing on the Cellular Transport of Antisense Oligonucleotide

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Antisense oligonucleotide represents an interesting tool for selective inhibition of gene expression. However, their low efficiency of introduction within intact cells remains to be overcome. Antisense-TGFβ (25 mer) and antisense-TNFα (18 mer) were used to study the cellular transport and biological function of antisense oligonucleotide in vitro. Since TGF and TNF play on important role in regulating the nitric oxide production from macrophages, the action of the above antisense oligonucleotides was easily monitored by the determination of nitrite. Poly-L-lysine, benzalkonium chloride and tetraphenylphosphonium chloride were used as polycations, which neutralize the negative charge of antisense oligonucleotide. The production of nitric oxide mediated by γ-IFN in mouse peritoneal macrophage was increased by antisense-TGFβ in a dose-dependent manner. Antisense-TNFα reduced the nitric oxide release from activated RAW 264.7 cells. Significant enhancement in the nitric oxide production was investigated by the co-treatment of poly-L-lysine with antisense-TGFβ. On the meanwhile, inhibition effect of antisense-TNFα is not changed by the addition of poly-L-lysine. These results demonstrate that control of expression of TGFβ and TNFα gene is achieved using antisense technology and the cellular uptake of antisense oligonucleotide could be enhanced by ion-pairing.

Key words : Antisense, TGFβ, TNFα, Nitric oxide, Polycation, Cellular uptake

INTRODUCTION

Antisense oligonucleotide has a sequence complementary to a specific gene or messenger RNA in order to block translation by forming hybrid complex with the target nucleotide sequence (Uhlman and Peyman, 1990). It represents an interesting tool for selective inhibition of gene expression in many areas of medical research (Agrawal *et al.*, 1988; Matsukura *et al.*, 1988; Leiter *et al.*, 1990; Simon *et al.*, 1992). However, the application of antisense oligonucleotide is considerably limited by the low efficiency of their penetration into intact cells and instability in a biological environment (Smith *et al.*, 1986; Gao *et al.*, 1990). The ineffective delivery of antisense oligonucleotide to a target site may be due to the relatively large size and polyanionic character of the molecules. In order to overcome these problems, some chemically modified oligonucleotide have been designed. Phosphotriester, phosphorothioates, and methylphosphonates have shown a much higher resistance toward exonucleases (Miller and McParland, 1981; Eckstein, 1985; Reed *et al.*, 1990; Agrawal *et al.*, 1991). Chemical conjugation with other chemicals is an interesting alternative as enhanced cellular uptake and stability (Aselline and Toulme, 1984; Stein *et al.*, 1988; Akhtar and Shoji, 1991). Another strategy recently developed consist of encapsulating oligonucleotides in liposome (Thierry *et al.*, 1990), use of biodegradable carriers such as nanoparticles (Chavany *et al.*, 1992).

Nitric oxide (NO) which is implicated as a antiviral and/or antinecrotic factor, is synthesized by the action of nitric oxide synthase (NOS). Inducible form of NOS is exited in macrophages, and activated by the various external stimuli (Stamler *et al.*, 1992). Some cytokine such as γ-IFN and LPS showed the increase mRNA level of inducible form of NOS in macrophage (Lyons *et al.*, 1992; Melkova and Esteban 1994). TNFα, cytokine which was induced by LPS-activated cells, also activated nitric oxide production. Since TNFα is a mediator of the LPS from LPS-activated cells, antisense-TNFα could inhibit the production of nitric oxide from activated cells. On the other hand, TGFβ can block the ability of γ-IFN to induce nitrite release in macrophage and expression of TGFβ mRNA is increased remarkably when macrophages are activated (Junquero *et al.*, 1992; Karupiah *et al.*, 1993; Vodovotz *et al.*, 1993). Therefore, it is expected that antisense-TGFβ, which consists of the sequence comple-

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mentary sequences against TGFβ mRNA, will block the effect of TGFβ on the production of nitric oxide mediated γ-IFN in macrophages.

In this paper we establish the method by which biological function of antisense-TGFβ and antisense-TNFα is measured based on nitric oxide release from activated macrophages. In order to develop a method to improve the cellular uptake of antisense, it is investigated how much the cations influenced on cellular uptake of antisense oligonucleotides by use of nitric oxide release. Here we present data that it is possible to inhibit selectively the expression of target gene with a antisense oligonucleotide, and to increase the effect of antisense oligonucleotide by addition of cations.

MATERIALS AND METHODS

Materials

The sequence of antisense-TGFβ (25 mer, phosphorothioate) is as follows;

5'-C*A*G*C*C*C*G*G*A*G*G*G*C*G*G*C*A*T*G*G*G*G*A*G-3')

Asterisk represents the position of sulfur-substituted base of 6S. The bases of 25S were fully substituted with sulfur. The sequence of antisense-TNFα (18 mer, phosphorothioate) is 5'-CTGTGCTCATGGTGTCTT'3'. Dulbecco's modified eagle medium (DMEM), thioglycolate medium and fetal bovine serum were purchased from Gibco BRL Life Technologies, Inc. (Grand Island NY, USA). Lipopolysaccharide from *E. coli* (LPS), tetraphenylphosphonium chloride (TPP), cetyltrimethyl ammonium bromide (CATB), poly-L-lysine (PLL), penicillin/Streptomycin, sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD), sodium nitrite, Trypan Blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). γ-IFN and T4 polynucleotide kinase was purchased from Genzyme Co. and Amersham Inc., respectively. Other chemicals were reagent grade and used without further purification.

Cells and Cultures

Peritoneal macrophage were elicited in male ICR mice (6~8 weeks). After introduction of peritoneal macrophage with 4% thioglycolate medium, peritoneal macrophage were taken out with hank's balanced salt solution. RAW 264.7 cells and mouse peritoneal macrophage were incubated in DMEM containing 10% FBS, penicillin (50 U/ml) and streptomycin (50 μg/ml).

Effect of antisense-TGFβ on production of nitrite mediated by γ-IFN

Peritoneal macrophage and RAW 264.7 cells were seeded in 96 well plates at a density of 2×10^5 cells/well. After 2~3 hr, the medium was replaced with fresh medium (DMEM without serum) and the cells were treated with the different concentrations of antisense-TGFβ (25 mer, phosphorothioate) in the presence of 5 units/ml of γ-IFN. After 42 hr of incubation, the supernatant (100 μl) was taken out for determination of nitrite.

In order to investigate the influences of cations on the effect of antisense-TGFβ, poly-L-lysine (PLL, 100 μg/ml), benzalkonium chloride (BZ, 500 nM), and tetraphenylphosphonium chloride (TPP, 500 nM) was coadministrated with the antisense-TGFβ.

Effect of antisense-TNFα on production of nitrite mediated by γ-IFN and LPS

Antisense-TNFα (18 mer, phosphorothioate) was used to study relations between TNFα and production of nitric oxide. RAW 264.7 cells were treated with the diverse doses (0, 50, 100, 200, 250 μg/ml) of antisense-TNFα in the presence of LPS (10 ng/ml) and γ-IFN (5 units/ml). After 42 hr, supernatant was removed for determination of nitrite.

Determination of nitrite concentration

Nitrite concentration was measured by Griess method as described. Briefly, 100 μl of conditioned medium was removed and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined by ELISA reader. Sodium nitrite was used as standard.

RESULTS AND DISCUSSION

Effect of antisense-TGFβ on production of nitrite mediated by γ-IFN

Antisense-TGFβ increased the production of nitric oxide in a dose-dependent manner in the rage of 0~50 μg/ml from peritoneal macrophage treated with γ-IFN (Fig. 1). At 25 μg/ml of antisense-TGFβ, the content of nitric oxide produced was about 5 times as much as the control, which was content of nitric oxide produced from peritoneal macrophage incubated with γ-IFN (5 units/ml) alone. After addition of 50 μg/ml of antisense-TGFβ with 5 units/ml of γ-IFN, the amount of nitric oxide was increased about 7 times. It is suggested that increase in nitrite production is resulted from antisense-TGFβ mRNA in a dose dependent manner within a given concentration range. On the other hand, addition of 100 μg/ml antisense-TGFβ reduced somewhat of the production of nitric oxide as compared with that of 50 μg/ml antisense-TGFβ. It might be due to influence involved in desensitization. It is known that TGFβ suppressed the synthesis of nitric oxide mediated by γ-IFN not by inhibition of tran-

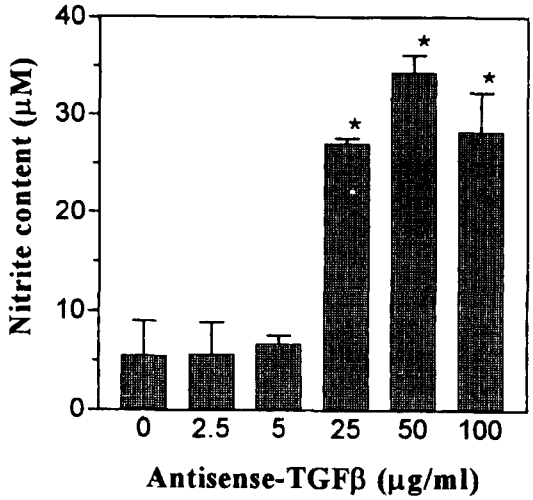

Fig. 1. Effect of antisense-TGFβ on the production of nitrite from mouse peritoneal macrophage mediated by γ-IFN. Peritoneal macrophages from mouse (2×10^5 cells/well) were incubated with 5 units/ml of γ-IFN in the presence of various amount of antisense-TGFβ. After 42 hr, the supernatant were collected and nitrite content was determined by Griess method. The nitrite content of blank control which was not treated with anything, was 0.160±0.001. Results are presented as the mean±SEM (n=3). Means are significantly different by one-way ANOVA ($p<0.0001$). *Significantly different from 0, 2.5, 5 μg/ml ($p<0.001$).

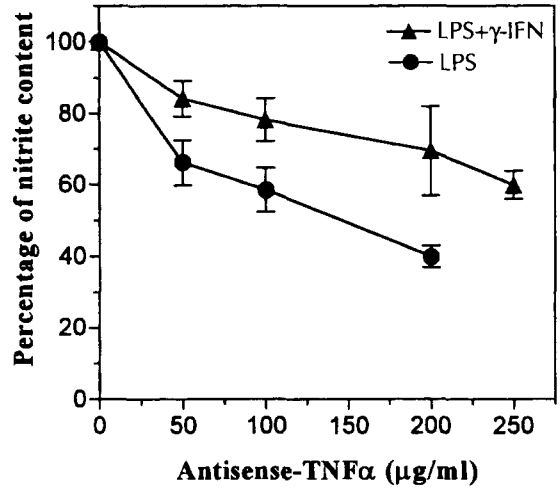

Fig. 2. Inhibition of the nitrite production by antisense-TNFα in activated RAW264.7 cells. RAW264.7 cells (2×10^5 cells/well) were treated with 10 ng/ml of LPS with (▲) and without (●) coadministration of 5 units/ml of γ-IFN in the presence of various amount of antisense-TNFα. After 42 hr, the supernatant were collected and nitrite content was determined by Griess method. Results are presented as the mean±SEM (n=3). Two groups are not significantly different from each other by student t-test.

scription but by decreasing iNOS mRNA stability (Vodovotz *et al.*, 1993). Therefore, these results demonstrated that highly expressed TGFβ mRNA by γ-IFN-stimulated state, has been efficiently inhibited by antisense-TGFβ. As a result of that, the nitric oxide production by γ-IFN was markedly increased.

Effect of antisense-TNFα on production of nitrite mediated by γ-IFN and LPS

In the range from 0 μg/ml to 250 μg/ml, antisense-TNFα reduced the release of nitric oxide from RAW cells incubated with LPS (10 ng/ml) and γ-IFN (5 units/ml). At doses of 50, 100, 200, 250 μg/ml of antisense-TNFα, the rate of decrement was about 6, 12, 21, 41 percent, respectively. In the same way, when the production of nitric oxide was induced by LPS (10 ng/ml) alone, nitric oxide released from RAW cells was decreased in a concentration dependent manner. By the addition of 50 μg/ml of antisense-TNFα, the production of nitric oxide was inhibited by 66%, while the production of nitric oxide was reduced to 40% when the doses of antisense-TNF were increased to 200 μg/ml (Fig. 2). The mean values of percent nitrite content from LPS-activated cells were lower than that from cells costimulated with LPS and γ-IFN at given amount of antisense-TNFα, but difference turn out to be statistically insignificant.

In this study, it is observed that nitric oxide production from activated RAW cells was decreased by addition of antisense-TNFα. It measns that TNFα, a mediator of the LPS-activated nitric oxide production, can be efficienty inhibited by antisense-TNFα, which resulted in a reduced production of nitric oxide from activated macrophage cell line.

Influences of various cations

In the presence of 25 μg/ml antisense-TGFβ, peritoneal macrophages were treated with various cations and then 5 units/ml of γ-IFN. After incubation for 42 hr nitrite concentration was determined as shown in Table I. Poly-L-lysine (PLL), benzalkonium chloride (BZ) and tetraphenylphosphonium chloride (TPP) were used as a counter cation. The effect of antisense-TGFβ on

Table I. Effect of the various cations on the production of nitrite by antisense TGFβ from activated mouse peritoneal macrophages

Cations	Concentration	Nitrite (mM)
-		27.109±0.306
PLL	100 μg/ml	40.042±2.273*
BZ	500 nM	24.364±1.242
TPP	500 nM	26.128±4.165

Mouse peritoneal macrophages (2×10^5 cells/well) were incubated with 5 units/ml of γ-IFN and 25 μg/ml of antisense TGFβ. Results are presented as the mean±SEM (n=3). Means are significantly different by one-way ANOVA ($p<0.0001$). *Significantly different from control group and TPP-treated group ($p<0.001$) and BZ-treated group ($p<0.01$).

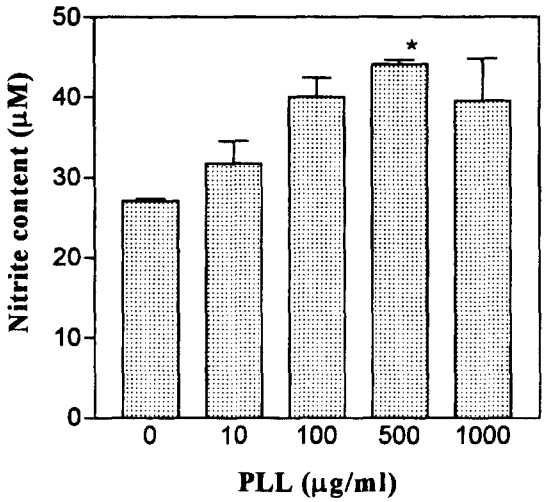

Fig. 3. Effect of poly-L-lysine on the production of nitrite mediated by γ-IFN and antisense-TGFβ in mouse peritoneal macrophage cells. Mouse peritoneal macrophages (2×10^5 cells/well) were incubated with 5 units/ml of γ-IFN and 25 μg/ml of antisense-TGFβ in the presence of various amount of poly-L-lysine. After 42 hr, the supernatant were collected and nitrite content was determined by Griess method. Results are presented as the mean ± SEM (n=3). Means are significantly different by one-way ANOVA (p=0.0113). *Significantly different from 0 μg/ml ($p<0.05$).

the production of nitric oxide mediated by γ-IFN, was increased about one half times by the addition of 100 μg/ml of PLL. No increment of production of nitric oxide was observed with cells treated with BZ and TPP, quaternary ammonium salts. In order to study the influences of PLL in detail, PLL was added in different doses. As shown in Fig. 3, poly-L-lysine (0~100 μg/ml) increased the release of nitrite from cultured peritoneal macrophages after 48 hr exposure in a concentration dependent manner. The effect of PLL may be the result of reduced polarity by neutralization of negative charge of oligomer with polycationic PLL, which resulted in enhanced stability to nucleases and followed by increment of permeation into cells. On the other hand, BZ and TPP did not affect on the effect of antisense-TGFβ. Since the positive charge contained of BZ and TPP was less than that of PLL, these were incapable of adequate neutralization of negative charge. BZ and TPP were surfactants, which can destruct the cells, so that these cannot be used above concentration of 500 nM. When the cell viability was measured by trypan blue extrusion method followed by exposure to these compounds, PLL did not affect on the cells in concentration treated in this experiments.

In conclusion, the biological effect of antisense-TGFβ and -TNFα was easily monitored by the nitric oxide production from activated macrophages. By using this system, effect of ion pairing on cellular uptake of antisense oligonucleotide was investigated. PLL increased the effect of antisense-TGFβ, showed its potential for being a counter cation for enhancement of the cellular uptake of antisense oligonucleotide. More experiments will be carried out in order to certify to the effect of PLL.

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REFERENCES CITED

Agrawal, S. Goodchild, J. and Civeira, M. P., Oligodeoxynucleoside phosphoramidates and phosphorothioates as inhibitors of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA*, 85, 7079-7083 (1988).

Agrawal, S., Temsamani, J. and Tang, J. Y., Pharmacokinetics, biodistribution, and stability of oligonucleotide phosphorothioates in mice. *Proc. Natl. Acad. Sci. USA*, 88, 7595-7599 (1991).

Akhtar, S. and Shoji, Y., Degradation and membrane transport characteristics of modified antisense oligonucleotides. *J. Cell. Biochem.*, 15D, 16 (1991).

Aselline, U. and Toulme, F., Oligodeoxynucleotides covalently linked to intercalating dyes to base sequence-specific ligands. Influence of dye attachment site. *EMBO J.*, 3, 795-800 (1984).

Chavany, C., Doan, T. L., Courvreur, P., Polyalkylcyanoacrylatenamopartcles as polymeric carriers for antisense oligonucleotides. *Pharmaceutical Res.*, 9, 441-449 (1992).

Eckstein, F., Nucleoside phosphorothioates. *Ann. Rev. Biochem.*, 54, 267-402 (1985).

Gao, W.-Y., Hanes, R. N., and Miguel, A., Vanzquez-Padua, Inhibition of Herpes Simplex Virus Type 2 growth by phosphorothioate oligodeoxynucleotides. *Antimicrobial Agents and Chemotherapy*, 34, 808-812 (1990).

Harel-Bellan, A., Ferris, D. K., Vinocour, M., Aselline, U., Toulme, F., Oligodeoxynucleotides covalently linked to intercalating dyes as base sequence-specific ligands. Influence of dye attachment site. *EMBO J.*, 3, 795-800 (1984).

Junquero, D. C. Scott-Burden, T., Schini, V. B. and Vanhoutte, P. M., Inhibition of cytokine-induced nitric oxide production by transforming growth factor-1 in human smooth muscle cells. 454, 451-465 (1992).

Karupiah, G., Xie, Q.-W., Buller, R. M. K., Inhibition of viral replication by interferon- -induced nitric oxide synthase. *Science*, 261, 1445-1448 (1993).

Leiter, J. M. E., Agrawal, S. and Palese, P., Inhibition of influenza virus replication by phosphorothioate oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA*, 87, 3430-3434 (1990).

Lyons, C. R., Orloff, G. J. and Cunningham, J. M., Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J. Biol. Chem.*, 267, 6370-6374 (1992).

Matsukura, M, Zon, G. and Shinozuka, K., Synthesis of phosphorothioate analogues of oligodeoxyribonucleotides and their antiviral activity against human immunodeficiency virus (HIV). *Gene*, 72, 343-347 (1988).

Melkova, Z and Esteban, M., Interferons-γ severely inhibits DNA synthesis of vaccinia virus in macrophage cell line. *Virology*, 198, 731-735 (1994).

Miller, P. S. and McParland, K. B., Biochemical and Biological effects of nonionic nucleic acid methylphosphonates. *Biochemistry*, 20, 1874-1880 (1981).

Reed, J. C., Stein, C. and Subasinghe, C., Antisense-mediated inhibition of BCL2 protooncogene expression and leukemic cell growth survival: Comparisons of phosphorothioate oligodeoxynucleotides. *Cancer Res.*, 50, 6565-6570 (1990).

Simon, M., Edelman, E. R. and DeKeyser, J.-L., Antisense *c-myb* oligonucleotides inhibit initial arterial smooth muscle cell accumulation *in vivo*. *Nature*, 359, 67-70 (1992).

Smith, C. C., Aurelian, L., Reddy, M. P., Antiviral effect of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type1 immediate early pre-mRNAs 4 and 5. *Proc. Natl. Acad. Sci. USA*, 83, 2787-2791 (1986).

Stalmer, J. S., Singel, D. J. and Loscalzo, J., Biochemistry of nitric oxide and its redos-activated forms. *Science*, 258, 1898-1901 (1992).

Stein, C. A., Moris, K. and Loke, S. L., Phosphorothioate and normal oligodeoxynucleotides with 5'-linked acridine: characterization and preliminary kinetics of cellular uptake. *Gene*, 72, 333-341 (1988).

Thierry, A., Drischilo, A. and Rahnan, A., Liposomal carrier system as a novel approach to transport oligonucleotides into cells. *Proc. Am. Assoc. Cancer Res.*, 31, 445 (1990).

Uhlman E. and Peyman, A., Antisense oligonucleotides: A new therapeutic principle. *Chemical Rev.*, 90, 543-584 (1990).

Vodovotz, Y., Bogdan, C. and Paik, J., Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. *J. Exp. Med.*, 178, 605-612 (1993).