Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 13–18

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

INHIBITION OF TRANSFORMING GROWTH FACTOR-β2 EXPRESSION WITH PHOSPHOROTHIOATE ANTISENSE OLIGONUCLEOTIDES IN U937 CELLS

Zhong-Jian Shen, Sang Kook Kim, Young Sup Lee, Jong Won Lee[1], Byung Jo Moon*

Department of Biochemistry, Kyungpook National University, Taegu 702-701 Korea
[1]Department of Biochemistry, School of Medicine, Catholic University of Taegu-Hyosung, Taegu 705-034, Korea

Received 3 September 1998; accepted 29 October 1998

Abstract: Four types of phosphorothioate antisense oligonucleotides for transforming growth factor-β2 were synthesized and tested for their antisense activity in U937 cells. The full-length phosphorothioate modified antisense analogues exhibited the highest inhibitory effects on the transforming growth factor-β2 expression in U937 cells.

Transforming growth factor-β(TGF-β) is a family of multifunctional cytokines that comprises at least five proteins, TGF-β1, β2, β3, β4 and β5 with closely related biochemical and functional features. They may act as either positive or negative regulators of cell proliferation. On cells of epithelial and endothelial origin, TGF-β acts as a strong growth inhibitor,[1,2] but on the mesenchme-derived cells such as fibroblasts and osteoblasts it acts as a powerful stimulator.[3,4] These opposing effects are likely controlled by the selective production and receptor modulation and differential susceptibility of target cells by TGF-β at various stages of development, maturation, and activation. In recent study, it has been found that autocrine TGF-β1 plays a role in colon cancer tumorigenicity and invasion.[5] It has also been shown that a relatively small decrease in levels of TGF-β1 was enough to markedly decrease colon carcinoma cell aggressiveness. Tumor promoting activity of TGF-β in some model systems in human breast cancer cells was also reported.[6] In contrast to the large number of studies of TGF-β2 on cell proliferation and differentiation,[7-10] much less is known about the effect of TGF-β2 on leukemia cell lines.[11] As part of our study on roles of cytokine in regulation of human leukemia cells, we tried to inhibit TGF-β2 expression in the human promonocytic leukemia U937 cells with antisense oligonucleotides. It has been reported that phosphorothioate antisense oligonucleotides designed to hybridize to target mRNA sequence inhibited the function of a number of cellular[12,13] and viral proteins.[14,15] For example, antisense oligonucleotides complementary to cytokine-specific-mRNA may selectively inhibit the synthesis of the target protein and therefore can be used as excellent tools for studying their effects in vitro and in vivo. The effects of

0960-894X/99/$ - see front matter
PII: S0960-894X(98)00675-1

antisense oligonucleotides on the expression and on the function of various cytokines have been reported by numerous investigators.[3,16-19]

In this study, unmodified, partially modified and full-length modified 20mer- phosphorothioate antisense oligonucleotides(dn) have been synthesized and tested for their inhibitory activities in U937 cells. The antisense oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems Model 391 DNA Synthesizer with CPG as a solid support. Four types of antisense oligonucleotides were designed to be complementary to the initiation codon region of mRNA of TGF-β2 since the site is known to be one of the most successful sites for translational inhibition by antisense oligonucleotides.[20] AS-1: unmodified oligonucleotide (Table 1); AS-2: full-length modified phosphorothioate analogue; AS-3 and AS-4: partially phosphorothioate derivatized in the middle and both ends of their nucleotides chain, with the remaining bases maintained in the natural state. Phosphorothioate linkages were prepared by using tetraethylthiuram disulfide in acetonitrile. Coupling, oxidation and/or sulfurization were performed according to manufacturers recommendations. Synthesized oligonucleotides were purified by reverse HPLC and determined by PAGE to adhere to the > 95% full-length.

Table 1. Oligonucleotides for TGF-β2 antisense experiments *

Initiation codon region of mRNA of TGF-β2	5′--UUUUUUAAAAAAUGCACUAC--3′
Antisense-1	3′--AAAAAATTTTTTACGTGATG--5′
Antisense-2	3′--**AAAAAATTTTTTACGTGATG**--5′
Antisense-3	3′--AAA**AAATTTTTTACGTG**ATG--5′
Antisense-4	3′--**AAA**AAATTTTTTACGTG**ATG**--5′
Missense	3′--**ATTACAAGTCAATATAGAAT**--5′
TGF-β1 antisense	3′--**GGCGGAGGGGGTACGGCGGG**--5′

* All the phosphorothioate derivatized oligonucleotides are boldfaced and underlined.

Monoblastic U937 cell lines, which secrets high levels of TGF-β2, were washed with serum-free RPMI-1640 medium without antibacterial agent, and seeded in 24 well multidish. Add increasing concentration of antisense oligonucleotides(0.1 μM - 10 μM) to the cell suspensions in the presence(80 μg/mL) or absence of Lipofectamine, mix gently to ensure uniform distribution, and incubate for 4 hours at 37°C in a CO_2 incubator. Following incubation, add complete RPMI-1640 growth medium to each well. Incubate cells at 37 °C in a CO_2 incubator for a total of 48 hours. The level of TGF-β2 expression in the cell culture media was determined by

ELISA and intracellular TGF-β2 level was evaluated by western blots. Under the incubation conditions described above, it has been found that, among the four antisense oligonucleotides, the AS-2 and AS-3 exhibited a concentration-dependent inhibition in target protein expression in the presence of Lipofectamine solution(Fig 1, A). Inhibition of TGF-β2 expression in cell culture media occurred from 0.4 μM oligonucleotides (20-29%)and maximized at 5 μM(88-94%). However, unexpectantly, chimeric AS-4 and unmodified AS-1 in the same experimental condition exhibited only a slight inhibition(28% and 15%, respectively) in TGF-b2 expression even at a high oligonucleotide concentration(10 μM). It has been reported that unmodified antisense oligonucleotides were undergone nearly complete intracellular degradation after cellular uptake within a few hours after incubation[21-24]. It has also been proved that end-capped phosphorothioate version of phosphorothioate oligonucleotides(dn) exhibited greater cellular uptake than fully modified analogues while exhibiting similar biological stability, but they were sensitive to nucleases during long-periods of incubation (>24h).[25] In our work it was demonstrated that FITC-labeled oligonucleotide were completely internalized into the U937 cells after 2-4 hours of incubation whatever phosphorothioate modifications exist in their backbone(data not shown). From our results described above, it is likely that the end-capped AS-4 and unmodified AS-1, although they have been liposomally transfected into cells, was easily degraded by the both intracellular endonucleases or/and

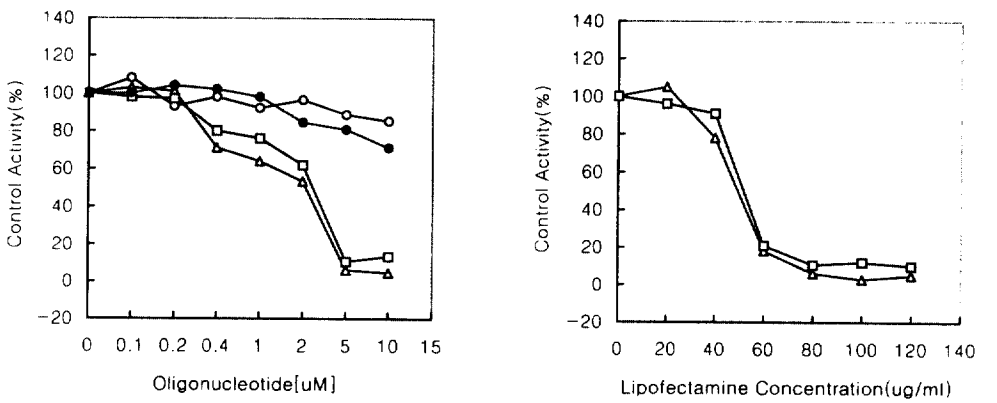

Fig 1. Effect of phosphorothioate antisense oligonucleotides on TGF-β2 expression. A: Cells were treated with increasing concentration of oligonucleotides as indicated concentration in the presence of serum free medium and 80 μg/mL Lipofectamine for 4hr. B: Cells were treated with increasing concentration of Lipofectamine as indicated concentration in the presence of serum free medium and 5μM oligonucleotides for 4hr. Following incubation(A,B), add appropriate complete growth medium and incubate cells for a total of 48hr. The TGF-β2 expression were determined by ELISA. Results are expressed as TGF-β2 protein % control(medium treatment) and the mean of three data points. The standard error of the mean was < 10% for each data point. Oligoncleotides used were AS-1(- ○ -), AS-2(- △ -), AS-3(- □ -), AS-4(- ● -).

exonucleases, especially by the endonucleases since AS-4 exhibited somewhat higher antisense activity than AS-1. Nevertheless, both the AS-2 and AS-3 exhibited the highest inhibiting activity at 5uM oligonucleotide concentration(by 94% and 90%, respectively). AS-3 which has natural phosphodiester linkages at their both ends could be cleaved from both the ends by exonucleases, but it was yet sufficient to exert their antisense activity. This indicates that antisense oligonucleotide 14-15 nucleotides in length may be enough to bind selectively to the target site of mRNA. The results from our control experiments also showed that the inhibitory effect of TGF-β2 antisense oligonucleotide was clearly sequence specific and specific for TGF-β2 expression, since a missense oligonucleotide and a TGF-β1 antisense oligonucleotide failed to prevent the expression of TGF-β2(data not shown).

In the presence of Lipofectamine, the antisense(AS-2 and AS-3) activities were easily detected at lower concentration(0.4 μM); but in the absence of Lipofectamine, the activity revealed only at high oligonucleotide concentration(>5 μM). While AS-1 and AS-4 exhibited a little activity(<14%) in the presence of Lipofectamine even at high oligonucleotide concentration(5 μM). The effect of Lipofectamine was found to closely parallel the effect of DOTMA-containing lipid vesicles which not only enhanced the rate of oligonucleotide uptake into cells but also markedly changed the subcellular distribution of the oligonucleotide, and eventually enhanced antisense activity.[26] When cells were treated with Lipofectamine only(>40 μg/mL), it produced a toxic effect on cells, and their toxic effect exhibited in a time and dose dependent manner. However, when Lipofectamine(20-120 μg/mL) formed complex with antisense oligonucleotides(5 μM), the toxic effect of Lipofectamine has not been observed(data not shown). The optimum concentration of Lipofectamine was determined by incubating cells in the presence of increasing concentrations of Lipofectamine plus 5μM antisense oligos(AS-2, AS-3). The inhibition effect of antisense oligonucleotides was dependent upon the concentration of Lipofectamine

FIg.2. Western blot analysis for TGF-β2 protein of U937 Celllysates. Antisense oligonucleotide treated (48 h, 5μM oligomers plus 80μg/mL Lipofectamine) and untreated cells were grown inRPMI-1640 medium containing 10% FBS. The cells were lysed and total cellular protein(20 μg) was loaded onto each lane of a 12% polyacrylamide sodium dodecyl sulfate gel. fractionated proteins were then electroblotted to a nitrocellulose membrane and probed with a polyclonal antibody to TGF-β2 protein employing standard techniques. Lane 6(medium treatment) and 5(Lipofectamine treatment) indicate antisense oligonucleotide-untreated blots; 4(AS-1), 3(AS-2), 2(AS-3), and 1(AS-4) indicate the antisense oligonucleotide-treated blots.

used(Fig.1, B). But the inhibition was not due to the presence of Lipofectamine alone, because there was not a significant difference in TGF-β2 expression between control cells and cells incubated in the presence of up to 80 μg/mL Lipofectamine(Fig.2). Additionally, the length of incubation time required to evaluate antisense activity was determined after treatment with Lipofectamine-antisense oligonucleotides complexes. Cells were incubated for various periods of time(12hr, 24hr, 36hr, 48hr) with 5 mM AS-2 and AS-3, in the presence of 80 μg/mL Lipofectamine. The expression of the TGF-β2 protein in the cell culture media, at each time point, was reduced by 0%(12hr), 8%(24hr), 87%(36hr), 98%(48hr) with AS-2; and 4%(12hr), 32%(24hr), 82%(36hr), 92%(48hr) with AS-3, respectively. Interestingly, the antisense effects were also observed by inhibiting cell proliferation in parallel with decrease of TGF-β2 expression (Fig 3). The growth of U937 cells transfected with AS-2 and AS-3 was inhibited by 80-95% when the cells were incubated for 48 hr with 5 μM oligo concentration, demonstrating pharmacological activity of phosphorothioate antisense oligonucleotides in this cells.

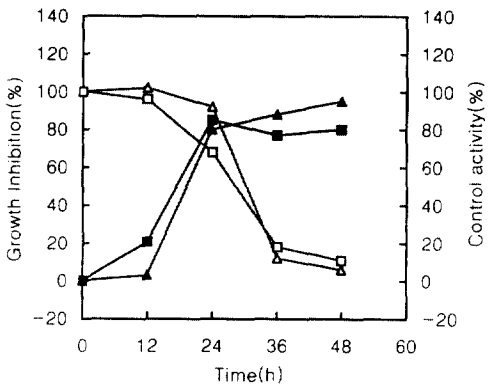

Fig 3. Effect of Treatment Time with Antisense Oligonucleotide on Inhibition of Cell Growth and Expression of TGF-β2. Cells were treated with 5 μM of AS-2(- △ -, - ▲ -) and AS-3(- □ -, - ■ -) and 80 μg/mL Lipofectamine for 4hr in the presense of serum free medium. Following incubation, to cells were added appropriate complete growth medium. Cells were incubated for a total of the indicated period of times. At the each time point, the cell number were counted in triplicate and the TGF-β2 expression in cell culture media was determined by ELISA. The standard error of the mean was < 10% for each data point(three determinations). Inhibition of cell growth were expressed with closed symbols; control activity with open ones.

In conclusion, the unmodified natural antisense oligonucleotide and the chimeric antisense analog(AS-4), which were designed to target the initiation codon region of mRNA of TGF-β2 , failed to significantly inhibit their target protein expression. In contrast, the phosphorothioathe antisense oligonucleotides(dn)(AS-2, AS-3) are capable of significantly inhibiting their target protein expression in U937 cells. These phosphorothioate antisense oligonucleotides may be useful as therapeutic agents against some leukemia diseases in which TGF-β2 plays a role. Furthermore, Lipofectamine provided a successful method for intracelluar delivery of phosphrothioate antisense oligonucleotides and enhanced specific antisense activity.

Acknowledgment. This work was supported by the Korea Science and Engineering Foundation through the Center for Biofunctional Molecules.

REFERENCES

1. Frater-Schroder, M.; Muller, G.; Birchmeier, W.; Bohlen, P. *Biochem. Biophys. Res. Commun.* **1986**, 137, 295.
2. Chinkers, M. J. *Cell. Physiol.* **1987**, 130, 1.
3. Kay, E.P.; Lee, M.S.; Seong, G.J.; Lee, Y.G. *Curr. Eye Res.* **1988**, 17, 286.
4. Okawa, M.; Hakeda, Y.; Wakatsuki, N.; Katoh, Y.; Higashino, K.; Kumegawa, M. *Meikai Daigaku Shigaku Zasshi.* **1990**, 19, 310.
5. Huang, F.; Newman, E.; Theodorescu, D.; Kerbel, R.S.; Friedman, E. *Cell Growth Differ.* **1995**, 6, 1635.
6. Sun, L.; Chen, C. *J. Biol. Chem.* **1997**, 272, 25367.
7. Boumediene, K.; Vivien, D.; Macro, M.; Bogdanowicz, P.; Lebrum, E.; Pujol, J.P. *Cell Prolif.* **1995**, 28, 221.
8. Zerath, E.; Holy, X.; Mouillon, J.M.; Farbos, B.; Machwate, M.; Andre, C.; Renault, S.; Marie, P.J. *Life Sci.* **1997**, 61, 2397.
9. Constam, D.B.; Schmid, P.; Aguzzi, A.; Schachner, M.; Fontana, A. *Eur. J. Neurosci.* **1994**, 6, 766
10. Fromigue, O.; Marie, P.J.; Lomri, A. *J. Cell. Biochem.* **1998**, 68, 411
11. Bruno, E.; Horrigan, S.K.; Van Den Berg, D.; Rozler, E.; Fitting, P.R.; Moss, S.T.; Westbrook, C.; Hoffman, R. *Blood.* **1988**, 91, 1917
12. Burch, R.M.; Mahan, L.C. *J. Clin. Invest.* **1991**, 88, 1190.
13. Simons, M.; Edelman, E.R.; Dekeyser, J.L.; Langer, R.; Rosenberg, R.D. Nature. **1992**, 359, 67.
14. Cohen, J.S. *Antiviral Res.* **1991**, 16, 121.
15. Agrawal, S. *In Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS;* Eric, Wickstrom, Ed.; Wiley-Liss: New Youk, **1991**; pp143-158.
16. Tzai, T.S.; Lin, C.I.; Shiau, A.L.; Wu, C.L. *Anticancer Res.* **1988**, 18, 1585.
17. Marzo, A.L.; Fitzpatrick, D.R.; Robinson, B.W.; Scott, B. *Cancer Res.* **1997**, 57, 3200.
18. Smyth, A.P.; Rook, S.L.; Detmar, M.; Robinson, G.S. *J. invest. Dermatol.* **1997**, 108, 523.
19. Potts, J.D.; Dagle, J.M.; Walder, J.A.; Weeks, D.L.; Runyan, R.B, *Proc Natl. Acad. Sci. U.S.A.* **1991**, 88, 1516.
20. Neckers, L.M.; Whitesel, L.; Rosolen, A.; Geselowitz, D. A. *Crit. Rev. Oncogenesis* **1992**, 3, 175
21. Harel, B.A.; Ferris, D.K.; Vinocour, M.; Holt, J.T.; Farrar, W.L. *J. Immunol,* **1988**, 140, 2431
22. Wickstrom, E.L.; Bacon, T.A.; Gonzalez, A.; Freeman, D.L.; Lyman, G.H.; Wickstrom, E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, 85, 1028
23. Yakubov, L.A.; Deeva, E.A.; Zarytova, V.F.; Ivanova, E.M.; Ryte, A.S.; Yurchenko, L.V.; Vlassov, V.V. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, 86, 6454
24. Woolf, T.M.; Jennings, C.G.B.; Rebagliati, M.; Melton, D.A.; *Nucleic Acids Res.* **1990**, 18, 1763
25. Alain, R.T.; Anatoly, D.; *Nucleic Acids Res.* **1992**, 20, 5691
26. Bennett, C.F.; Chiang, M.Y.; Chan, H.; Schoemaker, J.E.; Mirabelli, C.K. *Molecular Pharmacology* **1992**, 41, 1023