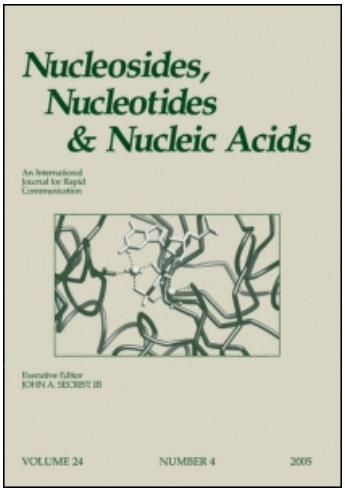

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A Novel Approach for Gene Medicine: Synthetic Poly-L-lysine/serine Copolymer Enhances Bioactivity of Antisense Oligonucleotides

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A NOVEL APPROACH FOR GENE MEDICINE ; SYNTHETIC POLY-L-LYSINE/SERINE COPOLYMER ENHANCES BIOACTIVITY OF ANTISENSE OLIGONUCLEOTIDES

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ABSTRACT : Antisense oligonucleotides have therapeutic potential as inhibitor of gene expression. Water soluble and nuclease resistant-polymers complexed with the antisense oligodeoxynucleotide which specifically inhibits the production of interleukin-1β were prepared using poly-L-lysine/ L-serine and its polyethylene glycol 5000 conjugate and evaluated their activity in vitro and in vivo. These complexes biologically inhibited interleukin-1β production in a dose-dependent and sequence-specific manner at nanomolar levels in lipopolysaccride-stimulated human macrophage-like, U937 cells. Moreover, in vivo studies using mouse endotoxin shock model, it had a strong efficacy in a dose-dependent and sequence-specific manner. The mice(n=10), administered with the antisense complex at 100mg/kg, survived for more than 7days. These carriers may give a promise for developing nucleic acid drug in clinical use.

Recently, the development of improved delivery systems for oligonucleotides (ONs) or plasmid expression vectors into mammalian cells have been a major pre-requirement for the therapeutic use of ONs in order to increase stability and cellular uptakes[1,2]. Numerous ON studies using unmodified phosphodiester(D-) have been tried, but resulted in fair, since D-ONs are rapidly hydrolyzed by cellular or serum nucleases. Then, it has been shown that some analogs such as phosphrothioate(S-) ONs can overcome these difficulties, however in many cases, these modified ONs are the subject of questions on the separation of isomers, non-sequence specificity[2,3]. An alternative approach would be to use molecular assemblies without any chemical modifications of ONs.

On the other hand, preclinical studies for use of the ONs (e.g., antisense RNA/DNA and ribozyme) as drugs have been actively performed in recent year[4]. However, it is known that the ONs, per se, due to their high molecular weight, are difficult to be delivered in required amounts into the cytoplasm which constitutes the site of exertion of effects, together with the fact that the ONs are prone to undergo degradation by various kinds of nucleases. To put the ONs into practical use as real drugs, therefore, it is required to overcome such issues, and numerous studies aimed to develop an effective drug delivery system for ONs have been attempted.

In this study, we investigated efficacy of the synthetic polymers(HLS) composed by hydrophilic serine residues and cationic lysine residues or its conjugates with

polyethyleneglycol-5000, as delivery systems of ONs in vitro and in vivo. It is strongly suggested that a pro-inflammatory cytokine, IL-1 β plays an important role in pathogenesis of rheumatoid arthritis(RA). Then, we also tried to develop a specific drug for treatment of patients with RA using antisense(ANS) methodology.

EXPERIMENTAL

D- and S-ANS 20mer oligodeoxynucleotide(ODN), complementary to the sequence including initiation codon of the human interleukin-1 β (hIL-1 β) gene, were automatically synthesized on ABI DNA synthesizer (380B; Applied Biosystems, Foster City California) . It is difficult to select the sequence in mRNA on target cytokines. So, the sequences in mRNA on target cytokine were focused on the structure of the antisense molecules in solution and the ability of hybrid formation with their complementary target sequences using computer simulation. Purification of these ODNs were performed by reverse-phase high performance liquid chromatography(PRP-3 reversed phase 300A column, Hamilton Co., Reno, N.V.) .

Amino acidic copolymers of L-lysine and L-serine were synthesized by the method reported previously[4] . Reference poly-L-lysine(PLL) and poly-D,L- lysine were purchased from Sigma Co. Ltd.

Collection and purification of the ODN and HLS complexes were carried out by ultrafiltration method. A solution that contained ^{32}P-labeled ODN was mixed with the solution of a poly peptide in 2mM phosphate buffer (pH7.2) with a molar ratio of ODN to the HLS polypeptide region of 1 to 1.5. Ultrafiltration method was used as a procedure to directly examine the complex formation between the ODNs and the HLSs, frequently. Ultrafiltration tube was purchased from MILIPORE Co.(Ultrafree C3LGC, Lot. N2DMBO51A). First, the ODNs were 5'-end labeled using γ -^{32}P-ATP and T4-polynucleotide kinase by the exchange reaction method. OND solution was mixed with the HLS solution of several concentration. The ultrafiltration tube with a menbrane fractioning capacity which enables to permeate only the free ODN was used to conduct centrifugation, and the amount of free ODN in the filtrate was then determined with a Bio-Imaging analyzer(Fuji film, BAS-2000) or on the based on the absorbance at 260nm or at 210nm. The amount of free ODN was subsequently detected from the total amount of ODN used to determine the amount of the complex. ^{32}P-labeled ODNs were mixed with the solutions that contained poly-cationic amino acids.

Circular Dichroism (CD) measurements were carried out at 20℃ with a Jasco J-720 spectropolarimeter. In these measurements, a phosphate buffer of pH 7.0 was used exclusively ; the ionic strength of the buffer was 0.014 for PLL, PDL, and PDLL solutions and 0.100 for HLS solutions. The relative proportions of secondary structures were estimated by curve fitting the CD spectrum, using the reference spectra of α-helix as determined by Chen et al.[5]. The simulation was carried out in the wavelength region 200-240nm at 1nm intervals. In the case of PLL addition, the simulated wavelength was shifted to 210-240nm with an increase in the concentration of PLL, since higher concentrations interfered in the CD measurement in the lower-wavelength region.

Concentrations of polypeptides were determined spectrophotometrically. Extinction coefficients of HLS and anotheir polypeptides were estimated on the basis of their concentrations determined by weighing the lyophilized samples of 50-100mg. The samples had beeen exhaustively dialyzed against pure water to removed the electrolytes composing the buffer and then they were lyophilized. The extention at 215nm was 60,000 for HLS.

The optimal proportions for complex formation between the HLS carriers and the oligonucleotides were determined using an poly-acrylamide gel electrophoresis retardation system[6]. A conjugate to anionic OND molar ration of 1.5:1(based on cationic lysine content of the HLS) was found to completely retard OND migration in the gel and form soluble complexes, and this ratio was used in all subsequent experiments. All complex were filterd through UltraFree C3-LGC(Millipore) membrane prior to injection to ensure that samples used did not contain

precipitates. Complexes found to be stable in above buffer at 37℃ for at least 2 days, and at 2℃ for at least 3 weeks.

The effects of antisense ODN and HLS complexes on human IL-1 β production in human macrophage-like cell ,U937 cells were detected by the methods, previously described elsewhere3).

12-o-Tetradecanoyl Phorbol Acetate(TPA) and lipopolysaccharide(LPS) were purchased from Wako Chemicals Co. Ltd. and Sigma Chemical Co. Ltd., respectively. Six southernd U937 cells were suspended in 0.3 ml of growth medium, then cultured in a 96-wells microtiter plate. One ng/ml of TPA, 10 μg/ml of LPS and various concentrations of ONs were added into the medium. In some experiments, ODs complexed with HLS, were used instead of uncomplexed ONs. ONs and HLS complexe solutions were prepared just prior to its addition to the medium.The human macrophage cell-line U937 was grown in RPMI1640 medium containing 10% heat-inactived fetal bovine serum, 10unit/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37℃ in 5 % CO_2 and air saturated with water vapor, and were maintained in their logarithmic growth phase. Viable cells were determined using a trypan blue-exclusion method assay and counted using a hemocytometer.

After incubation under conditions, microtiter plate was frozen and thawed in three times. The supernatant of each sample was collected and then hIL-1 β concentration was detected using an enzyme-linked immunosorbent (ELISA) system.

The BALB/c mice has served as a useful animal model for anaphylatic, burn, endotoxine, and septic shock. Female BALB/c mice were obtained from Charles River Breeding Laboratories,Inc., Portage, Mich., and were used between 9 and 11 weeks of age. The animals were given i.v. doses of ODNs as drugs at several concentrations. , 30min. prior to LPS injection. Control animals received isotonic sodium chloride solution alone. Lethal (20mg/kg) doses of endotoxine were then injected i.p., immediately. Mortality in all experiments groups was evaluated daily.

Turbidity of the complex

Figure 1 shows the spectroscopic evaluations of the complex-forming property of ODNs and HLS carriers according to the turbidity of the solution. An increasing in absorbance was noted at higher concentrations than a given level in case of PLL, while no changes were noted in absorbance at 360nm in case of HLS. The HLS and ODN complex which indicated no agreegation in the solution, indicating that the solubility of the HLS-ODN complex is extremely high

Complex dissociation

It is considered that the ODNs are required to be dissociation from the complex with in the target cells and to be delivered into the cytoplasm before their physiological activity. Figure 2 shows the results of pH-dependent dissociation of ODNs and HLS complexes . The ordinate represents the amount in percentage of ODNs dissociated from thecomplex at each pH. As results,although no dissociation of ODNs from HLS carriers was observed at pH7.0, the dissociation of ODNs finally reached 100% at pH3.0. These results indicate that the HLS and ODN complex dissociates at acidic pH range and simultaneously shows the possibility that theODNs ODNs might be effectively delivered into target cells, e.g., endocytosis, by use of the HLS-ODNcarrier system. Apparently, in terms of the binding of the ODNs with HLS, secondary structures of the ODNs had no effects (data not shown).

On the other hand, in the case of PLL as a poly-amino acid, the complex formation did not progress to completion when antisense OND that had adopted secondary structures (e.g., stem-loop structure) was used (data not shown). This demonstrates that the release of the ODNs, capable of adopting secondary structures with poly-amino acids, that have the main chain consisted of only lysine, is more difficult than the case of the ODNs that adopt a linear chain structure. Therefore, the insertion of serine residues into PLL resulted in promotion of the OND relese that has a secondary structure. It is to be stressed that poly-(D,L)-lysine synthetic

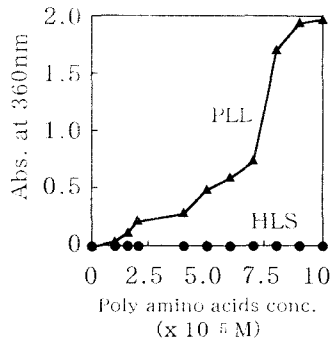


Figure 1. Concentration dependence of PLL or HLS on absorbance at 360 nm.

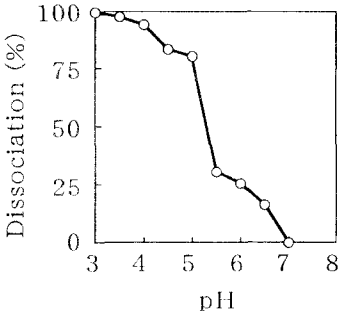


Figure 2. pH-dependent dissociation of ODN and HLS complexes.

copolymers failed to form complexes with ONDs (data not shown), an indication that an ionic interaction is not the sole driving force for the complex dissociation.

Following CD measurements hinted at the importance of some structural element such as α-helices in the formation of complexes between cationic peptides and ODNs. We therefore replaced parts of the poly-L-lysine chain with L-serine residues. The resultant synthetic copolymer, composed of L-lysine and L-serine had fewer cationic charges but had a greater tendency towards an α-helical configulation. When HLS was examined for its capacity to bind to ODNs, it did indeed form a tighter complex with ODNs than did PLL. Then, the stability of the complexes was examined in human serum.

Stability of ODNs with HLS in human serum

Figure 3 shows the time course of antisense ODN-degradation, in undiluted human serum, when formed complexes with the cationic peptides. In the case of naked ODNs, there were hardly degradation in seconds or within 1 min. In the caseof PLL-complexed ODNs, about 60% of each complex remained intact after 24hrs. in a form of complex with cationic peptides survived in undiluted human serum more than 24hours whereas naked ODN was degraded in seconds.

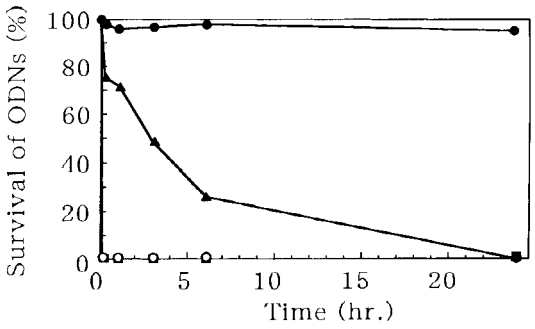


Figure 3. Effect of poly amino acids on stability of ODNs in undiluted human serum.

●: D-ODN with HLS ○: D-ODN alone
▲: D-ODN with PLL □: S-ODN alone

ELISA

The effects of antisense ODN with cationic peptide on inhibition of IL-1 β are shown in figure 4. Although S-antisense ODN of 10uM inhibited IL-1 β production by 20%, S-sense(SEN) ODN also inhibited IL-1 β production in considerable level. Surprisingly enough, low concentrations (nM levels) of antisense D-ODN:HLS complex dose-dependently inhibited IL-1 β production, and it was quite specific for its antisense sequence.

Further analysis revealed that antisense ONs were all released from the complex with HLS when the pH was decreased to 5.5 or lower (data not shown). Most probably, the release occurs because structures such as α-helices are destabilized at lower pH(upon the protonation of increasing numbers of lysine residues). This feature is important since the complex of HLS and a nucleic acid would be exposed to such a low pH(about 5.0)upon endocytosis and nucleic acids may be able to escape from the endosomes. Another advantage of using HLS as a carrier is that the ON / HLS complex dose not form a precipitate out at a high concentration. By contrast, ON/ PLL complex tends to precipitate out at a high concentrations. Complexes of nucleic acids and HLS by themselves cannot penetrate macrophage-like cells, requiring additional attachementof targeting molecules(data not shown). However, our preliminary data indicate that an antisense ON/HLS complex can exert an antisense effect in cells of macrophage origin.

It is clear that the PLL portion of cationic peptide carrier that has been used effectively in earlier experiments can be replaced by HLS since naked ODNs that are unstable in vivo can be completely protected from attack by DNases in human serum when they have formed a complex with HLS. The formation of this kind of complex provides a potentially powerful methodology for delivering any nucleic acids, including antisense molecules, into cells. Available data suggests that ODNs : poly-cationic amino acid complexes have high potential as carriers for ODN drugs.

Efficiency of HLS in mouse endotoxin shock model

IL-1 β has been implicated as playing a critical role in endotoxin shock.The appearance of IL-1 β in the serum of mice following infusion of endotoxin and the ability of anti-IL-1 β antibodies to protect the animals from death support the hypothesis that IL-1 β has a role in septic shock[8,9]. Figure 5 shows the effects of several ODNs on LPS lethality. Mortality as a result of LPS administration at 20mg/kg was apparent by 24hrs. most mice die[7]. S-ODNs treatment had no beneficial effect in the

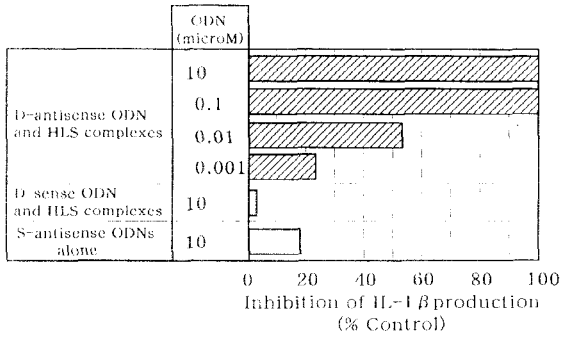


Figure 4. Inhibitory effect of the ODNs with or without HLS on IL-1 β secretion by human.

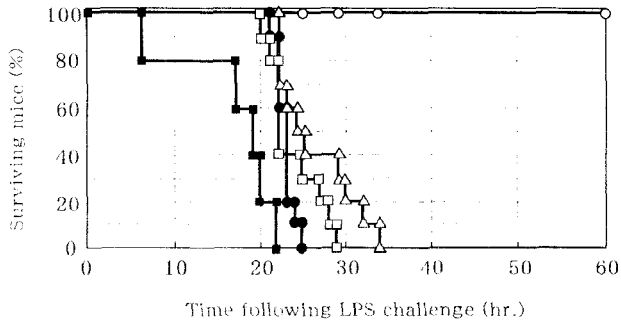
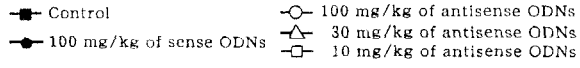


Figure 5. Effect of ODNs with HLS on LPS lethality.



endotoxin-shock model. High concentration of D-sense ODNs with HLS carriers were at 30 or 100mg/kg protected mice against endotoxine lethality, and these mice were long-time survivors, with no deaths occuring beyond 48hrs. In contrast, similar concentrations of ODN without HLS were ineffective in protecting BALA/c mice died, with kinetics similar to those observed for the non-complex- treated controls.

Powerful effects of the antisense D-ODN to IL-1 β gene and the HLS complexes suggest that this complex may be a new type of antiinflammatory agent in clinical use. The BALB/c has served as a useful animal model for anaphylatic, burn, endotoxine, and septic shock. Furthermore, the cationic copolymers may provide potentially strong methods for delivering antisense ODN targeted to other genes, and also for delivering other kinds of nucleic acid drugs or their analogs to the cells in various studies.

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