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Antisense Inhibition of Silica-induced Tumor Necrosis Factor in Alveolar Macrophages*

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Tumor necrosis factor-α (TNFα) has been shown to play an important role in the pathogenesis of silicotic fibrosis. In this study, antisense oligonucleotides targeted to TNFα mRNA were used to inhibit silica-induced TNFα gene expression in alveolar macrophages. To achieve macrophage-specific oligonucleotide delivery, a molecular conjugate consisting of mannosylated polylysine that exploits endocytosis via the macrophage mannose receptor was used. Complexes were formed between the mannosylated polylysine and oligonucleotides and added to the cells in the presence of silica. Enzyme-linked immunoadsorbent assay showed that the complex consisting of the conjugate and antisense oligomer effectively inhibited TNFα production, whereas the oligomer alone had much less effect. Reverse transcriptase-polymerase chain reaction analysis revealed that the reduction in TNFα secretion was associated with specific ablation of targeted TNFα mRNA. The conjugate alone or conjugate complexed with inverted or sense sequence oligonucleotide had no effect. The promoting effect of the conjugate on antisense activity was shown to be due to enhanced cellular uptake of the oligomer via mannose receptor-mediated endocytosis. Cells lacking mannose receptors showed no susceptibility to the conjugate treatment. These results indicate that effective and selective inhibition of macrophage TNFα expression can be achieved using the antisense mannosylated polylysine system.

Tumor necrosis factor-α (TNFα)[1] is a macrophage-derived peptide that has been shown to play an important role in the pathogenesis of pulmonary fibrosis (1–3). Several fibrogenic agents such as crystalline silica and asbestos stimulate TNFα mRNA expression and protein synthesis in macrophages (2, 3). Elevated TNFα levels stimulate fibroblast proliferation and production of collagen matrix, leading to fibrosis (2–4). Although the role of TNFα in the pathogenesis of pulmonary fibrosis has been well studied, relatively little is known about its molecular regulation during the fibrotic process. This information is important not only for the understanding of the molecular mechanism of disease pathogenesis but also for the development of potential therapeutic interventions.

The ability to regulate expression of an individual gene by the use of an antisense oligonucleotide (ON) complementary to a specific sequence of mRNA provides a powerful tool for elucidating the role of a particular gene and may allow therapeutic intervention when that gene is overexpressed (5). The strong binding affinity of ONs to their mRNA targets makes these compounds potent and specific inhibitors of gene expression (6). To take advantage of this specificity, however, the ONs must also be delivered selectively to the intended target cells, where they can find and bind to their target mRNA sequences. The lung represents a major target for antisense therapy for diseases such as cancer and fibrosis. However, the complex anatomy of the lung as well as the presence of numerous lung cell types make ON targeting in this organ difficult. Furthermore, ONs are structurally bulky and highly charged, so their cellular uptake is generally low (7) and must be enhanced.

Receptor-mediated endocytosis offers the potential to target a selected cell population and enhance cellular uptake of ONs. This method has been used successfully to aid ON delivery in selected cell types (8–11). For example, asialoorosomucoid-polylysine conjugates have been used to target ONs to HepG2 and chloramphenicol acetyltransferase cells (8, 9), and transferrin and folic acid conjugates have been employed to aid ON delivery to rapidly growing cells such as HL-60 cells (10, 11). In the lung, alveolar macrophages (AMs) represent a major target for dust-induced pulmonary fibrosis. These cells are also major sources of TNFα production in the lung. The AMs possess mannose-specific membrane receptors that can recognize and internalize glycoproteins bearing mannose residues (12, 13). On this basis, mannosylated glycoproteins could potentially be used to target and enhance ON uptake by the AMs. Targeted delivery of several cytotoxic drugs and antiviral agents by glycoproteins specific to macrophages has been demonstrated previously (14, 15). Moreover, 6-phosphomannosylated glycoproteins have also been used to target antisense ONs to peritoneal macrophages (16). In this study, we utilized mannosylated polylysine (MPL) as a drug-targeting vector to aid the cellular delivery of ONs to the AMs. The effectiveness of this system in promoting antisense activity and its mechanism of enhancement were evaluated.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—Nuclease-resistant phosphorothioate ON with a sequence complementary to the initiation codon of TNFα mRNA (17) (5′-TGTGCTCATGGTGTCTTT-3′, AS-ON), its inverted sequence (5′-TTTCTGTGGTACTCGTGT-3′, INV-ON), and sense sequence (5′-AAAGACACCATGAGCACA-3′, S-ON) were synthesized on an automated solid phase synthesizer using standard phosphoramidite chem-

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[1] The abbreviations used are: TNFα, tumor necrosis factor-α; AM, alveolar macrophage; BSA, bovine serum albumin; MPL, mannosylated polylysine; ON, oligonucleotide; RT-PCR, reverse transcriptase-polymerase chain reaction; AS-ON, antisense-ON with a sequence complementary to the initiation codon on TNFα mRNA; INV-ON, inverted sequence; S-ON, sense sequence.

istry (OligoTherapeutics Inc., Wilsonville, OR). A fluorescent label was sometimes attached to the terminal 5′ linkage group by the use of 5′-carboxyfluorescein phosphoramidite. The ONs were purified by high performance liquid chromatography and were >98% pure.

Synthesis of Mannosylated Polylysine—The MPL was synthesized according to the method previously described (18). Briefly, poly-L-lysine (Sigma, 0.25 μmol, $M_r \approx 10{,}000$) was dissolved in 0.15 M NaCl solution. The pH of the solution was adjusted to 9.0 using 0.1 N NaOH. 4-Isothiocyanatophenyl-α-D-mannoside (Sigma, 12.5 μmol) was added in small portions to the magnetically stirred protein solution. After a 6-h reaction, the resulting solution was refrigerated overnight, and on the following day the pH was adjusted to 7.0. Unreacted mannoside and polylysine were removed by centrifugal filtration through a dialysis membrane filter (Durapore™ CL3K, Millipore Corp.) at 5000 × *g* for 30 min. The degree of sugar substitution was determined by the resorcinol sulfuric assay (19). The percentage of sugar in MPL was determined using the equation $X\% = n \times M_o/(M_p + n \times M)$, where n is the number of mannose residues per polylysine molecule and M_o, M_p, and M are the molecular weights of mannose, polylysine, and isothiocyanatophenyl-mannoside, respectively. The MPL prepared under this condition was found to contain ≈25% sugar.

Oligonucleotide-mannosylated Polylysine Complex—In a typical experiment, the complex was prepared by adding 25 μl of 50 μg/ml MPL to 25 μl of 50 μg/ml ON in culture medium for 1 h prior to use. In studies designed to evaluate the dose effect of ON, various concentrations of ON (25–100 μg/ml) were used. Complex formation was verified by gel electrophoresis using a 6% polyacrylamide, 7 M urea gel. Using a dual fluorescence labeling technique, we previously found that both the ON and MPL, when prepared as a complex under this condition, were co-internalized by the AMs (20). In a separate study, the stability of ON and ON·MPL complex was studied in culture medium using gel electrophoresis. After a 7-h incubation at 37 °C, no detectable degradative products of ONs were observed.

Cell Preparations—The AMs were harvested from male Sprague-Dawley rats (200–250 g) by bronchoalveolar lavage. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (0.2 g/kg of body weight). The trachea was cannulated and the lungs lavaged 10 times with 8-ml aliquots of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4). Lavaged cell suspensions were centrifuged at 500 × *g* for 10 min at 4 °C. The cell pellets were washed twice by alternate resuspension and centrifugation in macrophage-serum-free medium, pH 7.4 (Life Technologies, Inc.). Cell number and purity of the macrophage preparations were determined using a Coulter electronic cell counter with a cell-sizing attachment (Coulter Instrument, Hialeah, FL). The average values for yield and purity were 6.2 ± 0.3 × 10^6 cells/rat and 92.5 ± 0.4%, respectively. Cell viability, measured via trypan blue exclusion, was >95%. Aliquots of 0.1 ml containing 10^5 cells were added onto a 96-well plate (Costar, Cambridge, MA) and incubated at 37 °C in a humidified atmosphere at 5% CO_2.

In studies involving the use of alveolar epithelial cells, the lungs were lavaged as described above. They were then excised and incubated for 20 min at 37 °C with phosphate-buffered saline containing elastase (40 units/ml, Type I, U. S. Biochemical Corp.) and DNase (0.006%, Sigma). After enzymatic digestion, the lungs were finely minced and the digestion was arrested by incubation for 5 min in phosphate-buffered saline containing 25% fetal bovine serum and 0.006% DNase (to help prevent cell clumping). The crude extract was sequentially filtered through 160- and 45-μm screens and centrifuged, and the resulting cell pellet was spun on a sterile Percoll density gradient. The second cell band from the surface was collected, washed twice, and resuspended in Dulbecco's modified Eagle's medium. The cell suspension yielded 15–20 × 10^6 cells/rat with viability greater than 95% as determined by the Coulter counter and trypan blue exclusion.

Cell Culture—J774.1 cells, a macrophage cell line deficient in mannose receptors (21), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 μg/ml streptomycin. They were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Prior to use, the cells were washed and resuspended in macrophage-serum-free medium culture medium.

Silica Exposure and Antisense Inhibition Studies—Cells were plated on a 96-well plate at the density of 10^5 cells/well. They were treated with heat-sterilized silica (Mil-U-Sil, endotoxin-free, Pennsylvania Glass and Sand Corp.) or titanium dioxide (a nonfibrogenic dust control) (Fisher), both at final concentrations of 10–100 μg/ml. To test the effect of ONs on TNFα expression, the cells were simultaneously treated with free oligomers (AS-ON, INV-ON, or S-ON) (25 μg/ml) or oligomers complexed with MPL (ON·MPL, 25:25 μg/ml). After a 7-h incubation at 37 °C, the culture medium was collected and analyzed for TNFα using a Genzyme TNFα enzyme-linked immunoadsorbent assay kit (Genzyme Corp., Cambridge, MA) according to the manufacturer's instructions. This assay is specific for TNFα and does not cross-react with other cytokines, as stated by the manufacturer.

Analysis of TNFα mRNA was also performed using RT-PCR assay. Total RNA was extracted from cells following treatments. Lysis buffer (500 μl) containing 0.15 M NaCl, 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 0.65% Nonidet P-40 was added to a cell pellet containing approximately 10^6 cells. The mixture was incubated for 5 min at 4 °C. After centrifugation, the supernatant was mixed with an equal volume of urea-SDS buffer (7 M urea, 10 mM Tris, pH 7.5, 10 mM EDTA, 1% SDS). Then protein precipitation was carried out in the presence of 2 M NaCl. The tubes were centrifuged and RNA was precipitated from the supernatant with 2 volumes of ethanol. Reverse transcription and PCR (2 μg of RNA) were performed using a Clontech RT-PCR kit according to the manufacturer's instructions (Clontech Laboratories). Reactions were performed on a Perkin-Elmer thermal cycler (model 480). As an internal standard, β-actin cDNA was also amplified. Samples were denatured for 2 min at 94 °C and then amplified for 25 cycles, with each cycle consisting of three heated segments: 94 °C for 30 s, 55 °C for 30 s, and 74 °C for 90 s. Samples were held for a further 10 min at 74 °C on the final cycle. The primers used were based on those previously reported (22). Reaction products were precipitated and separated on a 7% polyacrylamide gel and visualized by ethidium bromide staining.

Cellular Uptake Studies—Cellular uptake studies were conducted using fluorescently labeled AS-ON. The cells (10^5/well) were plated on a 96-well plate and incubated for 4 h at 37 °C in culture medium containing AS-ON (25 μg/ml) or AS-ON·MPL complex (25:25 μg/ml). The cells were washed with cold medium containing excess unlabeled ON and/or MPL to remove surface-bound labeled ON. The cells were then measured for their fluorescence intensity using a fluorescence microplate reader at the excitation and emission wavelengths of 490 and 520 nm, respectively. For competitive inhibition studies, cells were treated with the AS-ON·MPL complex in the presence of 1 mg/ml mannosylated BSA (a specific competitor for mannose receptors) (Sigma) or BSA (a non-specific competitor).

Cell Viability—Cell viability was determined using fluorescence propidium iodide assay. After specific treatments the cells were incubated with 1 μg/ml propidium iodide in culture medium for 10 min at 37 °C. Cellular fluorescence intensity was then measured at the excitation and emission wavelengths of 490 and 600 nm, respectively. Cell survival was calculated as percentage cell survival = 100 − percentage cell death.

$$\text{Percentage cell death} = \frac{\text{measured signal} - \text{minimum signal}}{\text{maximum signal} - \text{minimum signal}} \times 100\%$$

The maximum signal is the fluorescence signal obtained in the presence of Triton X-100 (0.1%), which was used to permeabilize the cells. The minimum signal is the background autofluorescence signal.

Statistical Analysis—Data were analyzed by analysis of variance with the use of the Newman-Keuls test for multiple comparisons. Statistical significance was considered at $p < 0.05$.

RESULTS

Stimulation of TNFα by Silica and Its Inhibition by Antisense Oligonucleotides—Exposure of the AMs to crystalline silica (10–100 μg/ml) resulted in a dose-dependent increase in TNFα secretion over nontreated controls (Fig. 1). Treatment of the cells with nonfibrogenic dust titanium dioxide at similar concentrations had no significant effect on TNFα secretion (Fig. 1). In order to assess the potential inhibitory effect of antisense ONs on silica-stimulated TNFα release, AS-ON (25 μg/ml) or AS-ON·MPL complex (25:25 μg/ml) was added to the cells simultaneously with silica (100 μg/ml). As controls, noncomplementary ON sequences (INV-ON and S-ON) and their respective MPL complexes (INV-ON·MPL and S-ON·MPL) at the same concentrations were also used. Our results, shown in Fig. 2, indicated that treatment of the cells with free AS-ON had no significant effect on TNFα level ($p < 0.05$). However, when the AS-ON was given as a complex (AS-ON·MPL), >90% of the TNFα secretion was inhibited. Non-antisense ONs, given either alone or in the form of complexes, had no effect on TNFα

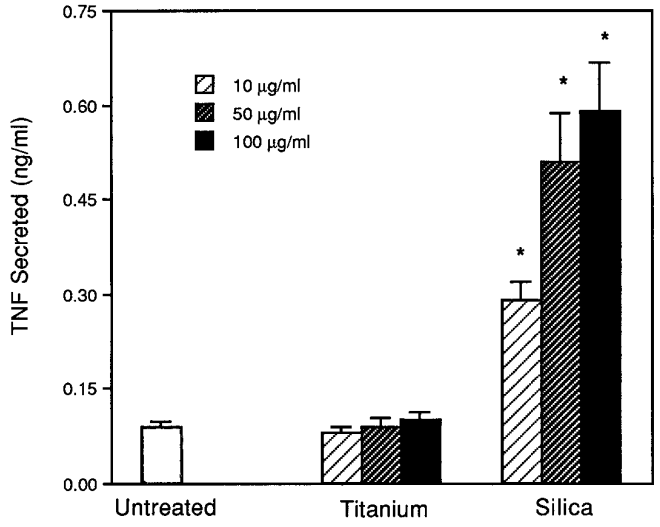

FIG. 1. **Release of TNFα by alveolar macrophages after exposure to silica or titanium dioxide.** AMs (10^5/well) were incubated with heat-sterilized silica or titanium dioxide (10–100 μg/ml) for 7 h in culture medium at 37 °C in 5% CO_2. After incubation, the culture medium was collected and analyzed for TNFα. The data represent mean ± S.E. of four measurements obtained from different cell preparations. *Asterisks* denote a significant difference from untreated and titanium-treated controls ($p < 0.05$).

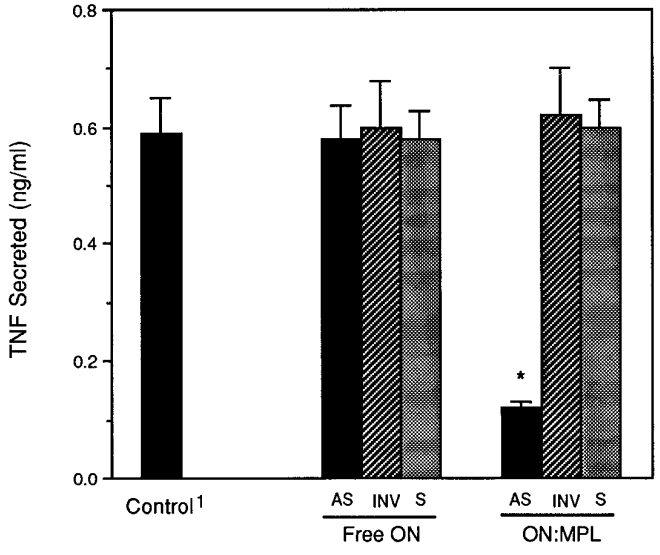

FIG. 2. **Antisense activity of ON mediated by MPL.** Cells (10^5/well) were incubated with silica (100 μg/ml) in the presence or absence (*Control*) of free ON (25 μg/ml) or ON·MPL complex (25:25 μg/ml). After a 7-h incubation at 37 °C, the culture samples were collected and analyzed for TNFα. The values represent mean ± S.E., $n = 4$. *Asterisk* indicates significant difference from control ($p < 0.05$).

level ($p < 0.05$). The latter results suggested that the inhibitory effect of AS-ON·MPL complex was not due to MPL and that the presence of AS-ON was required for the anti-TNFα effect. Studies using MPL alone (see below) further confirmed this point.

The ability of AS-ON·MPL complex to suppress TNFα production at the cellular level leads to the question of its molecular mechanism of TNFα inhibition. To address this query, AS-ON·MPL complex (25:25 μg/ml) was added to the AMs in the presence of silica (100 μg/ml), and 4 h later mRNA for TNFα was recovered and assessed by RT-PCR analysis. As controls, AS-ON (25 μg/ml), INV-ON (25 μg/ml), or MPL (25 μg/ml) alone were also tested (Fig. 3). The addition of AS-ON·MPL complex reduced the expression of silica-induced TNFα mRNA, whereas all other control treatments had no effect. These results are consistent with the earlier enzyme-linked immunoadsorbent assay studies and suggest that the mechanism of TNFα inhibition by the AS-ON complex is due to translational inhibition of TNFα mRNA.

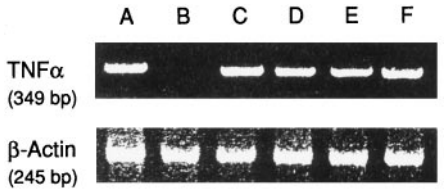

FIG. 3. **TNFα and β-actin mRNA expression in silica-exposed AMs.** Representative ethidium bromide-stained gels showing TNFα and β-actin products ($n = 3$) amplified from RNA of silica-treated AMs. The cells were treated with silica (100 μg/ml) for 4 h at 37 °C in the absence (*A*) or presence of AS-ON·MPL (25:25 μg/ml) (*B*), MPL (25 μg/ml) (*C*), AS-ON (25 μg/ml) (*D*), INV-ON (25 μg/ml) (*E*), and S-ON (25 μg/ml) (*F*). Total RNA was extracted and reversed transcribed, and the resulting cDNA was amplified and electrophoresed as described under "Experimental Procedures."

The lack of the inhibitory effect of free AS-ON is somewhat surprising but may be attributed to its poor cellular uptake and the inability to reach its intracellular target. Because previous studies have established that cellular uptake of ONs is dose-dependent (6), we therefore investigated the effect of ON concentration (25–100 μg/ml) on silica-induced TNFα production (Fig. 4). Our result showed that an increase in ON concentration (AS-ON), as expected, resulted in a corresponding increase in TNFα inhibition. A significant inhibitory effect (≈35%) was observed at the AS-ON concentration of 75 μg/ml ($p < 0.05$). At the highest concentration tested (100 μg/ml), ≈50% inhibition was observed. The control INV-ON had no significant effect on TNFα production at all concentrations tested except at 100 μg/ml, at which concentration a minimal but significant effect was observed (≈15%) ($p < 0.05$). This latter result suggested the possible nonsequence-specific effect of ON, which has been reported in other systems when high concentrations of ONs are used (23, 24). The potential cytotoxic effect of ONs at high concentrations was also examined using fluorescence propidium iodide assay. The percentage of cells viable after treatment with 100 μg/ml AS-ON was 94 ± 5 and 92 ± 4% for INV-ON. These values were not significantly different from that of the nontreated control (95 ± 4%) ($p < 0.05$, $n = 4$).

Antisense Oligonucleotide Cellular Uptake Mediated by Mannosylated Polylysine—To further examine the promoting effect of MPL on antisense activity of AS-ON, cellular uptake studies were conducted. In these studies, 5′ fluorescently labeled AS-ON was used to allow direct detection of cellular uptake. AS-ON (25 μg/ml) or AS-ON·MPL complex (25:25 μg/ml) was added to the AMs, and after indicated time intervals the cells were washed and analyzed for their fluorescence intensity. As shown in Fig. 5, the MPL greatly promoted the cellular uptake of AS-ON. At 4 h of incubation, the uptake of the complex was found to be ≈17-fold greater than that of the free AS-ON. This result indicated that the MPL carrier system was highly effective at promoting the cellular uptake of AS-ON and that the AS-ON alone was poorly taken up by the AMs. The saturable nature of the complex uptake is indicative of receptor-mediated endocytosis.

To test whether the cellular uptake of the MPL complex occurred via mannose receptor-mediated endocytosis, cellular uptake of the AS-ON·MPL complex in the presence of competition for mannose receptors was carried out. In these experiments, the AS-ON·MPL complex was incubated with the AMs in the presence of an excess amount of a specific mannose receptor competitor, mannosylated BSA, or a nonspecific competitor, BSA (Fig. 6). These results demonstrated that the

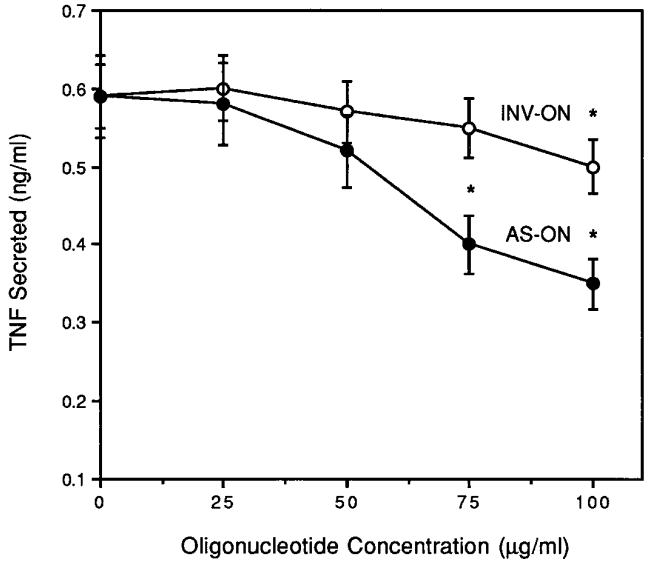

FIG. 4. **Effect of free ON concentration on silica-induced TNFα release.** Cells (10^5/well) were incubated with silica (100 μg/ml) in the presence of varying amounts of free ONs (25–100 μg/ml) in culture medium. After a 7-h incubation at 37 °C, the culture samples were collected and analyzed for TNFα. The values represent mean ± S.E., $n = 4$. *Asterisks* indicate significant differences from non-ON-treated control ($p < 0.05$).

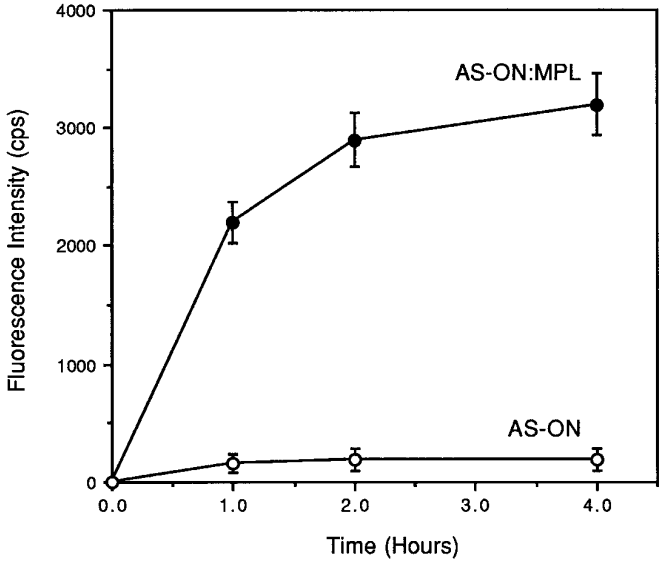

FIG. 5. **Comparison of cellular uptake of free ON or ON complexed with MPL.** Cells (10^5/well) were incubated with free AS-ON (25 μg/ml) or AS-ON·MPL complex (25:25 μg/ml) for 4 h in culture medium at 37 °C in 5% CO_2. After incubation, the cells were washed with fresh medium containing excess unlabeled ON and/or MPL to remove surface-bound labeled ON. The cells were then measured for their fluorescence intensity using a fluorescence microplate reader at the excitation and emission wavelengths of 490 and 520 nm, respectively. The values represent mean ± S.E., $n = 4$.

cellular uptake of the AS-ON·MPL complex was inhibited by mannosylated BSA but not by BSA, thus indicating that the ON uptake mediated by MPL occurred via the mannose receptor pathway.

To further confirm the mechanism of AS-ON complex uptake and to test the potential targeting ability of the MPL system, uptake studies were repeated in the J774.1 macrophage cell line, which is known to express a low level of mannose receptors (21), and in alveolar epithelial cells, which lack mannose receptors (Fig. 6). Both cell types exhibited a much lower complex uptake compared with the AMs ($p < 0.05$), thus confirming

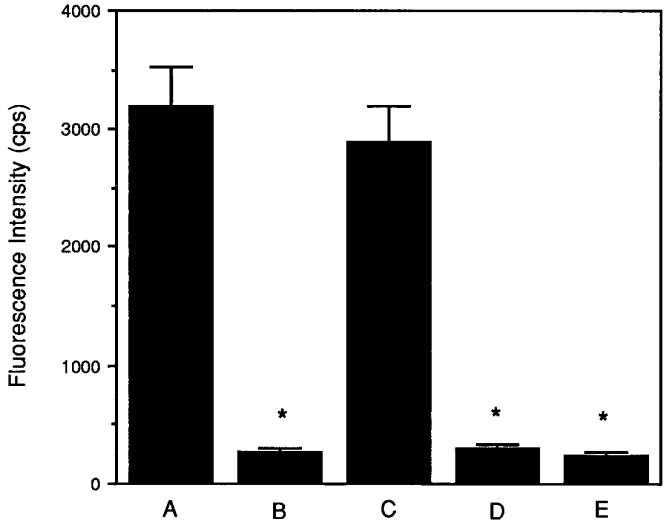

FIG. 6. **Mechanism of ON uptake mediated by MPL.** AMs (10^5 cells/well) were incubated with AS-ON·MPL complex (25:25 μg/ml) at 37 °C for 4 h in the absence (*A*) or presence of mannosylated albumin (*B*, 1 mg/ml) as a specific competitor for mannose receptor or albumin (*C*, 1 mg/ml) as a nonspecific competitor. Experiments were also conducted using J774.1 (*D*) or alveolar epithelial cells (*E*) (both at 10^5/well) in the presence of AS-ON·MPL (25:25 μg/ml). Cellular uptake was monitored fluorometrically as described in Fig. 5. The values represent mean ± S.E., $n = 4$. *Asterisks* indicate significant differences from control (*A*) ($p < 0.05$).

the mannose receptor-mediated complex uptake and the targeting ability of the MPL carrier system.

DISCUSSION

Pulmonary macrophages are known to be sources of inflammatory mediators and fibrogenic factors and are one of the first cells to respond to inhaled particles in the lung. Several studies have shown that TNFα plays an important role in the pathogenesis of silicotic fibrosis. Driscoll *et al.* (1) demonstrated an increase in TNFα production by the AMs in rats treated with silica. Piguet *et al.* (2) later showed that this increase in TNFα production was associated with increased expression of TNFα mRNA in the lung, although the cellular origin of TNFα was not identified in this study. The work by Piguet *et al.* (2) also demonstrated that pretreatment of the animals with anti-TNFα IgG or exogenous recombinant TNFα reduced and augmented, respectively, silica-induced fibrosis. Thus, these studies strongly indicate the role of TNFα in silicotic fibrosis.

There are, however, a number of studies that have reported contradictory results. Mohr *et al.* (25) and Lemaire (26) showed that silica treatment in rats had no effect on TNFα production by the AMs. Bissonnette and Rola-Pleszczynski (27) similarly reported the lack of silica effect in mice. The discrepancies between test results by different groups may be attributed to differences in experimental conditions, *i.e.* the dose and type of silica used and the mode of silica instillation. With regard to the dose, Driscoll *et al.* (1) showed that silica stimulation of TNFα release occurred only at doses greater than 50 mg/kg. Lemaire (26) also reported the lack of silica effect on TNFα release at a low silica dose of 15 mg/kg. In agreement with these *in vivo* data, our *in vitro* results indicated a dose-dependent effect of silica on TNFα production by the AMs. Silica directly stimulated TNFα secretion and mRNA expression, effects that were not observed with the nonfibrogenic dust titanium dioxide. These results confirm and extend those of Gosset *et al.* (28) and Driscoll *et al.* (29), who reported a direct effect of silica on TNFα production in isolated human and rat AMs, and those of Savici *et al.* (30), who reported an elevated TNFα gene

expression in THP-1 myelomonocytic cells following silica treatment.

The regulatory role of TNFα in fibrogenesis and its potential therapeutic intervention can be studied at the molecular level using antisense ONs. These compounds have the potential to interfere selectively with cellular protein synthesis by sequence-specific hybridization to DNA or RNA molecules. Since the gene sequence encoding TNFα had been previously identified (17), we predicted that specific antisense sequences could be synthesized and used to inhibit TNFα expression and therefore fibrosis. To investigate this possibility, we utilized an antisense ON specific to the initiation codon of the TNFα mRNA (AS-ON) and tested its effect on the macrophage cytokine gene expression. Our results indicate that AS-ON can be used to inhibit TNFα expression. However, when used alone this compound is relatively ineffective, and high concentrations are required to elicit the effect. At a high concentration of 100 μg/ml, AS-ON causes ≤50% inhibition of TNFα production. Because high concentrations of ONs are commonly associated with nonsequence-specific effects (23, 24), innovative approaches to improving potency of ONs are desirable.

It has been reported that ONs, due to their polyanionic nature, poorly permeate cell membranes to reach their intracellular targets (31, 32). Several research groups (23, 33, 34) also observed that in the absence of appropriate delivery systems ONs exhibited no antisense activity, whereas in the presence of these delivery systems, *i.e.* liposomes, ONs showed strong activity. These observations suggest that ONs do not normally enter the cells to a significant extent, and therefore approaches to improve their cellular uptake are important for their effective use. In agreement with previous findings, our results indicated that the cellular uptake of free ON by the AMs is very low, and this may account for the observed low activity of the AS-ON. This conclusion is supported by a dramatic and parallel increase in cellular uptake and anti-TNFα activity of the AS-ON when given as a complex with the MPL. At a low concentration of AS-ON (25 μg/ml), the MPL (25 μg/ml) promoted a 17-fold increase in cellular uptake of the ON and caused ≥90% TNFα inhibition. At this concentration level, the AS-ON alone had no significant effect on TNFα. The effect of AS-ON·MPL complex on TNFα activity was associated with specific ablation of targeted mRNA as shown by RT-PCR. Control studies also showed that the promoting effect of MPL was not associated with loss of cell viability as determined by fluorescence propidium iodide assay. Furthermore, MPL alone or complexed with non-antisense ONs (INV-ON and S-ON) had no effect on TNFα activity. Thus, these results suggested that the promoting effect of MPL was most likely due to enhanced ON uptake.

To further confirm these observations and to test the mechanism of ON uptake mediated by the MPL, a series of cellular uptake experiments was conducted. Our results indicated that MPL promoted the cellular uptake of AS-ON via mannose receptor-mediated pathway since competition for mannose receptors by mannosylated BSA inhibited the uptake of the AS-ON·MPL complex and the noncompetitor BSA had no effect. Furthermore, cells lacking mannose receptors (epithelial cells) or bearing few receptors (J774.1 cells) did not appreciably take up the AS-ON·MPL complex. The latter results also indicated the potential targeting ability of the MPL carrier system for the AMs.

In conclusion, we have demonstrated that antisense ON targeted to the TNFα mRNA can be used to inhibit TNFα expression in AMs. The findings in this study have a direct implication on the therapeutic utilization of ONs in pulmonary fibrosis. A similar strategy may be employed to aid the study of specific cytokine functions and their role in disease pathogenesis. Effective utilization of antisense ONs requires development of appropriate delivery systems capable of effective and selective transfer of ONs to the target cells. With regard to the AMs, we have shown that the MPL delivery system is effective and selective in promoting the cellular delivery and antisense activity of ON. The system exploits the efficient receptor-mediated endocytosis to achieve cell-specific antisense delivery. Because the system utilizes a naturally occurring cellular process, it is potentially nontoxic. Although our *in vitro* studies indicate the relative safety of this system, further *in vivo* studies are needed. Demonstration of its safety and efficacy will allow further development of this system for therapeutic purposes.

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