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Inhibition of LPS-induced nitric oxide production by transduced Tat-arginine deiminase fusion protein in Raw 264.7 cells

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Arginine deiminase (ADI), an arginine-degrading enzyme, has anti-proliferative and anti-tumor activities and is capable of inhibiting the production of nitric oxide (NO). Modulation of nitric oxide (NO) production is considered a promising approach for the treatment of various diseases including cancer, inflammation and neuronal disorders. In this study, an ADI gene was fused with an HIV-1 Tat peptide in a bacterial expression vector to produce an genetic in-frame Tat-ADI fusion protein. When added exogenously to the culture media, the expressed and purified Tat-ADI fusion proteins were efficiently transduced into macrophage Raw 264.7 cells in a time- and dose-dependent manner. Furthermore, transduced Tat-ADI fusion proteins markedly increased cell viability in cells treated with lipopolysaccharide (LPS). This increase in viability was mediated by an inhibition of NO production. These results suggest that this Tat-ADI fusion protein can be used in protein therapies of NO-related disorders such as cancer, inflammation and neuronal diseases. [BMB reports 2009; 42(5): 286-292]

INTRODUCTION

Arginine deiminase (ADI) is a mycoplasma enzyme that catalyzes the imine hydrolysis of arginine to produce citrulline and ammonia. ADI is a key enzyme of the ADI pathway, which utilizes arginine as a major nonglycolytic energy source (1). The ADI pathway involves three enzymes: the first, ADI, hydrolyzes arginine to citrulline and ammonia, the second, ornithine transcarbamylase, converts citrulline into carbamoyl phosphate and ornithine, and the last, carbamate kinase, transfers a phosphate from cabarmoyl phosphate to ADP, resulting in a single ATP molecule (2). Several studies have reported that ADI inhibits the proliferation of human cancer cells and that it has antitumor activity (3-8). Arginine significantly influences the inflammation process and carcinogenesis because it is involved in polyamine synthesis, creatine production and NO generation (2).

It is well known that amino acid deprivation results in the starvation of cancer cells and can be a strategy for cancer therapy. For example, asparaginase has been used for treating acute lympoblastic leukemia since these cancer cells lack asparagine synthetase and are thus auxotrophic for asparagines. ADI, an arginine-degrading enzyme, is in clinical trials for the treatment of arginine-requiring cancers such as hepatocellular carcinoma (HCC) and metastatic melanoma (9-12). Moreover, Ni *et al*. (2008) suggest that ADI has several advantages when compared to traditional chemotherapy, including high specificity for targeting malignant cells and low toxicity for patients.

The inflammatory process involves the activation of monocytes and macrophages, which secrete inflammatory mediators such as nitric oxide (NO). Inducible nitric oxide synthase (iNOS) is one of three enzymes generating NO, which mediates many physiological events. It is well known that the overproduction of iNOS is associated with various human diseases including inflammatory and neuronal disorders (13-20). Lipopolysaccharide (LPS) is the main component of endotoxin and is formed by a phosphoglycolipid that is covalently linked to a hydrophilic heteropolysaccharide (21). LPS arrests macrophage proliferation and activates them to produce proinflammatory factors, which play important roles in the immune response (22, 23).

Previously, many researchers have demonstrated the successful delivery of full-length Tat fusion proteins using protein transduction technology. Several small regions of proteins, called protein transduction domains (PTDs), have been developed to allow for the delivery of exogenous proteins into living cells (24-27). By using this protein transduction technology, we have reported that various fusion proteins were efficiently transduced into mammalian cells and animal tissues. These transduced fusion proteins markedly protected cells and

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tissues against reactive oxygen species (28-31). Recently, we demonstrated that PTD fusion proteins or PTD signal peptides can be efficiently transduced into cells and maintain their enzymatic activities (32-34).

In the present study, we designed a Tat-ADI fusion protein for transduction and showed that this Tat-ADI fusion protein can be directly transduced into Raw 264.7 cells and that it efficiently protects them against LPS-induced cell death. Therefore, we suggest that the Tat-ADI fusion protein may be useful as a potential therapeutic agent for inflammatory skin diseases and NO-related diseases. In addition, the Tat-ADI fusion protein will accelerate the development of ADI as a therapeutic protein for the treatment of cancer.

RESULTS AND DISCUSSION

Construction and purification of Tat-ADI fusion proteins

To generate a cell-permeable expression vector, Tat-ADI, an ADI cDNA, was subcloned into the pET-15b plasmid that had been reconstructed to contain the Tat peptide. The Tat-ADI expression vector thus contained consecutive cDNA sequences encoding ADI, HIV-1 Tat peptide and six histidine residues at the amino-terminus (Fig. 1A). We also constructed the ADI expression vector to produce a control ADI protein without PEP-1 transduction peptides (data not shown).

Following induction, the Tat-ADI fusion proteins were purified. The fusion proteins were expressed in *E. coli* and the clarified cell extracts were loaded onto a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column. Fractions containing the fusion protein were combined and salts were removed using a PD10 column. The crude cell extracts obtained from *E. coli* and the purified Tat-ADI fusion proteins were separated by 10% SDS-PAGE. The expression and purification results are shown in Fig. 1B. As shown in Fig. 1B, Tat-ADI fusion proteins were highly expressed and the Tat-ADI fusion proteins were found to be nearly homogenous and greater than 95% pure, as determined by SDS-PAGE. The expressed and purified proteins were further characterized by Western blot analysis using an anti-rabbit polyhistidine antibody. Tat-ADI was detected in the corresponding bands seen in Fig. 1C. The purified Tat-ADI fusion protein had a specific activity of 76.2 unit/mg. Noh *et al.* (2002) demonstrated that purified recombinant ADI protein had a 72.3 unit/mg of ADI activity. Also, purified recombinant ADI had a molecular mass of 45 kDa, as determined by SDS-PAGE (8). Our results indicate that the purified Tat-ADI fusion protein is similar to that of the recombinant ADI.

Transduction of Tat-ADI fusion protein into Raw 264.7 cells

To evaluate the transduction ability of Tat-ADI fusion proteins, purified Tat-ADI fusion proteins were added to the culture media of Raw 264.7 cells for various times and concentrations and analyzed by Western blotting. As shown in Fig. 2A, the Tat-ADI fusion proteins were transduced into Raw 264.7 cells in a dose-dependent manner. Tat-ADI fusion proteins were added to the cell cultures at various concentrations (1-3 μM) for 1 h and the levels of transduction were analyzed by Western blotting. Next, the time-dependency of Tat-ADI fusion protein tranduction was further analyzed. Tat-ADI (3 μM) fusion proteins were

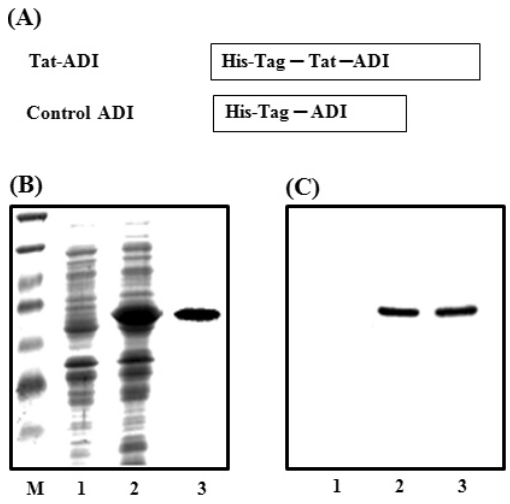

Fig. 1. Expression and purification of the Tat-ADI fusion protein. A schematic representation of the Tat-ADI fusion protein is presented in panel A. Protein extracts from cells and purified fusion proteins were analyzed by 10% SDS-PAGE (B) and subjected to Western blot analysis with an anti-rabbit polyhistidine antibody (C). The lanes in B and C are as follows: lane 1, non-induced; lane 2, induced Tat-ADI; lane 3, purified Tat-ADI.

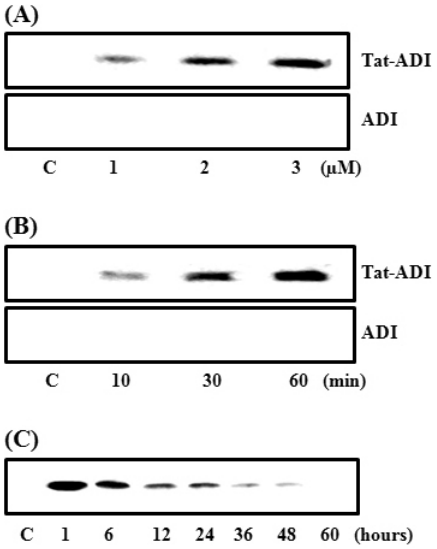

Fig. 2. Transduction of Tat-ADI fusion proteins. Prior to Western blot analysis, Tat-ADI proteins (1-3 μM) were added to the culture media for 1 h (A), Tat-ADI (3 μM) were added to the Raw 264.7 cell culture media for 10-60 min (B), the cells were pre-treated with 3 μM Tat-ADI and incubated for 1-60 h (C).

added to the cell cultures for various times (10-60 min) and the levels of transduction were analyzed by Western blotting. As shown in Fig. 2B, the intracellular concentration of Tat-ADI transduced into cells gradually increased over time. In addition, we examined the stability of transduced Tat-ADI fusion protein in the cells by Western blotting. Raw 264.7 cells were incubated with 3 μM Tat-ADI for 1 h, free Tat-ADI was removed by washing and the cells incubated for up to 60 h. The levels of intracellular Tat-ADI declined gradually over the period of observation, however significant levels of transduced Tat-ADI fusion protein persisted in the cells for 48 h. It was reported that a Tat-β-galactosidase fusion protein was rapidly transduced into HepG2 cells, reaching near-maximum intracellular concentrations in less than 15 min (26). These small differences in time course stability may be a result of the properties of the transduced Tat fusion proteins, such as the degree of folding, polarity and the molecular shape of the protein.

The intracellular delivery of Tat-ADI into Raw 264.7 cells was confirmed by direct fluorescence. To exclude the possibility that cell fixation with paraformaldehyde affects Tat-ADI transduction using direct fluorescence, we transduced a FITC-labeled Tat-ADI protein into non-fixed cells. Immunofluorescence staining using a rabbit anti-histidine polyclonal antibody revealed that the Tat-ADI fusion protein was transduced into cells, whereas fluorescence signals were absent in cells not treated with the Tat-ADI fusion protein. In addition, the fluorescence patterns of fixed cells were similar to those of non-fixed cells. These results indicate that cell fixation is not required for Tat-ADI fusion protein transduction (data not shown).

HIV-1 Tat and $(Arg)_9$ PTD fusion proteins are not transduced across the cell membrane of living cells and any apparent entry is an artifact caused by fixation. Fixation disrupts the cell membrane and therefore cannot be reliably used to study membrane-translocating proteins since peptides and fusion proteins are internalized by endocytosis. Thus, fixation should be avoided in studies of protein transduction into living cells (35). However, we were unable to detect any differences in the distribution of fluorescence in non-fixed and fixed cells transduced with Tat-ADI. Similar observations indicate that artifacts of protein transduction are not induced by paraformaldehyde fixation (36). We have also observed that the transduction of various PTD-fusion proteins into cells was not affected by paraformaldehyde fixation (29, 31).

The effect of transduced Tat-ADI fusion proteins on LPS-induced iNOS expression and cell viability

Macrophages play crucial roles in the initiation and maintenance of inflammation. Since the level of NO is important in addressing the extent of inflammation and cell viability, the effects of this Tat-ADI fusion protein on the inhibition of NO expression and cell viability were investigated. To determine whether transduced Tat-ADI fusion protein is cytotoxic to Raw 264.7 cells, the cells were pretreated for 1 h with various concentrations (1-3 μM) of Tat-ADI fusion proteins followed by 12 h additional incubation. The Tat-ADI fusion protein was not significantly cytotoxic to the cells (data not shown). It is well known that NO plays a key role in the pathophysiology of many diseases. To determine the effects of Tat-ADI on NO production, Raw 264.7 cells were incubated for 12 h with LPS (100 ng/ml) in the presence or absence of various concentrations of this Tat-ADI fusion protein. As shown in Fig. 3, the Tat-ADI fusion protein suppressed LPS-induced NO production and the expression levels of iNOS in Raw 264.7 cells in a dose-dependent manner. Although the exact mechanism of Tat-ADI transduction on NO synthesis remains to be elucidated, the suppression of iNOS in cells transduced with the Tat-ADI fusion protein was observed. Similar observations have been reported indicating that recombinant ADI protein supressed iNOS and NO production by depleting L-arginine in the medium (13, 37, 38).

To determine whether the transduced Tat-ADI fusion proteins have a functional role in the cells, we tested the effects of transduced Tat-ADI fusion proteins on cell viability. As shown in Fig. 4, when the cells were exposed to LPS (50 μg/ml) for 12 h without the Tat-ADI fusion protein, only 40-45% of the cells

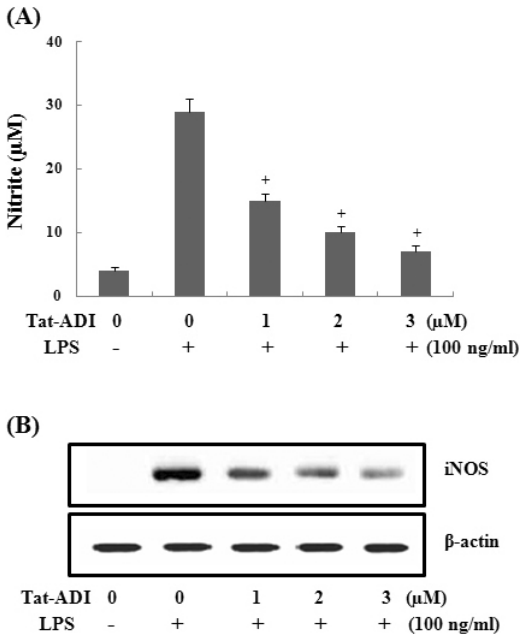

Fig. 3. The effect of transduced Tat-ADI on LPS-induced NO production (A) and iNOS expression levels (B). Raw 264.7 cells were pretreated with Tat-ADI for 1 h prior to LPS (100 ng/ml) incubation for 12 h. Nitrite levels in the culture media of LPS-stimulated cells were measured using the Griess reaction (A) and iNOS expression levels were analyzed by immunoblotting using an anti-iNOS antibody (B). Each bar represents the mean ± SEM obtained from five experiments. Crosses (+) denote statistical significance at $P < 0.01$ compared to treatment with LPS alone. The statistical analysis was performed using the Student's *t*-test.

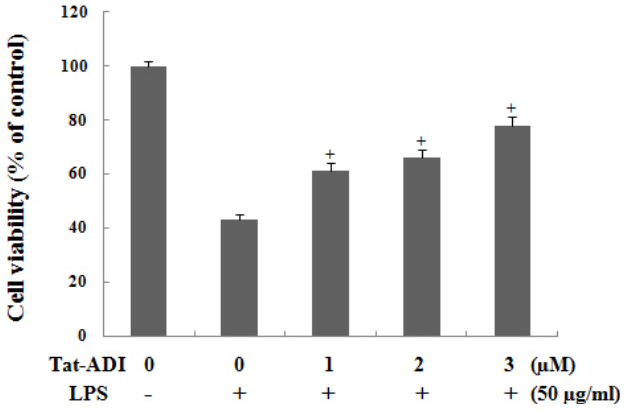

Fig. 4. The effect of transduced Tat-ADI on the viability of Raw 264.7 cells. LPS (50 μg/ml)-exposed cells pre-treated with 1-3 μM of Tat-ADI for 1 h. Cell viabilities were estimated with a colorimetric assay using MTT. Each bar represents the mean ± S.E.M. obtained from five experiments. Crosses denote statistical significance at $P < 0.01$. The statistical analysis was evaluated using the Student's *t*-test.

were viable. However, the viability of the cells pretreated with Tat-ADI fusion protein increased in a dose-dependent manner, reaching over 78% at the maximum concentration used. The increased viability of cells transduced with this Tat-ADI fusion protein suggests that this fusion protein has a critical protective effect against LPS-induced inflammation in these cells. These results indicate that the transduction of Tat-ADI could counteract LPS-induced cell death by inhibiting NO expression. Recently, a recombinant ADI protein with a modified polyethylene glycol was developed as an antitumor drug for patients with hepatocellular carcinoma and melanoma (39). Although ADI is considered to be a potential therapeutic protein for treating NO-related diseases, its inability to enter cells limits its use for this purpose. The inhibition of NO may constitute an effective therapeutic strategy for the prevention of inflammatory- and NO-related diseases. Therefore, our results demonstrated that this Tat-ADI fusion protein can be used in protein therapy for NO-related disorders. Recently, Park *et al*. (2008) demonstrated that ADI enhances radiosensitivity by inducing changes in the expression of cell cycle-related protein such as p21 and p27. These proteins are well known and are representative inhibitors of cyclin-dependent kinases (CDKs) attenuating cell growth arrest. Moreover, several reports demonstrated that ADI enhances the suppressive effects against radiation-induced tumor growth *in vivo* and significantly delays the radiation-induced growth in rat hepatoma cells (40-42).

In summary, we demonstrated for the first time that ADI fused with HIV-1 Tat peptide (Tat-ADI) can be efficiently transduced into Raw 264.7cells. This Tat-ADI fusion protein markedly inhibited LPS-induced NO expression and cell death. Although the detailed mechanism is not completely understood, our success in transducing the Tat-ADI protein may be beneficial as a topical application against inflammatory skin disorders such as Atopic dermatitis (AD). In addition, we suggest that this Tat-ADI fusion protein can be a potential clinical drug candidate for cancer therapy.

MATERIALS AND METHODS

Materials

Ni^{2+}-nitrilotriacetic acid sepharose superflow was purchased from Qiagen (GmbH, Germany). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co (Haarlen, Netherlands). Fetal bovine serum (FBS), DMEM, and penicillin-streptomycin antibiotics were purchased from Gibco BRL (Grand Island, USA). The rabbit anti-histidine polyclonal antibody was purchased from the Santa Cruz Biotechnology company (Santa Cruz, CA, USA). All other chemicals and reagents were the highest analytical grade available.

Expression and purification of Tat-ADI fusion proteins

Construction of the HIV-1 Tat fusion protein plasmid was previously described (28). The *Mycoplasma arginini* arginine deiminase (ADI) gene was amplified by PCR with the following primers. The sense primer had a sequence of: 5'-CTCGAGATG TCTGTATTTGACAGTAAATTT-3' and contained an *Xho*I restriction site, and the antisense primer had a sequence of: 5'-G GATCCCTATCACTTAACATCTTTACGTGA-3' and contained a *Bam*HI restriction site. The PCR products were purified and cloned into a TA cloning vector. After digestion with *Xho*I and *Bam*HI, the Tat-ADI gene was ligated into the expression vector pET-15b in frame with six histidine residues to generate the expression vector. Next, this expression vector was transformed into *E. coli* BL21 (DE3) cells, and transformants were selected on LB plates containing ampicillin. The selected colonies were cultured in LB medium containing ampicillin at 37°C with shaking at 250 rpm. After cells grew to an $O.D_{600}$ of 0.5-0.6, protein expression was induced by adding IPTG to a final concentration of 0.5 mM and incubating the cultures for an additional 3-4 h. The cells were harvested and 5 ml of a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 6 M urea was added followed by sonication. After centrifugation, supernatants containing Tat-ADI were immediately loaded on a 2.0 ml Ni^{2+}-nitrilotriacetic acid Sepharose column. After the column was washed with 10 volumes of binding buffer and 6 volumes of washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), the fusion protein was eluted using an elution buffer (0.5 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The salts in the purified fractions were removed by PD10 column chromatography. The protein concentrations were estimated using the Bradford procedure with bovine serum albumin as a standard (43).

Transduction of Tat-ADI into Raw 264.7 cells

The murine macrophage Raw 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20

mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C under humidified conditions of 95% air and 5% CO_2. For the transduction of Tat-ADI, Raw 264.7 cells were grown to confluence on a 6-well plate. Next, the culture medium was replaced with 1 ml of fresh solution. After this, the cells were treated with various concentrations of the Tat-ADI fusion protein for 1 h, treated with trypsin-EDTA (Gibco, Grand Island, NY, USA) and washed with phosphate-buffered saline (PBS). The cells were harvested for the preparation of cell extracts and these extracts were analyzed by Western blotting.

The intracellular stability of transduced Tat-ADI fusion protein was estimated as follows: first, Raw 264.7 cells were treated with 3 μM Tat-ADI for 1 h, the cells were washed and fresh culture medium was added to remove the Tat-ADI that was not transduced. Cells were then further incubated for 60 h, followed by the preparation of cell extracts for Western blot analysis.

Enzymatic assay of ADI and western blot analysis

The enzyme activity of Tat-ADI was assayed by colorimetric determination of the enzyme reaction product, citrulline (8). The reaction mixture (0.1 M potassium phosphate pH 6.5, 10 mM L-arginine and 0.1 ml enzyme solution in a final volume of 1 ml) was incubated at 25°C for 5 min and the amount of citrulline was determined with diacetyl-monoxime (44). Under these defined conditions, one unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of L-arginine to L-citrulline per min.

For the Western blot analysis, the transduced Tat-ADI fusion proteins on the polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% tween-20 (TBST) for 2 h followed by incubation for 1 h at room temperature with anti-histidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:400) in TBST. After washing, the membrane was incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase diluted to 1:10,000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ, USA).

Determination of NO production

Raw 264.7 murine macrophage cells were incubated in 24-well plates at 70% confluence for 12 h. After incubation, the cells were pretreated with the Tat-ADI fusion protein (1-3 μM) for 1 h before 12 h of LPS (100 ng/ml) treatment, and the culture medium was harvested. The levels of NO production were determined by measuring the abundance of nitrite, the metabolite of NO oxidation, as described previously (45, 46). Briefly, 100 μl of cell culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and incubated at room temperature for 10 min. Next, the absorbance at 540 nm was measured using a microplate reader with $NaNO_2$ as the standard.

Under the same conditions, the expression levels of iNOS protein were determined by Western blotting using an anti- iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

MTT assay

The biological activity of the transduced Tat-ADI fusion proteins was assessed by measuring the cell viability of Raw 264.7 cells treated with LPS (50 μg/ml) for 12 h. The cells were then seeded into a 6-well plate at 70% confluence. The cells were first pre-treated with Tat-ADI (1-3 μM) for 1 h followed by treatment with LPS. Cell viability was estimated with a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) staining.

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