Establishment of a New Interleukin-6 (IL-6) Receptor Inhibitor Applicable to the Gene Therapy for IL-6–Dependent Tumor

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Abstract
Interleukin-6 (IL-6) is a key molecule involved in the pathogenesis of several inflammatory diseases and malignancies. Treatments that inhibit IL-6 mitigate the clinical conditions of such diseases. Here, we report on the development of a new receptor inhibitor of IL-6 (NRI) by genetically engineering tocilizumab, a humanized anti-IL-6 receptor monoclonal antibody which specifically blocks IL-6 signaling. This NRI consists of VH and VL of tocilizumab in a single-chain fragment format dimerized by fusing to the Fc portion of human immunoglobulin G1. The binding activity to IL-6 receptor and the biological activity of the purified NRI were found to be similar to those of parental tocilizumab. Because NRI is encoded on a single gene, it is easily applicable to a gene delivery system using virus vehicles. We administered an adenovirus vector encoding NRI to mouse i.p. and monitored the serum NRI level and growth reduction property on S6B45, an IL-6–dependent multiple myeloma cell line, in vivo. Adequate amount of the serum NRI level to exert anti-IL-6 action could be obtained by the NRI gene introduction combined with adenovirus gene delivery, and this treatment inhibited the in vivo S6B45 cell growth significantly. These findings indicate that NRI is a promising agent applicable to the therapeutic gene delivery approach for IL-6–driven diseases. [Cancer Res 2007;67(3):871–5]

Introduction
The expansion of genetic engineering technologies has circumvented various disadvantages observed in the application of original therapeutic monoclonal antibodies. Originally, chimeric antibodies consisting of mouse Fab and human Fc immunoglobulin structures were developed to mitigate the host immunoreaction induced by the administration of mouse antibodies. Food and Drug Administration–approved chimeric antibodies such as rituximab and infliximab have been well-tolerated treatments for malignancies and chronic inflammatory diseases (1, 2).

To decrease the immunogenicity of murine monoclonal antibodies, humanized antibodies containing over 95% human amino acid sequences have been developed. These contain murine sequences for only three pairs of complementarity-determining regions in the antigen-binding region. Humanized antibodies such as bevacizumab or trastuzumab have drastically improved the prognosis of malignant tumors refractory to the conventional treatments (3, 4). Costs associated with these treatments have greatly increased, however. One strategy to reduce these costs is gene therapy.

Interleukin-6 (IL-6) is involved in cell proliferation and in the production of vascular endothelial growth factor by several cancers, including multiple myeloma, mesothelioma, cervical cancer, and renal cell carcinoma (5–8). Additionally, IL-6 is a key agent in the pathogenesis of various inflammatory diseases, such as Castleman’s disease, rheumatoid arthritis, juvenile idiopathic arthritis, and Crohn’s disease (9). These diseases are often refractory to conventional, nonspecific anti-inflammatory chemotherapy such as corticosteroids and antineoplastic agents. Tocilizumab (previously, MRA), a humanized anti-IL-6 receptor monoclonal antibody which specifically blocks IL-6 cell-to-cell signaling, has shown greater promise in treating inflammatory diseases. Given its success in treating these diseases, tocilizumab may also prove useful in treating IL-6–related cancers.

To broaden the therapeutic arsenal of anti-IL-6 agents, we have explored the use of a transgenic adenovirus vector carrying an anti-IL-6 recombinant antibody. Tocilizumab would be a desirable candidate as an anti-IL-6 agent for this project. The heavy chain and light chain of tocilizumab are encoded by separate genes, however, which poses a serious obstacle to generating recombinant antibodies. Therefore, we modified the tocilizumab genetic material into a form that is more feasible for adenovirus-mediated gene delivery. We designed a new receptor inhibitor of IL-6 (NRI) consisting of VH and VL of tocilizumab in a single-chain fragment format (scFv) dimerized by fusing to the Fc portion of human immunoglobulin G1 (IgG1). In this study, we investigated the biological activity of recombinant NRI in vitro by using two IL-6–dependent cell lines: S6B45 (multiple myeloma–derived B cells) and KT-3 (Lennert’s lymphoma–derived T cells).

We additionally tested the efficacy of NRI in an in vivo animal model for multiple myeloma by treating the mice with NRI using an adenovirus gene delivery system. We chose multiple myeloma as the model system because IL-6 is a critical cytokine for the development and proliferation of multiple myeloma cells, and high IL-6 secretion is related to the poor prognosis of the disease (5). There was thus reason to suspect that our IL-6–specific agent would have a biological effect on these cells.

Materials and Methods

Cytokines and antibodies. Recombinant human IL-6 (rIL-6) and recombinant soluble IL-6 receptor (sIL-6R) were provided by Ajinomoto (Kawasaki, Japan) and Tosoh (Kanagawa, Japan), respectively. Chugai Pharmaceutical Co., Ltd. (Roche Group, Tokyo, Japan) kindly provided us two types of antibodies recognizing sIL-6R, namely, tocilizumab and MT-18.

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Tocilizumab is a humanized anti-IL-6R monoclonal antibody. MT-18 is a monoclonal antibody recognizing a distinct tocilizumab epitope in sIL-6R structure. Anti-asialo GM1 antibody was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

**Cells and cell culture.** S6B45 was established by transfecting a human IL-6 gene to an IL-6–dependent human myeloma cell line MMS1. As such, IL-6 acts as an autocrine growth factor for S6B45 (10). Lennert’s lymphoma–derived T cells (KT-3) also shows IL-6–dependent cell growth (11). Both cell lines were cultured in RPMI 1640 supplemented with 10% FCS and rIL-6 (S6B45, 1 ng/mL; KT-3, 4 ng/mL). The 293 cell line was purchased from the American Type Culture Collection (Manassas, VA) and was cultured in DMEM/F12 supplemented with 10% FCS. Cell culture medium and supplements except rIL-6 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

**Construction of a new receptor inhibitor of IL-6.** Complementary DNAs encoding the light and heavy chains of tocilizumab were provided from Chugai Pharmaceutical. First, tocilizumab cDNA templates were used to construct an scFv having the binding capacity to the IL-6R. The VH and VL nucleotide sequences appropriate for this scFv construction were amplified by PCR and linked with a 20-amino-acid linker (G4S)(GGRAS)(G4S)2 by the method reported previously (12). At the second step, the COOH terminus of this resultant scFv was fused to the hinge and Fe portions of human IgG1, producing the agent we named NRI (Fig. 1A). This NRI gene was inserted into the multicloning site of pShuttle/CMV (Qbiogene, Irvine, CA), and the resultant plasmid was denoted pShuttle/CMV/NRI. After confirming the secretory property of NRI by measuring the Fc concentration in the supernatant of 293 cells transfected with pShuttle/CMV/NRI, NRI was purified from the supernatant using protein A beads (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom). Purified NRI was applied to the 10% SDS-PAGE and then stained with Coomassie blue. In vitro cell proliferation assay. S6B45 and KT-3 cells were plated in 96-well plates in triplicate at a density of 10,000 and 5,000 cells per well, respectively, and then incubated in complete medium supplemented with rIL-6 (S6B45, 1 ng/mL; KT-3, 4 ng/mL) in the presence of various concentrations of NRI, tocilizumab, or hIgG were mixed with 10 ng/mL of IL-6 in the presence of immobilized IL-6R for 1 h at room temperature, followed by the addition of biotinylated anti-IL-6 antibody (R&D Systems, Inc., Minneapolis, MN). The wells were then incubated with horseradish peroxidase-conjugated streptavidin (CHEMICON, Temecula, CA). This assay was done in triplicate.

**Binding and inhibition assay of NRI.** Ninety-six–well ELISA plates were coated with 5 µg/mL of monoclonal antibody MT-18 and incubated overnight at 4°C. After bovine serum albumin blocking, 100 ng/mL of sIL-6R was added for 1 h at room temperature. This procedure was followed by the reaction with various concentrations of NRI or tocilizumab or hIgG to investigate the NRI binding activity compared with parental tocilizumab. This assay was done in triplicate. To evaluate the ability of NRI to inhibit the binding between IL-6 and IL-6R, various concentrations of NRI or tocilizumab or hIgG were mixed with 10 ng/mL of IL-6 in the presence of immobilized IL-6R for 1 h at room temperature, followed by the addition of biotinylated anti-IL-6 antibody (R&D Systems, Inc., Minneapolis, MN). The wells were then incubated with horseradish peroxidase-conjugated streptavidin (CHEMICON, Temecula, CA). This assay was done in triplicate.

**In vitro cell proliferation assay.** S6B45 and KT-3 cells were plated in 96-well plates in triplicate at a density of 10,000 and 5,000 cells per well, respectively, and then incubated in complete medium supplemented with rIL-6 (S6B45, 1 ng/mL; KT-3, 4 ng/mL) in the presence of various concentrations of NRI, tocilizumab, or hIgG. After a 3-day culture, cell

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**Figure 1.** A, structure of NRI. NRI consists of VH and VL of tocilizumab in an scFv format dimerized by fusing to the Fc portion of human IgG1. The nucleotide sequence of NRI indicates that there are two disulfide bonds in the hinge region. **B**, NRI molecular weight. One microgram of purified NRI was applied to each lane in 10% SDS-PAGE gel. After running the gel, it was stained with Coomassie. **Left lane**, nonreduced; **right lane**, reduced; a 55-kDa band consistent with the formula weight of NRI monomer.
proliferation was evaluated by (\(^{3}H\))-thymidine incorporation. A 6-h (\(^{3}H\))-thymidine pulse was followed by measurement of radioactivity using a liquid scintillation counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA).

**Animal model.** We used S6B45 cells for making an *in vivo* model because S6B45 shows tumorigenicity in severe combined immunodeficiency (SCID) mouse pretreated with anti-asialo GM1 antibody. Female SCID mice C.B-17/Scid-SCID Jcl (6 weeks old) were purchased from Nippon Clea Co., Inc. (Tokyo, Japan). The mice received an i.p. pretreatment with 1 mg of anti-asialo GM1 antibody. The next day, the mice received s.c. injection of 1 \(\times\) 10^7 S6B45 cells. The tumor-inoculated mice were injected i.p. with 100 \(\mu\)L of PBS containing 10^9 plaque-forming units (pfu) of viruses (Ad/CMV/NRI or control vector; Ad/CMV/Luc). The NRI concentration in blood and size of tumor were monitored.

**Statistical analysis.** The Wilcoxon-Mann-Whitney test was used for the statistical comparison of tumor volumes. Student's *t* test was used for comparing cell proliferation assays and tumor weights. Statistical significance was set at *P* < 0.05.

**Results**

**Characterization of NRI.** A schema of the NRI structure is depicted in Fig. 1A. The molecular weight of dimerized NRI is \(\sim\) 110 kDa, consistent with the putative size calculated on the NRI gene construct (Fig. 1B). The figure also shows that the supernatant of 293 cells transfected with NRI expression vector (pShuttle/CMV/NRI) contains a NRI monomer as well as a dimerized NRI.

In the ELISA assays, NRI recognized the human IL-6R and bound to it in a dose-dependent fashion (Fig. 2A). This binding was comparable in magnitude to that of parental tocilizumab. Control hlgG did not show binding activity at all doses. Furthermore, NRI inhibited binding between IL-6 and IL-6R in a dose-dependent manner, and 90% inhibition was observed at 100 nmol/L of NRI (Fig. 2B). This inhibitory effect was comparable in magnitude to that of tocilizumab.

**Inhibitory activity of NRI on the cell growth of an IL-6–dependent cell line *in vitro*.** We next investigated the effect of NRI on the growth of S6B45 cells and KT-3 cells using the (\(^{3}H\))-thymidine incorporation method. NRI inhibited KT-3 proliferation in a dose-dependent manner similarly to tocilizumab, whereas control hlgG did not suppress the cell growth (Fig. 3A). These results indicate that the biological activity of NRI is comparable to that of tocilizumab. The cell proliferation of S6B45 cells was suppressed by both NRI and tocilizumab in a dose-dependent manner (Fig. 3B). NRI and tocilizumab both produced a milder suppression of S6B45 compared with their robust inhibition of KT-3 cells. This may have resulted from the differences between KT-3 and S6B45 in the extent of growth dependency on IL-6.

**Serum levels of NRI achieved through adenovirus-mediated gene delivery.** We injected 1 \(\times\) 10^9 pfu of Ad/CMV/NRI i.p. and sampled murine blood on days 0, 3, 7, 14, 28, and 56 from the tail to measure the concentration of serum NRI. The concentration of serum NRI reached 167.2 \(\pm\) 9.5 nmol/L (mean \(\pm\) SE) at 72 h after injection of Ad/CMV/NRI and was maintained to at least over 100 nmol/L for more than 2 months (Fig. 4A).

**Growth inhibitory effect of NRI on IL-6–dependent tumor *in vivo*.** The growth inhibitory effect of NRI delivered with Ad/CMV/NRI was examined in the SCID mouse model, which was inoculated with S6B45 cells. The tumor was first detected on days 14 to 21 after S6B45 cell inoculation and grew rapidly until day 35. As shown in Fig. 4B, administration of 1 \(\times\) 10^9 pfu Ad/CMV/NRI suppressed tumor growth significantly (*P* < 0.05) compared with groups of control vector and PBS administration. It also reduced final tumor weight (Fig. 4C; *P* < 0.05).

![Figure 2](https://www.aacrjournals.org/873/Cancer Res 2007; 67: (3). February 1, 2007)

**Discussion**

We have developed a new IL-6 receptor inhibitor, NRI, that is comparable to parental tocilizumab in terms of inhibitory activity on the IL-6–dependent cell growth. The structure of NRI consists of the scFv component derived from tocilizumab antigen-binding region and human IgG1 Fc region. The rationale for fusing the Fc to the scFv component having potential IL-6R binding activity was that it would increase secretion from the transduced cells, improve the avidity of Fc dimerization, and extend the half-life of the resultant IL-6 antagonist *in vivo* (14). Because it is encoded by a single gene, NRI is amenable to delivery by a genetically engineered vector. Indeed, here we successfully delivered NRI to mice using an Ad/CMV/NRI vector.
We showed that the administration of Ad/CMV/NRI resulted in the achievement of the therapeutic concentration of NRI in circulation, which was sustained for more than 2 months. In the tocilizumab treatment, it has been reported that maintenance of therapeutic concentration, not in the peak level but the trough level, is important to block the IL-6 signal completely (15). Therefore, in vivo continuous production of NRI shown in this study is preferable for the IL-6 blockade. In addition to its utility for the systemic administration of therapeutic agents, Ad/CMV/NRI is also suitable for local injection, where a high amount of IL-6 is produced and contributes to local inflammation such as is seen in malignant pleural effusion of mesothelioma (16). Furthermore, the

Figure 3. Effect of NRI on IL-6–dependent cell proliferation in vitro. After a 3-d culture, cell proliferation was evaluated by (3H)-thymidine incorporation. About 18.5 kBq per well of (3H)-thymidine (925 GBq/mmol; GE Healthcare) was added for the last 6-h incubation. After incubation, the cells and medium were transferred to 96-well filtration system plates (Millipore, Billerica, MA). The radioactivity was determined in a liquid scintillation counter. This assay was done in triplicate. A, KT-3 cell growth suppression by NRI or tocilizumab in the presence 4 ng/mL of IL-6. Points, mean number of cells of triplicate samples; bars, SD. A concentration of 100 nmol/L NRI completely inhibited the induction of cell growth, as did 100 nmol/L tocilizumab. Addition of control hIgG did not impact cell proliferation. B, S6B45 cells treated with NRI or tocilizumab. Points, mean number of cells of triplicate samples; bars, SD. Both NRI and tocilizumab showed significant suppression of cell growth compared with those treated with IgG at 100 nmol/L; *, P < 0.05. NRI did not significantly differ from tocilizumab.

Figure 4. Effect of transgene of NRI gene in vivo. A, NRI concentration in mouse serum by Ad/CMV/NRI administration. The serum concentration of NRI after injection of 1 × 10^9 pfu Ad/CMV/NRI i.p. was monitored over 2 mo. Points, data obtained from seven mice; bars, SD. The peak concentration was observed 3 d after adenovirus injection and was maintained to at least 100 nmol/L during 2 mo. B, Ad/CMV/NRI treatment group (n = 10) showed significantly reduced tumor size compared with the control vector group (n = 9) and the PBS group (n = 10). *, P < 0.05 at day 35. C, tumor weights at day 35. Mice were sacrificed at day 35, and s.c. tumors were harvested and measured. Columns, mean tumor weights; bars, SD. Weights in the Ad/CMV/NRI treatment group were significantly lower than those in the two control groups; *, P < 0.05.
introduction of a tumor-specific promoter, such as the cyclo-oxygenase 2 promoter, to the adenovirus vector expression cassette can control and optimize the NRI expression, synchronizing with the activity of the neoplastic cells (17). Alternatively, the introduction of a tetracycline-controllable expression system or a Cre/lox p system may improve control over NRI expression in case of adverse reaction due to NRI overexpression and, thus, lead to greater safety and efficacy (18).

To estimate the adverse consequences by Ad/CMV/NRI treatment, we sampled serum and tissues of mouse treated with Ad/CMV/NRI, Ad/CMV/Luc, or PBS administration on the final day of monitoring to evaluate their reactions. We did not detect any reactions due to sustained NRI expression or adenovirus injection in SCID mouse treatment model, however. Liver aminotransferases, blood urea nitrogen, and creatinine levels in the serum of each group remained normal, and each group showed no significant pathologic changes in liver, kidney, and lung microscopically (data not shown).

Repeated treatment using adenovirus causes the appearance of antibodies against adenovirus and its gene product. Adenovirus infection also induces acute inflammation in the host. This reaction may have been diminished by our use of an IL-6 antagonist because IL-6 stimulates antibody production on B cells and also promotes inflammation (9). Although we used an adenovirus delivery system to show the usefulness of NRI, gutless adenovirus or adeno-associated virus may be more feasible for the gene therapy (19, 20).

Antibody treatments based on molecular targeting are currently under intense investigation. Several have shown efficacy in clinical trials in humans. Keeping costs down remains a persistent barrier to the economic viability of these agents, however. We hypothesize that the strategy used in this study, employing continuous expression of a therapeutic agent mediated by adenovirus administration, may significantly curtail production expenses. In general, we hope that using transgenic vectors to deliver pathophysiologically specific antibody therapeutics will achieve their promise as cost-effective treatments of malignancies.

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References


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