

VEGF Targeting in Mesotheliomas Using an Interleukin-6 Signal Inhibitor Based on Adenovirus Gene Delivery

YASUO ADACHI¹, NAOKO YOSHIO-HOSHINO², CHIEKO AOKI¹ and NORIHIRO NISHIMOTO¹

¹Laboratory of Immune Regulation, Wakayama Medical University, 105 Saito Bio Innovation Center, 7-7-20, Saito-Asagi, Ibaraki-City, Osaka, 567-0085, Japan;

²Laboratory of Immune Regulation, Graduate School of Frontier Biosciences, Osaka University, Yamada-oka, 1-3, Suita City, 565-0871, Japan

Abstract. *Background:* Malignant mesotheliomas reportedly secrete interleukin-6 (IL-6) which augments production of vascular endothelial growth factor (VEGF) from mesothelioma cells. We previously reported the development of a new receptor inhibitor of IL-6 (NRI) by genetically engineering tocilizumab, a humanized anti-IL-6 receptor monoclonal antibody. Since NRI is encoded on a single gene, it is easily applicable to a gene delivery system using virus vehicles. *In this study, we report VEGF targeting through NRI expression based on adenovirus-mediated gene delivery in mesothelioma cells. Materials and Methods:* We constructed an NRI expression vector in the context of a tropism-modified adenovirus vector that had enhanced infectivity in mesothelioma cells. *Results:* This virus effectively induced NRI secretion from mesothelioma cells. This virus infection also reduced the VEGF production in mesothelioma cells. *Conclusion:* These results indicate that NRI shows potential as an agent in the treatment of mesotheliomas.

Abbreviations: IL-6, Interleukin-6; TCZ, tocilizumab; VEGF, vascular endothelial growth factor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; NRI, new receptor inhibitor of IL-6; sIL-6R, soluble IL-6 receptor; FCS, fetal calf serum; VH, heavy chain variable region; VL, light chain variable region; Fab, fragment of antigen binding; Fc, fragment of crystallizable; Ad, adenovirus; ELISA, enzyme linked immunosorbent assay; CDR, complementary determining region.

Correspondence to: Norihiro Nishimoto, Laboratory of Immune Regulation, Wakayama Medical University, 105 Saito Bio Innovation Center, 7-7-20, Saito-Asagi, Ibaraki-City, Osaka, 567-0085, Japan. Tel: +81 726468039, Fax: +81 726468140, e-mail: norichan@wakayama-med.ac.jp

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Interleukin-6 (IL-6) is a multifunctional cytokine involved in the acute phase response of inflammatory process (1). This pro-inflammatory cytokine plays a pivotal role in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis (2, 3). Clinical data have shown that anti-IL-6 receptor antibody provides an effective and safe anti-IL-6 therapy (tocilizumab, TCZ) (4, 5). Recently, the application of anti-IL-6 has not been confined only to immunological disorders, but has been extended into use in oncological fields (6, 7). Immunologists as well as oncologists have recently taken interest in IL-6-related tumors, one type of which is malignant mesothelioma (8-10). This malignancy is still refractory to current chemotherapy, however the introduction of a new therapeutic agent pemetrexed achieved significant improvements in patient response rates and mean survival time of these patients (11, 12). Our group reported that IL-6 induces both tumor cell growth and vascular endothelial growth factor (VEGF) production in malignant mesotheliomas (10). The Janus kinase-signal transducers and activators of transcription (JAK-STAT3) pathway are involved in VEGF induction by means of IL-6 stimulation. It has been reported that VEGF induces angiogenesis and acts as a mitogen for mesothelioma cells (13). These results suggested that an anti-IL-6 approach should be considered as a feasible therapeutic candidate for this incurable malignancy.

TCZ is a humanized anti-IL-6 receptor monoclonal antibody which specifically blocks IL-6 signaling (14, 15). To explore the new therapeutic modality regarding anti-IL-6 strategy, we constructed a new receptor inhibitor of IL-6 (NRI) (16). NRI consists of a heavy chain variable region (VH) and a light chain variable region (VL) of TCZ in a single chain fragment format (scFv) dimerized by fusion to the Fc portion of human IgG1. The effectiveness of NRI was comparable to parental TCZ with respect to IL-6 signal blocking strength. Compared to parental TCZ, NRI is more amenable to the gene technology due to its applicability to therapeutic viral vector-mediated gene transfers. In this

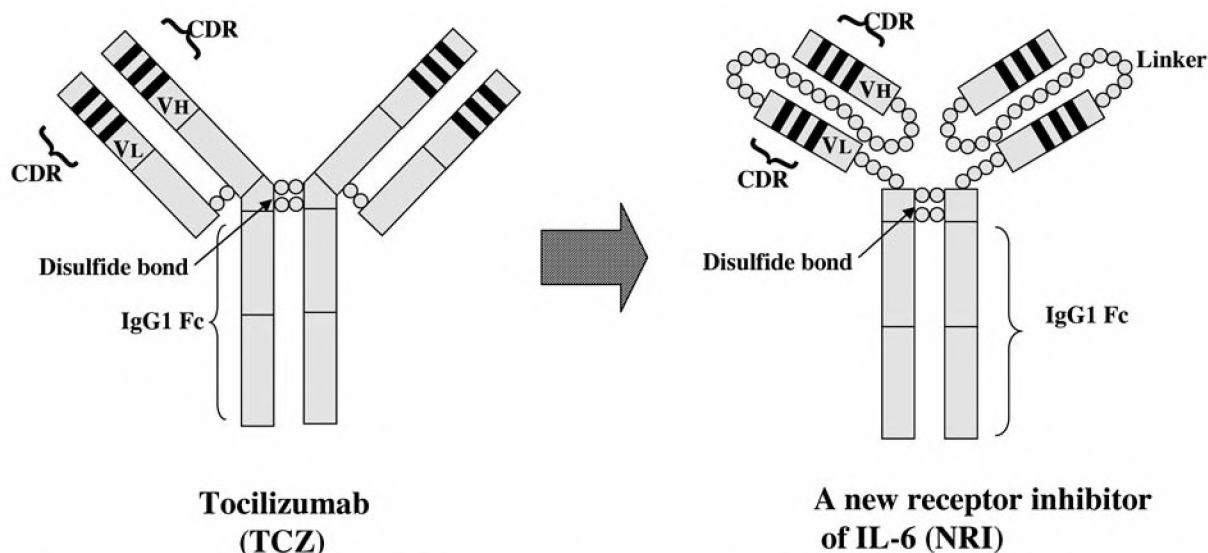


Figure 1. Structure of tocilizumab (TCZ) and new receptor inhibitor of IL-6 (NRI). A humanized monoclonal antibody TCZ preserved the complementary determining region (CDR) in the anti-IL-6 receptor mouse monoclonal antibody PM-1. NRI consists of VH and VL of TCZ in a scFv format dimerized by fusing to the Fc portion of human IgG1. The molecular weight of dimerized NRI is approximately 110 kDa.

study, we present VEGF targeting through NRI expression by a therapeutic gene delivery system mediated by recombinant adenovirus (Ad) for mesothelioma 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 Co., Inc. (Kawasaki, Kanagawa, Japan) and Tosoh Corporation (Ayase, Kanagawa, Japan), respectively. Chugai Pharmaceutical Co., Ltd. (Roche Group, Tokyo, Japan) kindly provided us with a humanized anti-IL-6R monoclonal antibody (TCZ). Purified human IgG (I4506) was purchased from Sigma (St. Louis, MO, USA). VEGF human ELISA Kit (Quantikine, R&D Systems, Minneapolis, MN, USA) was available for the measurement of VEGF concentration.

Cells and cell culture. H2052 mesothelioma cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA), and maintained in RPMI1640 supplemented with 10% fetal calf serum (FCS). The 293 cell line was purchased from ATCC and was cultured in DMEM/F12 supplemented with 10% FCS. Cell culture medium and supplements were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Construction of a new receptor inhibitor of IL-6 (NRI). We described the construction of NRI in detail in our previous study (16). Briefly, the VH and VL nucleotide sequences from the fragment of antigen binding (Fab) region in TCZ were linked with a 20 amino acid linker to construct a new single chain format (scFv). Subsequently, the ensuing scFv was fused to the hinge and fragment of crystallizable (Fc) portions of human IgG1 to construct the NRI. A schema of the NRI structure is depicted in Figure 1. The NRI gene was introduced into an adenovirus expression system. In this system, the NRI was

expressed under the control of a cytomegalovirus enhancer/promoter. Because it was previously shown that a fiber-modified adenovirus vector (Ad5/3) was beneficial for gene transfer in mesothelioma cells, we constructed the NRI expression vector in the context of Ad5/3, which an Ad serotype 5 with a chimeric fiber composed of the tail and shaft domains of Ad serotype 5 and the knob domain of Ad serotype 3 (17, 18).

The supernatant of the 293 cells infected with the NRI expression adenovirus (Ad5/3NRI) was collected for the purification of NRI. Protein A beads (GE Healthcare UK Ltd., Buckinghamshire, UK) were used in the purification process.

Enzyme linked immunosorbent assay (ELISA) for measurement of NRI concentration. After overnight coating of 96-well microplates with 5 µg/ml of rabbit anti-human IgG Fcγ (code: 309-006-008, Jackson ImmunoResearch Laboratories, Inc., Baltimore, MD, USA) followed by 5% of bovine serum albumin blocking (Sigma, St. Louis, MO, USA), samples were incubated for 1 hour at room temperature. This was followed by incubation with anti-human IgG/HRP rabbit F(ab')₂ (Dako, Glostrup, Denmark) for 1 hour. Color development was carried out with SIGMA FAST o-phenylenediamine dihydrochloride Tablet Sets (SIGMA), and then measured at 450 nm with a microplate reader (Microplate Manager 5.2 PC; Bio-Rad, Hercules, CA, USA).

Statistical analysis. Student's *t*-test was utilized to compare the concentration of NRI or VEGF in the supernatant. Statistical significance was set at *p*<0.05.

Results

NRI suppresses VEGF expression induced by IL-6/sIL-6R stimulation in H2052 mesotheliomas. H2052 cells were plated in 48 well plates in triplicate at a cell density of

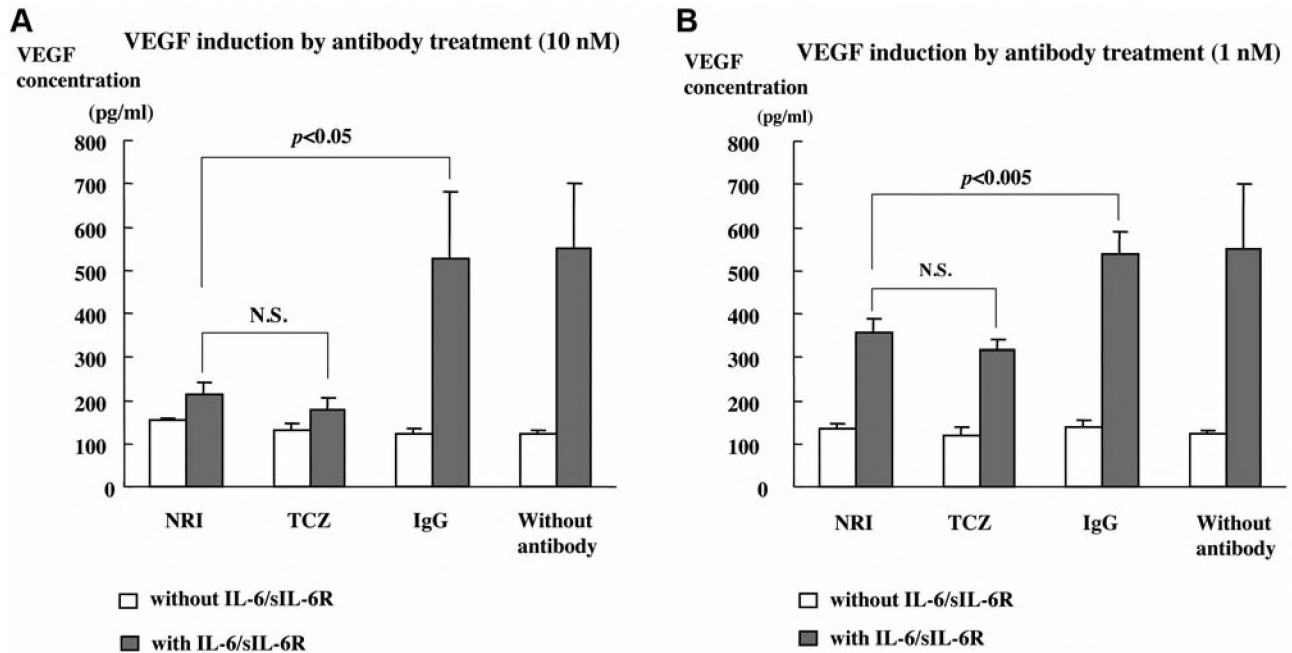


Figure 2. NRI suppresses VEGF production induced by IL-6/sIL-6R in mesothelioma cells. A concentration of NRI purified using protein A beads was measured by Fc detection ELISA. Suppression by (A) 10 nM or (B) 1 nM of recombinant NRI is depicted. IL-6 (10 ng/ml)/sIL-6R (100 ng/ml) stimulation augmented the VEGF production in each group. NRI or TCZ treatment significantly reduced the VEGF induction by IL-6/sIL-6R compared with that of the IgG treatment group. There was no significant difference between the NRI and the TCZ groups in VEGF level induced by IL-6/sIL-6R.

2.0×10^4 /well. After overnight culture, H2052 cells were treated with NRI, TCZ, or human IgG. Simultaneously, IL-6/sIL-6R stimulation was initiated. The final concentrations of each antibody, IL-6 and sIL-6R were 10 nM, 10 ng/ml and 100 ng/ml, respectively. The volume of the culture medium was 500 μ l/well. After a 48 hour culture, VEGF concentration in the supernatant was measured (Figure 2). Compared to the antibody-free group or control IgG treatment group, IL-6/sIL-6R stimulation generated a four-fold greater VEGF production effect. Ten nM of TCZ treatment suppressed VEGF induction by IL-6/sIL-6R stimulation. NRI treatment also suppressed VEGF induction, to an extent comparable to that of TCZ. When 1 nM of TCZ treatment was utilized, VEGF induction by IL-6/sIL-6R was reduced to approximately a half of that found in the IgG treatment group. For this reason it appears to be a dose-dependent inhibition.

Supernatant levels of NRI achieved through adenovirus-mediated gene delivery. We investigated the NRI production from H2052 cells infected with Ad5/3NRI. H2052 cells were plated in 24 well plates in triplicate at a cell density of 5.0×10^4 /well. After overnight culture, cells were infected with Ad5/3NRI at 1.0×10^3 or 5.0×10^3 vp/cell for 2 hours. Medium was replaced with 1 ml of complete media

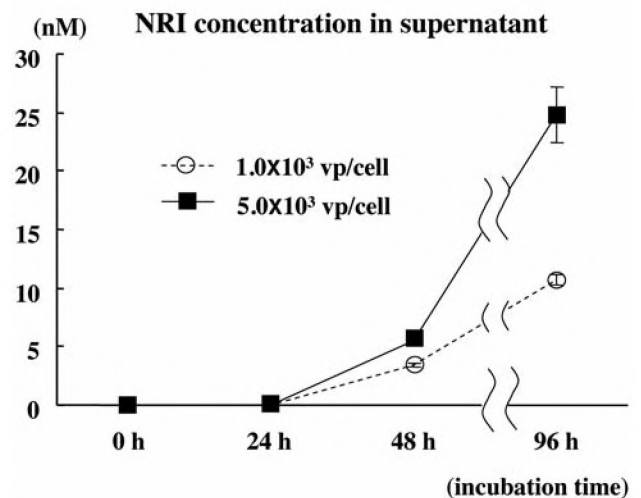


Figure 3. NRI secretion from mesothelioma cells infected with NRI expression adenovirus (Ad5/3NRI). Following Ad5/3NRI infection, supernatant NRI level was monitored. NRI concentration was measured by Fc detection ELISA. Data are expressed as mean $\pm$ SD of triplicate samples. Forty-eight hours after infection, the NRI level reached 3.5 ± 0.2 nM in the 1.0×10^3 vp/cell infection group and 5.5 ± 0.4 nM in the 5.0×10^3 vp/cell infection group. Ninety-six hours after the infection, NRI level reached 10.7 ± 0.5 nM in the 1.0×10^3 vp/cell infection group and 24.8 ± 0.2 nM in the 5.0×10^3 vp/cell infection group.

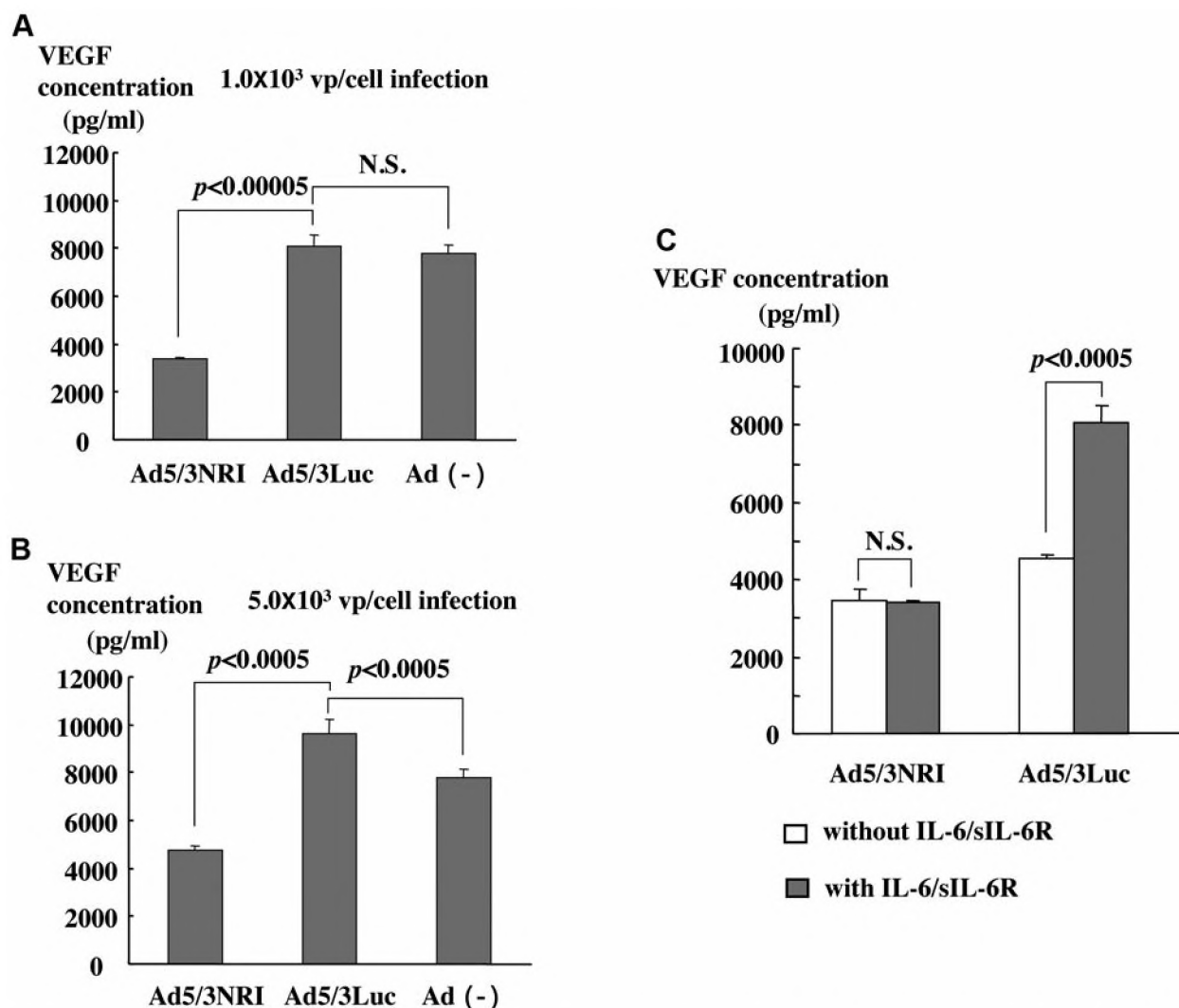


Figure 4. Transgene of NRI suppresses VEGF production induced by IL-6s/IL-6R in mesothelioma. Seventy-two hours after Ad5/3NRI infection, supernatant VEGF level was measured using VEGF human ELISA Kit (Quantikine). Data are expressed as mean $\pm$ SD of triplicated samples. A: In cells infected at 1.0×10^3 vp/cell, VEGF levels reached 8060 ± 480 pg/ml in the control Ad infection group and 3410 ± 70 pg/ml in the Ad5/3NRI infection group. There was no significant difference in VEGF production between Ad5/3Luc infection group and non-infection group. B: In cells infected at 5.0×10^3 vp/cell, VEGF levels reached 9600 ± 630 pg/ml in the control Ad infection group and 4730 ± 180 pg/ml in the Ad5/3NRI infection group. Compared with the non-infected group, Ad5/3Luc infection at 5.0×10^3 vp/cell significantly augmented VEGF production from mesothelioma cells. C: IL-6/sIL-6R stimulation did not augment VEGF induction on Ad5/3NRI infection.

following infection. The supernatant was sampled at 24, 48, and 96 hours after medium replacement to evaluate the VEGF level (Figure 3). NRI levels in the 5.0×10^3 vp/cell group were 5.5 ± 0.4 nM at 48 hours' incubation and 24.8 ± 2.4 nM at 96 hours' incubation. The levels of NRI in the 1.0×10^3 vp/cell group were 3.5 ± 0.2 nM at 48 hours' incubation and 10.7 ± 0.5 nM at 96 hours' incubation. These results showed that after 48 hours from Ad5/3 infection, the NRI level reached the concentration indicative of suppressing the VEGF induction in H2052 by IL-6s/IL-6R.

Inhibitory activity of Ad5/3NRI treatment on the VEGF production of H2052. We assessed the feasibility of the therapeutic application of the NRI expression vector on IL-6-related mesotheliomas. H2052 plating and Ad5/3NRI infection followed the aforementioned procedure. Infected H2052 cells were incubated with or without IL-6/sIL-6R stimulation. Supernatant was harvested 72 hours after Ad infection. In cells infected at 1.0×10^3 vp/cell, no significant difference was observed between the control Ad (Ad5/3Luc) infection group and non-Ad infection group in

VEGF production upon addition of IL-6/sIL-6R (Figure 4A). The VEGF level in the supernatant from the Ad5/3NRI infected group was reduced by approximate 60% compared to that of the Ad5/3Luc infection group under IL-6/sIL-6R stimulation. In cells infected at 5.0×10^3 vp/cell (Figure 4B), a significant increase in VEGF production was observed in the Ad5/3Luc infection group compared to that in the non-Ad infection group under IL-6/sIL-6R treatment ($p < 0.0005$). These results suggest that a high dose Ad treatment may induce VEGF production. However, in cells infected at 5.0×10^3 vp/cell, VEGF production in the Ad5/3NRI treatment group was reduced by approximately 40% compared to that of in the non-Ad treatment group.

In H2052 cells infected with Ad5/3NRI at 1.0×10^3 vp/cell, there was no significant difference in VEGF production between the IL-6/sIL-6R-stimulated group and the non-stimulated group (Figure 4C). In the Ad5/3Luc infection group, a significant increase in VEGF production was observed by IL-6/sIL-6R stimulation. These results would suggest that NRI is a possible therapeutic option for mesotheliomas, and that direct transfer of the NRI gene to tumors is a highly capable anti-angiogenic strategy.

Discussion

We developed an NRI that is comparable to parental TCZ in its inhibitory activity on IL-6 signaling (16). The rationale for fusing the Fc to the scFv component derived from TCZ is based on the notion that this would increase secretion from the transduced cells and extend the half-life of the resultant anti-IL-6 agent *in vivo*. The advantage of NRI over TCZ is that it is encoded by a single gene and is easily applicable toward delivery by genetically engineered vectors. In this study, we present the advantage of the NRI expression vector in terms of its ability to suppress VEGF production in mesothelioma cells. Although the NRI level secreted from Ad5/3NRI infected cells was relatively low (below 10 nM), it was enough to suppress VEGF induction. Reportedly, it requires 25 µg/ml of TCZ (approximately 170 nM) to achieve a complete block of membrane-bound IL-6 receptors (19). One mechanism of IL-6 signal transduction through the binding of IL-6 and sIL-6R is known as trans-signaling, and it is responsible for the inflammation of the synovial fibroblast in patients with rheumatoid arthritis (20-22). This trans-signaling mechanism is thought to play an important role in VEGF induction by IL-6 stimulation, which was revealed in this study. Compared with the block of membrane-bound IL-6 receptors, inhibition of IL-6 trans-signaling by targeting soluble forms of IL-6 receptor may need less than a tenth of the required amount of anti-IL-6 receptor agents.

Since mesothelioma is a highly malignant neoplasm, many innovative approaches, including virus-based treatments, have been reported (23). Within this context, we confirmed that Ad infection augmented the VEGF induction. Conditionally replicative viruses that selectively destroy target tumor cells have been well studied as one of the most effective treatments for lethal malignancies (24). However, administration of oncolytic viruses have often revealed little efficacy for tumor regression *in vivo* despite findings that showed prominent and specific oncolytic activity *in vitro*. Therefore, many oncolytic virus strategies supplemented with therapeutic molecules, such as interferon or transforming growth factor-beta type II receptor, were introduced (25, 26). It is important for future oncolytic virus strategies to consider the potential integrating capabilities that avoid early detection and eradication by the immune system (24). The recruitment of cytokines induced by virus infection is a possible reason that undermines the oncolytic efficiency *in vivo*. Thus, the oncolytic virus strategy combined with anti-IL-6 is a promising approach in achieving better control of the immune system. In addition, because angiogenesis is one of the main progression factors for tumor growth, a virus approach combined with anti-angiogenic agents, including NRI is a rational application *in vivo*.

In order to explore a new modality for cancer treatments, it is important to develop novel therapeutic agents that are applicable in the system. The establishment of NRI widens the possibility of the therapeutic window for IL-6 related disorders.

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