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Adenovirus-mediated gene transfer of a human IL-6 antagonist

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IL-6 is a pleiotropic cytokine and plays a major role in inflammation and in the immune response. Altered serum levels of IL-6 have been described in several pathologies such as myeloma, EBV-lymphoma and chronic autoimmune disease. Here we report data on the utilization of a hIL-6 receptor superantagonist with a gene therapy approach. The superantagonist used in this work possesses very high affinity for the hIL-6 receptor, and is therefore an excellent candidate for the treatment of IL-6-dependent diseases. To obtain an efficient in vivo delivery method, we constructed a recombinant adenovirus expressing the IL-6 receptor superantagonist by inserting the cDNA, controlled by the RSV promoter, into a first generation replication-incompetent adenoviral vector. Recombinant virus allowed correct expression of the transgene in vitro. Supernatants of infected cells specifically inhibited IL-6-induced transcriptional activation in hepatoma cells and blocked the IL-6-dependent proliferation of human myeloma cells. After intravenous injection of the recombinant virus into mice, nanomolar amounts of antagonist were produced in the serum, and these were able completely to inhibit IL-6 bioactivity. Gene transfer of such an antagonist offers a practical means of imposing long-term blockade of IL-6 activity in vivo for investigational and therapeutic purposes.

Keywords: *human interleukin 6; gene therapy; adenovirus; antagonists*

Introduction

Interleukin 6 (IL-6) is a multifunctional helical cytokine and plays a central role as a differentiation and growth factor of hematopoietic precursor cells, B cells, T cells, neuronal cells, osteoclasts and endothelial cells,[1] and modulates transcription of several liver-specific genes during acute inflammation.[2] Sequential interaction of IL-6 with the receptor subunits gp80 and gp130, induces a cascade of events, mediated by the family of JAK kinases, leading to the activation of specific transcription factors.[3] Altered IL-6 serum levels have been reported in several pathologies, such as multiple myeloma,[4] Castleman's disease,[5] osteoporosis,[6] EBV positive lymphomas[7] and rheumatoid arthritis.[8] In multiple myeloma, IL-6 clearly plays an autocrine and/or paracrine role in the growth of malignant cells.[4]

Anti-IL-6 monoclonal antibodies (mAbs) have been proposed as therapeutic tools for IL-6-related pathologies.[4,7,9] However, it has been shown in multiple myeloma that antibodies can stabilize IL-6 in the form of circulating complexes, creating the so-called 'paradoxical effect'.[10,11] The use of receptor antagonists rather than mAbs, is an innovative and promising approach for the treatment of cytokine-mediated disease states. These molecules act by specifically binding cytokine receptors in competition with their natural ligand. A natural IL-1 antagonist was isolated in 1990[12] and has been used successfully in several models of IL-1-related pathologies, such as rheumatoid arthritis.[13]

'Artificial' hIL-6 receptor-superantagonists were obtained by incorporating two sets of aminoacid substitutions in regions of the molecule responsible for receptor binding: (1) antagonistic substitutions required to selectively impair interaction between the protein and the signal transducing subunit of the receptor, gp130; and (2) superbinder substitutions required for higher binding affinity to the IL-6 receptor subunit, gp80.[14] IL-6 receptor superantagonists are of great therapeutic potential, especially because of their potency, that permits efficient IL-6-blocking at low doses. The use *in vivo* of therapeutic proteins is limited by the short half-life of the molecules and by the high doses required for clinical purposes. Further hindrance is that the therapeutic action of the protein cannot be directed to a specific tissue. Adenovirus gene therapy seems the most suitable system for delivery of such therapeutic genes *in vivo*, as it has been shown to be a very effective vector for transient gene transfer of both intracellular and secretable proteins. A wide range of cells are susceptible to Ad infection, high virus titers are readily obtainable and extremely efficacious gene transfer has been described in several animal models.[15]

We used defective adenovirus, deleted in the E1 and E3 regions, as a vector for the construction of a recombinant particle containing IL-6 receptor superantagonist cDNA, under the control of the strong RSV promoter. In this report, we provide data on the use of this recombinant virus for *in vitro* and *in vivo* gene transfer studies.

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Results

Expression of hIL-6-α in virally infected cells

The recombinant adenoviral vector AdRSVhIL6α was constructed by inserting the cDNA coding for the hIL-6 receptor antagonist Sant1 (Y31D, G35F, S118R, V121D, Q175I, S176R, Q183A hIL-6),[15] with wild-type (wt) IL-6 leader peptide sequence, under the control of the strong RSV promoter, into a viral backbone devoid of most E1 and E3 sequence. To test antagonist expression, human embryonic kidney 293 cells were infected with 5 p.f.u. per cell of a purified AdRSVhIL6α stock. After 24 h, conditioned medium was collected, concentrated and analyzed by Western blot. As shown in Figure 1, a single band of the expected molecular mass was detected in the supernatants of AdRSVhIL6α-infected cells. No detectable signal was observed in the supernatants of cells infected with AdRSVβgal, or in mock-infected cells. Antagonist production in the supernatants of infected cells was quantified by ELISA. Twenty-four hours after infection of 293 with AdRSVhIL-6α at MOI 10, the concentration of antagonist in conditioned medium ranged from 0.5 to 1 μg/ml (data not shown).

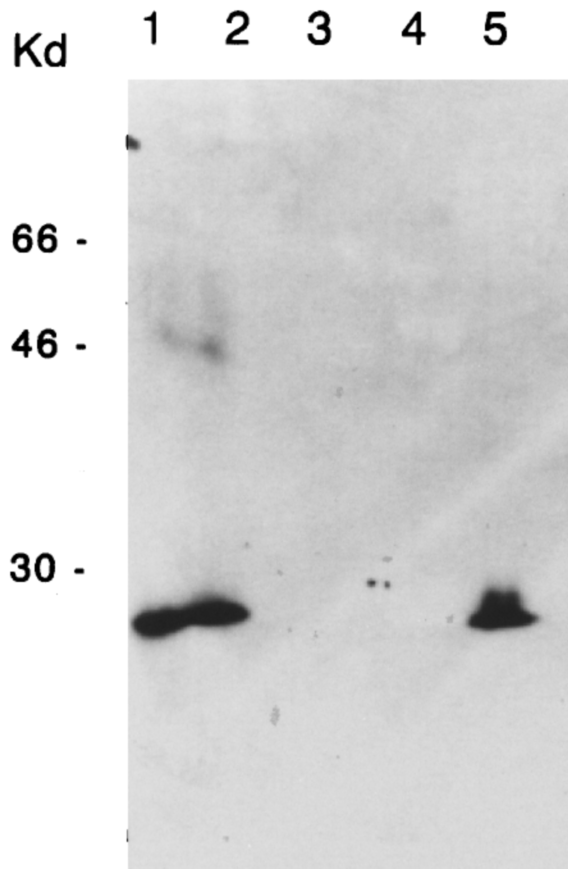

Figure 1 *Immunoblot showing hIL-6α production in 293 cells. Cells were infected at MOI 5 with AdRSVhIL6α (lane 5), or AdRSVβgal (lane 4), or mock infected (lane 3). Supernatants collected 24 h after infection were concentrated five-fold and loaded on acrylamide gel. The gel was blotted and hybridized with an anti-IL-6 polyclonal serum. As controls, 265 ng of purified hIL-6 and 400 ng of hIL-6α were used (lanes 1 and 2).*

Bioactivity of hIL-6-α expressed in vitro: experiments on liver cells

Liver cells are a major target for IL-6. During inflammation, many liver-derived proteins are increased in concentration to provide enhanced protection against invading micro-organisms, limit tissue damage and promote rapid return to homeostasis. These proteins, called acute phase proteins (APP) include serum amyloid protein, C reactive protein (CRP) and haptoglobin. The transcription of APP genes is under the control of cytokine-inducible promoters.[16] Conditioned medium from 293 cells infected with 10 p.f.u. per cell of AdRSVhIL6α, was analyzed on two human hepatic cell lines, Hep3B and HepG2. Cells were transfected with a construct containing as reporter gene a secretable form of alkaline phosphatase (SEAP), under the control of the inducible CRP gene promoter. After treatment of cells with cytokines, SEAP activity was monitored. Hep3B cells were treated with 4 ng/ml of wild-type hIL-6 in the presence or absence of conditioned 293 medium, SEAP activity was measured in cell supernatants. As shown in Figure 2a, the medium of cells infected with AdRSVhIL6α was able to inhibit hIL-6-induced CRP promoter activity in a dose-dependent manner. In the same bioassay, the effect obtained using equivalent doses of Sant1 produced in *E. coli* and purified to homogenicity, is comparable. The inhibitory effect observed when using supernatants of infected cells was not due to toxicity since in the presence of a molar excess of hIL-6 promoter activity was rescued up to 100% (data not shown). The specificity of the effect of AdRSVhIL6α-conditioned medium on CRP promoter activation was analyzed in the same bioassay, testing the inhibitory potential of conditioned medium on human oncostatin M (hOSM)-induced activation of the same promoter in the hepatic line HepG2. OSM is a member of the IL-6 cytokine family also as leukemia inhibitory factor, ciliary neurotrophic factor and interleukin 11. This group of cytokines uses the trasmembrane protein gp130 as common signal transducer, and different receptor α subunits to mediate the specificity of bioactivity;[3] therefore only if the interaction of the antagonist is highly specific for the IL-6 receptor α chain, the inhibitory effect should not be observed on OSM-induced cell activation. As shown in Figure 2b, CRP activity can be stimulated in HepG2 cells by comparable amounts of hIL-6 or of hOSM (4 ng/ml), but, as expected, supernatants of cells infected with AdRSVhIL6α inhibited only hIL-6-induced activation. Specificity of conditioned media bioactivity was further analyzed on HepG2 cells, studying the activation of the IL-6-type cytokine-induced acute phase transcription factor (APRF), STAT3. Following the interaction of the cytokines belonging to the IL-6 family with their receptors, one of the first steps in the intracellular signalling pathway, is tyrosine phosphorylation of APRF. Upon phosphorylation, APRF acquires the ability to bind specific DNA sequences (APREs) and migrates to the nucleus.[17] In Figure 3 are shown the results of a band-shift assay performed on HepG2 cells. Cells were treated with hIL-6 or hOSM in the presence or absence of hIL-6 receptor superantagonist, both in the form of purified protein (Sant1) and as supernatant of cells infected with AdRSVhIL6α (conditioned medium). Cell extracts were prepared and APRF activation was monitored by gel retardation. As shown in the Figure, the antagonist effect of the purified protein and of conditioned medium is

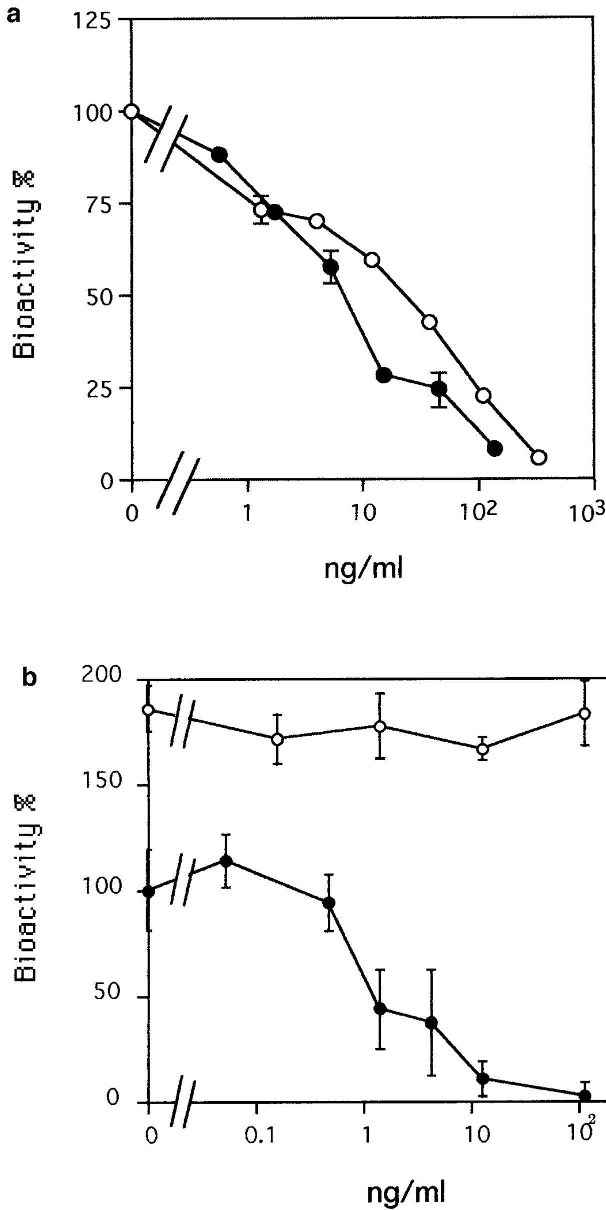

Figure 2 Bioactivity of supernatants of AdRSVhIL6α-infected 293 on liver cells. 293 Cells were infected with AdRSVhIL6α at MOI 10. Supernatants were collected at 24 h and quantified. (a) Conditioned media (black circles) and purified Sant1 (white circles), were tested at different dilutions on Hep3B cells transfected with the IL-6-inducible CRP-SEAP construct and induced with 4 ng/ml wt hIL-6. SEAP activity was quantified and expressed as a percentage of the transcriptional efficiency in cells incubated with 4 ng/ml wt hIL-6 alone, after substraction of the background reading given by the control. Each point is the average of duplicate measurements and the standard error is shown. (b) HepG2 cells transfected with the IL-6-inducible CRP-SEAP construct were induced with either 4 ng/ml hIL-6 (black circles) or with 4 ng/ml hOSM (white circles), both in the presence of increasing concentration of supernatant of AdRSVhIL6α-infected cells. SEAP activity was quantified and expressed as a percentage of the transcriptional efficiency in cells incubated with 4 ng/ml wt hIL-6 alone or 4 ng/ml hOSM alone, after substraction of the background reading given by the control. Each point is the average of duplicate measurements and the standard error is shown.

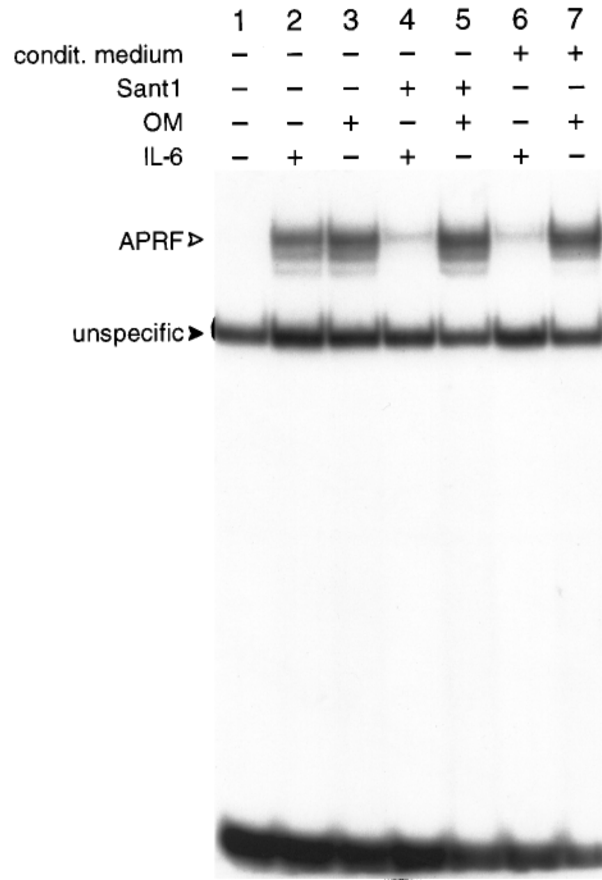

Figure 3 Specificity of the bioactivity of supernatants of AdRSVhIL6α-infected 293 on APRF activation in HepG2 cells. The effect of supernatants of 293 cells infected with AdRSVhIL6α at MOI 10, collected at 24 h and concentrated (condit medium, lanes 6 and 7) or purified protein (Sant1, lanes 4 and 5) was tested on HepG2 cells stimulated either with 2 ng/ml hIL-6 (lanes 2, 4 and 6) or 2 ng/ml hOSM (lanes 3, 5 and 7). Whole extracts were prepared as described in Materials and methods. APRF binding to the high affinity SIE m67 oligonucleotide and electromobility shift assays were performed essentially as previously described.[43]

specific for IL-6-induced activation of APRF. The results shown in Figures 2 and 3 clearly prove that AdRSVhIL6α mediates the production of a protein that possesses an antagonist activity that is highly specific for hIL-6-induced cell activation.

Bioactivity of hIL-6-α expressed in vitro: experiments on myeloma cells

To test the potential of the recombinant virus to inhibit IL-6-dependent proliferation of tumor cells, conditioned medium of cells infected with AdRSVhIL6α at MOI 10, was tested on the Xg1 cell line. These cells were obtained from a multiple myeloma terminal patient. They are dependent on hIL-6 for their growth and represent an important model for *in vitro* testing the effect of an IL-6 antagonist on the proliferation of myeloma cells.[18] Figure 4 shows the results of a proliferation assay. Xg1 cells were treated with a fixed dose of wild-type hIL-6, 0.1 ng/ml, in the presence or absence of increasing dilutions of 293 cells conditioned medium. As shown in the Figure, supernatants of 293 infected with the recombinant virus expressing hIL-6 antagonist were able to specifically

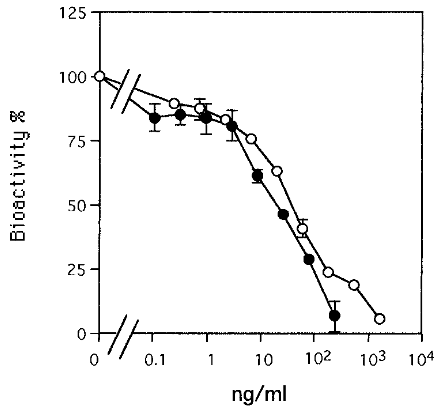

Figure 4 Bioactivity of supernatants of AdRSVhIL6α-infected 293 on human myeloma cells. Supernatants of 293 cells infected with AdRSVhIL6α at MOI 10, collected at 24 h (black circles) or purified protein (white circles) were tested on myeloma cells induced with 0.1 ng/ml of wt hIL-6. After 7 days of culture, the cell number was evaluated by colorimetric determination of hexosaminidase levels and expressed as a percentage of the number of cells incubated with 0.1 ng/ml of wt hIL-6 alone, after subtraction of the background reading given by the control. Each point is the average of duplicate measurements and the standard error is shown.

inhibit hIL-6-induced proliferation of the human myeloma cell line in a dose-dependent manner. The effect is not due to toxicity of the supernatant, since Xg1 proliferation was rescued by treating the cells with a molar excess of hIL-6 (data not shown). As shown in the Figure, results obtained with the purified protein produced in *E. coli* are comparable to those obtained with supernatants of infected cells.

In vivo expression of hIL-6α

C57Bl/6 and CB17-*scid* mice were injected with 2×10^9 p.f.u. of recombinant AdRSVhIL6α in the orbital vein. Sera were collected at different times after the injection and analyzed in ELISA. As shown in Figure 5, the transgene is expressed in immunocompetent mice up to 21 days, and in immunodeficient mice at least 50 days after the injection. The concentration of antagonist in the sera reaches a peak of 1–2 ng/ml in both animal models. As described in the literature, adenovirus-mediated *in vivo* expression of the transgene in immunocompetent animals is limited to 2–10 weeks, depending on the mice strain, in immunodeficient mice it is of several months, because of the absence of the immunological response, normally responsible for the elimination of adenovirus-infected cells.[19] Since the order of magnitude of expression of antagonist in the two animal models is comparable, it seems that the immune response present in immunocompetent mice has an effect on the length but not on the level of transgene expression.

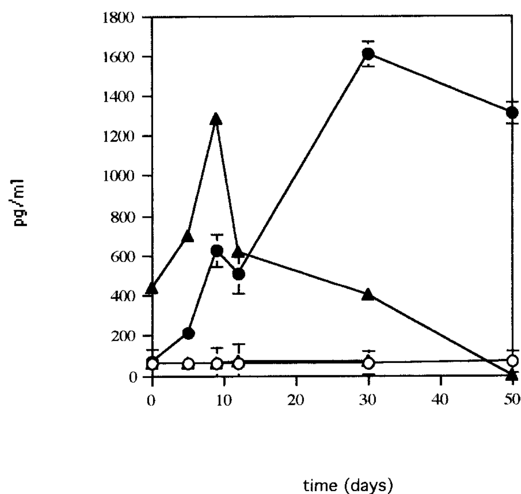

Figure 5 In vivo expression of hIL-6α. C57Bl/6 (circles) or CB17-scid mice (triangles) were i.v. injected with 2×10^9 p.f.u. of AdRSVhIL6α (black) or with 2×10^9 p.f.u. of AdRSVhααt (white). Blood was collected at different times after injections and tested in ELISA. Each point is the average of duplicate measurements of each animal; four to six animals were used for each experimental group, the standard error is shown.

Ex vivo bioactivity of mice sera

Sera of CB17-*scid* mice injected as above or with 2×10^9 p.f.u. of AdRSVhααt were collected at day 40, concentrated and analyzed *in vitro* to verify the bioactivity of the *in vivo*-produced antagonist. Figure 6 shows the results of the effect of infected sera on APRF activation in hIL-6-induced cells. Xg1, treated with 0.2 ng/ml of hIL-6, were incubated for 15 min in the presence or absence of mice sera, whole extracts were prepared and APRF activation was monitored by gel retardation. As this Figure demonstrates, sera of mice injected with AdRSVhIL6α specifically inhibited APRF activation. The inhibitory effect observed in this bioassay is not due to serum toxicity, as IL-6 bioactivity can be rescued by adding an excess dose of the cytokine (data not shown). These results show that AdRSVhIL6α mediated the direct *in vivo* production of a bioactive antagonist. This is important information for the validation of the potential of this system for therapeutic applications to IL-6-dependent diseases.

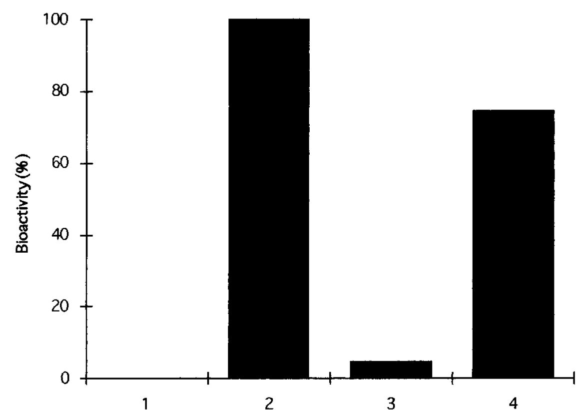

Figure 6 Ex vivo bioactivity of mice sera. Mice were i.v. injected with 2×10^9 p.f.u. of AdRSVhIL6α or of AdRSVhααt, sera were collected 40 days after injection and concentrated 1.5-fold. Xg1 cells were incubated (15 min) with no cytokine (column 1), or with 0.2 ng/ml wt hIL-6 in the presence of control mouse serum (column 2), serum of mice injected with AdRSVhIL6α (column 3), or serum of mice infected with AdRSVhααt (column 4). Whole extracts were prepared as described in Materials and methods. APRF binding to the high affinity SIE m67 oligonucleotide and electromobility shift assays were performed essentially as previously described.[43] Percentage of APRF activity was determined as the ratio between APRF activity in the presence of wt hIL-6 and 50% control serum and the activity in the presence of wt hIL-6 and sera of mice injected with recombinant adenovirus.

Discussion

In this report we provide data on the adenovirus-mediated expression and bioactivity of a human IL-6 receptor antagonist. Gene transfer and expression were accomplished with an adenoviral vector, a vector system generally accepted as having clinical potential for therapy of human diseases. The authenticity and biological activity of the human IL-6 receptor antagonist expressed from this vector were verified through *in vitro* and *in vivo* analysis. The specificity of antagonist activity was analyzed extensively: the inhibitory activity of conditioned medium was observed on cells activated with hIL-6 but not on cells stimulated with a cytokine belonging to the same family, hOSM, in two different bioassays. *Ex vivo* testing of infected sera on a human multiple myeloma cell line shows the potential efficacy of AdRSVhIL6α for therapeutic purposes. The superantagonist used in this study is specific for the human receptor, and thus no functional competition could be shown directly *in vivo* on a murine IL-6 receptor. None the less, we are currently testing *in vivo* the recombinant virus in an animal model of a human IL-6-related disorder.

Acute inflammation induced by excess doses of inflammatory cytokines in the serum is the primary or secondary cause of clinical symptoms in several pathologies, and AdRSVhIL6α is an important candidate for therapy of acute inflammation-related diseases. As shown in this report through the analysis on liver cells, adenovirus-mediated expression of the hIL-6 receptor antagonist specifically inhibited inflammation-related phenomenona, ie stimulation of the acute phase protein promoter, CRP, and activation of the acute phase transcription factor, APRF. The insertion of an IL-6 receptor antagonist gene in Ad vectors for gene transfer is also a promising strategy for improving adenovirus-mediated gene therapy itself. When adenovirus is injected *in vivo*, acute liver inflammation is observed; since it constitutes the first response of the host to Ad, it represents a major factor limiting transgene expression.[20,21] In order to reduce this adenovirus-induced response, the use of immunosuppressors[22] such as cyclosporin[23] or mAbs directed towards different components of the immune system,[24,25] have been proposed. McCoy and co-workers[21] recently described the implementation of AdRSVIL1rα in mice. This failed to reduce adenovirus-induced pulmonary inflammation, but transgene expression was observed for longer than other recombinant adenovirus. In the case of adeno-induced inflammation, an IL-6 antagonist is more specific and potentially more effective than an IL-1rα. In fact, when adenovirus is injected into animals, IL-6 is the first cytokine to be detected, and its levels in the sera are higher than those of other soluble mediators of inflammation such as IL-1 and TNF-α.[25–27]

Anti-IL-6 strategies would also appear very promising for the treatment of IL-6-dependent malignancies. As described in the literature, IL-6 plays a critical autocrine and paracrine role in the growth of several tumours.[1,28] Anti-IL-6 mAbs have been used in phase one and two clinical trials for therapy of multiple myeloma, and have been proven, at least in some patients, to be effective.[9,29] mAbs, and more generally cytokine-binding proteins, have however some limitations in therapy. In fact the immunogenicity of mAbs and the so-called 'paradoxical effect' induced by cytokine-binding proteins, significantly reduce their therapeutic potential. Receptor binding antagonists appear to be more promising effectors for the therapy of cytokine-related diseases. The mutant described in this work is a hIL-6 receptor superantagonist, ie the molecule is not bioactive, but binds to hIL-6 receptor α subunit with higher affinity than the wild-type protein.[14] Hence low molar excess of the antagonist over wt IL-6 is sufficient to block cytokine activity. In the perspective of therapeutic application, these characteristics would be highly advantageous, not only because of the high doses of IL-6 present in the sera of patients affected by IL-6-related diseases, but also because of the increase in the levels of circulating soluble IL-6-receptor observed in some patients.[30,31] In this report we show that AdRSVhIL6α-infected cells produce significant amounts of hIL-6 antagonist both *in vitro* and *in vivo*, and are capable of inhibiting IL-6 activity in an IL-6-dependent myeloma human cell line. After injection of 2×10^9 p.f.u., the *in vivo* concentration of the transgene product, both in immunocompetent and immunodeficient mice, is in the nanomolar range, which exceeds serum IL-6 levels measured in animal models of IL-6-related diseases.[7] Furthermore since recombinant first generation adenovirus can be readily obtained at titers of 10^{11}–10^{12} p.f.u. per milliliter, AdRSVhIL6α could be clinically applied to myeloma and post-transplant lymphoma patients where the concentration of circulating hIL-6 in the serum is in the picomolar range.[32,33]

As shown in this report the antagonist can be expressed *in vivo* for several weeks in immunocompetent animals. Gene therapy thus seems a suitable system for delivering such a molecule for therapeutic purposes. It has to be underlined that when microgram amounts of purified protein are injected intravenously into mice, the antagonist's half-life is of only a few hours; hence even repeated injections of the protein would probably be ineffective for treatment of diseases characterized by a continuous cytokine-mediated stimulation of malignant cell proliferation, such as multiple myeloma.

As shown for other transgenes, adenovirus mediates efficient but transient expression in immunocompetent animals. In the context of this study, this could be advantageous for two reasons. The first is that, as discussed above, the therapeutic targets for a cytokine antagonist are not genetic diseases, and the second is that IL-6 plays a physiological role in the organism. Studies on IL-6-deficient mice have shown that, even though these mice develop normally, specific functions of the immune system are impaired.[34] Therefore temporary rather than lifetime inactivation of this cytokine is more suitable for clinical purposes.

The gene therapy approach potentially allows for a controlled and intracellular expression of the antagonist, the latter being of interest for the inhibition of cytoplasmic interaction between ligand and receptor. This interaction would seem to play an important role in the autocrine loop of IL-6-dependent cell proliferation.[35]

The combination of the specific features of the antagonist used in this work, with the adenovirus gene therapy approach, that allows efficient *in vivo* bioactive transgene expression, appears to be a very promising strategy for the therapy of IL-6-related pathologies.

Materials and methods

Recombinant adenoviruses

For the construction of AdRSVhIL6α, (Y31D, G35F, S118R, V121D, Q175I, S176R, Q183A) hIL-6 cDNA[14] was subcloned together with hIL-6 leader sequence in the adenoviral vector pAdRSVβgal restricted *Sal*I/*Eco*RV.[36] Recombinant plasmid was cotransfected together with *Cla*I-restricted AdRSVβgal genome into 293 cells (ATCC, Rockville, MD, USA). Recombinant plaques were isolated and amplified as previously described.[37,38] DNA pattern of recombinant AdRSVhIL6α was analyzed by multiple restriction.

AdRSVβgal and AdRSVhααt construction has been described previously.[36,39]

All viral stocks were prepared in 293 cells and purified twice on isopicnic CsCl gradient. Desalting was performed using Pharmacia G50 columns (Orsay, France). Viruses were divided into aliquots and kept in PBS-15% glycerol at –80°C. Titers, calculated by plaque analysis on 293 cells, varied from 1 to 2×10^{11} p.f.u. per milliliter.

Animals

C57Bl/6 mice or CB-17-*scid* mice 6 to 8 weeks old were purchased from IFFACREDO (L'Arbresle, France). The animals were treated according to the institutional guidelines. Mice were injected in the orbital vein with purified virus, diluted in 100 μl of PBS. Sera were collected by puncturing the orbital vein after treating the animal with ether. After short centrifugation blood supernatants were kept at –20°C. Balb/c mice were bred at IRBM (Pomezia, Italy). Serum was collected by intracardiac puncture, after the death of the animals.

Immunodetection of IL-6 antagonist

Western blot: 35-mm Falcon plates of subconfluent 293 cells were infected with AdRSVhIL6α or AdRSVhααt, at MOI 5 in 3 ml of medium. Twenty-four hours after infection, supernatants were collected and concentrated fivefold with centrex UF filters (Scleicher and Schuell, Ecquevilly, France). Twenty-five microliters of concentrated supernatants were loaded on acrylamide gel. The gel was electroblotted and nitrocellulose membrane was blocked overnight in TBS-5% milk-0.05% Tween 20. After washing in TBS-0.05% Tween 20, the membrane was incubated 2 h with a polyclonal serum anti-hIL-6 (hIL-6 ELISA kit, R&D, Oxford, UK), coupled to horseradish peroxidase, diluted 1:2, v/v, in TBS-5% milk-0.05% Tween 20. After washing with TBS-0.05% Tween 20, the membrane was revealed with ECL bioluminescence kit (Amersham, UK). hIL-6, hOSM and Sant1 purified from bacteria, were prepared as previously described.[14]

ELISA: ELISA was performed on supernatants of infected cells and on mice sera with the IL-6 detection kit purchased from R&D.

Bioassays

35-mm Falcon plates of subconfluent 293 cells were infected with AdRSVhIL6α or AdRSVβgal, at MOI 10 in 3–5 ml of medium. Twenty-four hours after infection, supernatants were collected, eventually concentrated in centrex UF filters, quantified in ELISA, and tested, at different concentrations, on Hep3B (ATCC), on HepG2 (ATCC), or on the myeloma cell line XG-1 (a kind gift of Dr B Klein, Montpellier, France).

CRP promoter assay

Transcriptional activation of the CRP gene promoter in Hep3B or HepG2 cells was determined and quantified as described.[40] Briefly, 1×10^5 HepG2 or 5×10^4 Hep3B cells were plated in 24 multiwell plates (Falcon, SIAL, Rome, Italy). After 5 h cells were induced with 4 ng/ml hIL-6 or 4 ng/ml hOSM, in the presence or absence of supernatants of infected cells or of purified antagonist. SEAP activity in the supernatant of cells was assayed, as previously described, with a colorimetric reaction using a substrate *p*-nitrophenylphosphate (Sigma, Milan, Italy). The A405 of the reaction mixture was recorded with a Bio-Rad model 450 automatic plate reader (Bio-Rad, Ivry-sur-Seine, France).

XG-1 proliferation assay

XG-1 cells were cultured as described[19] in the presence of 2 ng/ml of hIL-6. To test supernatants of cells infected with AdRSVhIL6α or of purified Sant1 for antagonistic behavior, cells (6×10^3 per microwell, in 96-well microtiter plates; Falcon) were cultured in 200 μl of RPMI 1640 supplemented with 10% fetal calf serum and 5×10^{-5} M β-ME with serial dilutions of supernatant or Sant1, in the presence of 0.1 ng/ml of wt hIL-6. After 7 days, cell numbers were evaluated by colorimetric determination of hexosaminidase levels, according to Landegren.[41]

ElectroMobility Shift Assays (EMSAs)

For HepG2, 10^6 cells per well were plated in 6-multiwell plates (Falcon). Cells were induced with 2 ng per well hIL-6 or 2 ng/ml hOSM, in the presence or absence of 600 ng/ml antagonist, either in the form of pure protein obtained from bacteria, or as conditioned medium from AdRSVhIL6α-infected cells. For Xg1, cells were washed twice with culture medium, incubated for 4–5 h at 37°C in culture medium without hIL-6 and washed twice again in 1 × PBS. 2×10^6 Cells in 400 μl of culture medium were added to each sample (500 μl of serum +100 μl RPMI with or without hIL-6 0.2 ng/ml). For the control samples (no hIL-6 and hIL-6 0.2 ng/ml), sera from control BALB/c mice were used. Both control sera from BALB/c mice and sera from adeno-injected *scid* mice were concentrated 1.5-fold in centrex UF filters prior to their utilization. HepG2 or XG-1 cells were collected after 15 min of induction at 37°C, rinsed with ice-cold PBS containing 5 mM NaF, centrifuged and cell pellets were frozen in liquid nitrogen. Total cell extracts were prepared as previously described.[15] STAT activation was monitored by binding to the high affinity SIE m67 oligonucleotide,[42] which was labeled by filling in 5′ protruding end with T7 DNA polymerase (Boehringer Mannheim, Meylan, France), using α-^{32}P-dATP and α-^{32}P-dCTP (3000 Ci/mmol). EMSA were performed according to Sadowsky and Gilman.[43]

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