Targeting of Interleukin 2 to Human Ovarian Carcinoma by Fusion with a Single-Chain Fv of Antifolate Receptor Antibody

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ABSTRACT

To provide a new tool for the immunotherapy of human ovarian carcinoma, we constructed a fusion protein between interleukin-2 (IL-2) and the single-chain Fv (scFv) of MOV19, a monoclonal antibody directed against α-folate receptor (α-FR), known to be overexpressed on human nonmucinous ovarian carcinoma. This was accomplished by fusing the coding sequences in a single open reading frame and expressing the IL-2/MOV19 scFv chimera under the control of the murine immunoglobulin κ promoter in J558L plasmacytoma cells. The design allowed the construction of a small molecule combining the specificity of MOV19 with the immunostimulatory activity of IL-2. This might improve the tissue penetration and distribution of the fusion protein within the tumor, reduce its immunogenicity, and avoid the toxicity related to the systemic administration of IL-2. The IL-2/MOV19 fusion protein was stable on purification from the cell supernatant and was biologically active. Importantly, this construct was able to target IL-2 onto the surface of α-FR-overexpressing tumor cells and stimulated the proliferation of the IL-2-dependent CTLL-2 cell line as well as that of human resting peripheral blood lymphocytes. In a syngeneic mouse model, IL-2/MOV19 scFv specifically targeted α-FR gene-transduced metastatic tumor cells without accumulating in normal tissues, due to its fast clearance from the body. Prolonged release of IL-2/MOV19 scFv by in vivo transplanted J558-EF6.1 producer cells protected 60% of mice from the development of lung metastases caused by an i.v. injection of α-FR gene-transduced tumor cells. Moreover, treatment with IL-2/MOV19 scFv, but not with recombinant IL-2, significantly reduced the volume of s.c. tumors. The pharmacokinetics and biological characteristics of IL-2/MOV19 scFv might allow us to combine the systemic administration of this molecule with the adoptive transfer of in vitro retargeted T lymphocytes for the treatment of ovarian cancer, thereby providing local delivery of IL-2 without toxicity.

INTRODUCTION

IL-2 has been used for the immunotherapy of a variety of human malignancies; however, its rapid clearance, severe toxicity when given at high doses, and suboptimal concentration in the tumor site have limited its efficacy in vivo. Genetic manipulation of tumor cells or fibroblasts allowed IL-2 to be secreted in situ. In vivo, such IL-2-secreting cells induce an inflammatory reaction that may result in the elimination of tumor cells, the generation of a systemic immune response, and immunological memory (1-3). However, data from animal models indicate that immunotherapy with IL-2-secreting cells is only effective for limited tumor loads, suggesting its optimal use in the treatment of minimal residual disease. Furthermore, on gene transduction, IL-2 secretion may vary according to the vector used, the strength of the promoter driving the transgene expression, and the efficiency of protein synthesis in the targeted cell.

The selective delivery of IL-2 at the tumor site would avoid the toxicity associated with systemic administration, overcome the concerns due to ex vivo genetic manipulation of autologous or allogeneic cells, and solve the difficulties of transducing tumor cells in vivo. Taking advantage of the targeting specificity of mAbs directed to TAAs, fusion proteins have been constructed to allow IL-2 targeting at the tumor microenvironments and the activation of antitumor immune responses in murine models (4-8). Targeting of IL-2 to tumor sites is expected to sustain in situ the antitumor cytotoxicity of both T lymphocytes activated by direct immunization and lymphocytes infused in the context of adoptive immunotherapy.

Among human tumors, ovarian carcinoma has insidious dissemination patterns and is often diagnosed at advanced stages. Although platinum combination chemotherapy improved the response rate of these patients, satisfactory long-term survival has not been achieved (9). Immunotherapeutic approaches can be envisioned for these ovarian carcinoma patients, because their tumors express a panel of known TAAs, thereby providing a target for antitumor vaccination (10, 11). Moreover, mAbs have been raised against ovarian carcinoma antigens that can be used for delivery drugs and radionucleides for either diagnosis or therapy (12-14). Finally, the natural course of the disease includes a long-term phase of i.p. growth, providing the rationale for locoregional immunotherapy. After the chemotherapy of refractory ovarian cancer, immunotherapy treatment with mAbs or tumor necrosis factor can lead to the reduction of malignant ascites (15). Partial responses were obtained when IL-2 was given i.p. in combination with lymphokine-activated killer cells or tumor-infiltrating lymphocytes. In fact, signs of immune activation such as an increase of CD8+, CD16+, and CD56+ cells and the secretion of IFN-γ, granulocyte macrophage colony-stimulating factor, and IL-10 in the peritoneal cavity were observed in these patients as well as in patients treated with i.p. IL-2 only (16-18).

Recently, i.p. tumor regression was achieved after adoptive immunotherapy with T lymphocytes retargeted in vitro by the bsAb OC/TR (19) directed to CD3 and to the α-FR, a M., 38,000 glycoprotein TAA overexpressed in more than 90% of human nonmucinous ovarian carcinomas (12). The need for IL-2-dependent preactivation of T lymphocytes in vitro provides the rationale for targeting IL-2 to tumor sites to sustain the activation and function of retargeted effector cells in a combinational immunotherapy. To this end, we constructed a fusion protein between human IL-2 and the scFv of mAb MOV19 that specifically recognizes the α-FR. MOV19 is not internalized on binding to the α-FR (20) and therefore allows the IL-2 portion of the fusion protein to remain on the cell surface.

Here we show that the IL-2/MOV19 scFv fusion protein was expressed at high levels in murine myeloma cells, and that once purified, it exhibited the functional activity of its two components, i.e., specific binding to α-FR-expressing tumors and the stimulation of lymphocyte proliferation. To assess the activity of IL-2/MOV19 scFv

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4 The abbreviations used are: IL, interleukin; scFv, single-chain Fv; mAb, monoclonal antibody; α-FR, α-folate receptor; TAA, tumor-associated antigen; Ab, antibody; HPLC, high-performance liquid chromatography; PBL, peripheral blood lymphocyte; bsAb, bispecific Ab; %ID/g, the percentage of injected dose per gram of tissue.

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in vivo, a murine tumor model was prepared by the retroviral transduction of the gene coding for the human α-FR into murine colon carcinoma C26 (C26/α-FR). The pharmacokinetics and biodistribution of IL-2/MOV19 scFv showed a rapid clearance after i.p. administration and a specific retention within C26/α-FR but not in C26 or in normal tissues. Moreover, IL-2/MOV19 scFv impaired C26/α-FR outgrowth, thus suggesting that a therapeutic application of this reagent is warranted.

**MATERIALS AND METHODS**

**Construction and Expression of IL-2/MOV19 scFv.** The full-length human IL-2 cDNA sequence was amplified by PCR from the Lh2SN vector (21) with primers containing the suitable restriction sites HindIII-BamHI and cloned into the pCDNA3 vector (Invitrogen, Leek, the Netherlands). The sequences encoding the variable chains of mouse mAb MOV19 were reverse-transcribed from the producing hybridoma, amplified, and assembled to encode the scFv fragment using PCR essentially as described previously (22, 23). The assembled MOV19 scFv was cloned into pHE1 to be screened by phage panning on cells overexpressing the α-FR (23), and then the full-length DNA coding sequence was amplified with primers containing the EcoRI and XhoI sites and cloned 3' to the IL-2 sequence into the corresponding sites of vector pCDNA3. The synthetic linker GGTTGACCTCTGTTAGCCCAGTATCATCTCTGAAAGCAGGT was then ligated in-frame into the BamHI-EcoRI sites between IL-2 and MOV19 scFv to encode a 13-amino acid spacer that has been shown to facilitate the correct folding of the fusion protein. The entire open reading frame was sequenced and transfected into J558L myeloma cells (24) by protoplast fusion. Clones of G418-resistant J558L cells were screened for the secretion of IL-2/MOV19 scFv by ELISA for human IL-2 (Duoset kit; Genzyme, Cambridge, MA) according to the manufacturer's instructions. To increase the production of the fusion protein, the expression cassette was further modified and cloned into the phCry III vector (25). This expression vector encodes the constant portion of murine κ light chain (Cκ) under the immunoglobulin κ promoter and contains the murine immunoglobulin H and immunoglobulin κ enhancers. Using an appropriate PCR primer, the stop codon was removed from the 3' end of the MOV19 Vκ and substituted with a splicing consensus sequence to ensure the fusion between IL-2/MOV19 scFv and Cκ. The modified cassette was sequenced, blunt-ended, and cloned into the phCry-κll vector. Plasmid pHIL2/MOVSD was transfected into J558L cells by protoplast fusion, and cells were selected by G418. The supernatants of resistant cells were screened by ELISA for human IL-2, and the most positive clones were subcloned by limiting dilution to obtain two high-expressing colonies, J558-EF6.1 and J558-EF6.2.

The molecular size and immunoreactivity of the fusion protein in the supernatant of the expressing clones were tested by Western blotting. Total supernatant proteins (50 μg) were subjected to 10% SDS-PAGE and electroblotted onto Immobilon membranes (Millipore Corp., Bedford, MA). Filters were hybridized to biotinylated goat antiserum Cκ Ab (Southern Biotechnology Associates, Birmingham, AL; [0.1 μg/ml]) or rabbit antihuman IL-2 Ab (Genzyme; 10 μg/ml) in 5% BSA/PBS/0.1% Tween 1 for 1 h, washed several times in PBS/0.1% Tween 20, and incubated for 1 h with either Avidin Biotin Complex (Vector Laboratories, Burlingame, CA) or horseradish peroxidase-conjugated goat antirabbit Ab (0.05 μg/ml) in 5% BSA/PBS/0.1% Tween 20. After extensive washing, the filters were treated with enhanced chemiluminescence detection solutions (Amity; Amersham Life Science, Buckinghamshire, United Kingdom) and exposed to Hyperfilm (Amity; Amersham Life Science) from 10 s to 5 min.

**Functional Assays for IL-2/MOV19 scFv.** The IL-2 biological activity of IL-2/MOV19 scFv was tested in the supernatant of transduced J558L cells and as a purified fusion protein in the supernatant of IL-2-dependent CTLL-2 cells. A serial dilution of supernatant from the J558-EF6.1 and J558-EF6.2 clones or purified fusion protein and recombinant human IL-2 (Proleukin; Chiron Corp., Emeryville) were incubated for 48 h with 5 x 10^4 CTLL-2 cells that had been starved of IL-2. One μCi of [3H]thymidine (Amity; Amersham Life Science) was added to 200 μl of cell culture for the last 18 h, and cell proliferation was measured by [3H]thymidine incorporation.

The MOV19 binding reactivity of the IL-2/MOV19 scFv fusion protein was assayed by immunofluorescence on tumor cells expressing or not expressing the α-FR; 5 x 10^5 cells were incubated for 1 h on ice with serial dilutions of supernatant and with 5 μg/ml purified IL-2/MOV19 scFv or MOV19 mAb and diluted in 2% BSA/PBS/0.05% sodium azide. Cells were then washed four times with the same buffer and incubated with either biotinylated antiserum Cκ (Southern Biotechnology Associates) or biotinylated antihuman IL-2 (Genzyme) at 2.5 μg/ml for 1 h on ice. After repeated washing, the cells were incubated for 40 min with streptavidin-phycocerythrin conjugate (5 μg/ml; Pharmingen, San Diego, CA) in ice, washed, and analyzed using a FACScan (Becton Dickinson). For competition assays, 100 μg/ml chimeric MOV19 (26) were added to the IL-2/MOV19 scFv in the first incubation, and biotinylated antiserum Cκ was used as the secondary Ab.

**Purification of IL-2/MOV19 scFv Fusion Protein.** The rat antiserum Cκ mAb 187.1 (ATCC number HB58) was purified from hybridoma supernatant by affinity chromatography on Hi Trap Protein G (Pharmacia Biotech, Uppsala, Sweden) and coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech) according to the manufacturer's instructions. The EF6.1 cell supernatant was dialyzed against 0.1 M Tris-HCl (pH 8.0), filtered, and loaded on the column. The fusion protein was eluted by acid buffer, equilibrated with Tris-HCl (pH 9.5), and dialyzed extensively in 0.9 g/liter NaCl or in 3.4 mM PBS-EDTA. After purification, the fusion protein was analyzed on SDS-PAGE and by HPLC gel filtration on a Superose12 HR 10/30 column (Pharmacia Biotech), and its activity was assayed as described above for the cell supernatant.

**Murine Tumor Model and Cell Lines.** To analyze the in vivo antitumor effect of the fusion protein in a syngeneic context, the C26 adenocarcinoma of the BALB/c strain was transduced with the human α-FR gene by means of a retroviral vector to obtain the C26/a-FR clone. A 910-bp EcoRI fragment encoding the human α-FR was excised from pBluescript K3+ (27) and cloned into the corresponding site of retroviral vector LXSIN to obtain the Lgp38SN vector. The recombinant retroviral particles were obtained by the transfection of the amphotropic packaging cell line gp+AM12 and the infection of the ectopic packaging cell line gp+E68 as described previously (21). Subconfluent plates of the murine adenocarcinoma cell line C26 were infected with viral supernatant in the presence of polybrene as described previously (21), and colonies of transduced cells were isolated by G418 selection and subcloned by limiting dilution to obtain clones displaying homogeneous expression of the α-FR, as measured by immunofluorescence using mAb MOV19. Clone 5A6 that expressed the transgene at a high level was chosen for additional experiments and was designated C26/a-FR.

The cell lines INT.Ov-1, INT.Ov-2, INT.Ov-3, and INT.Ov-7 were established in our laboratory as described previously (11) from primary and metastatic ovarian carcinomas that were either serous or mucinous. IGROV-1 cells were kindly provided by Dr. J. Bénard (Institute Gustave Roussy, Villejuif, France; Ref. 28); SK-CO-1 and HT-29 colon carcinoma cell lines as well as the SK-BR3 breast carcinoma cell line were from the American Type Culture Collection. α-FR expression was assayed by immunofluorescence using the MOV19 mAb.

PBLs were collected from normal donors and stored frozen until use. All human tumor cell lines were maintained in RPMI 1640 supplemented with 10% FCS or 10% pooled human AB serum, whereas murine cell lines were grown in DMEM supplemented with 5% FCS, and transduced cells were grown in DMEM supplemented with 0.8 mg/ml G418.

**Tumor Targeting and IL-2 Bioactivity in Vitro.** Irradiated (20,000 rads) tumor cells with or without α-FR expression were incubated with purified IL-2/MOV19 scFv (5 μg/ml) for 30 min on ice, washed repeatedly with fresh medium, plated in triplicate in serial dilutions into 96-well plates, and incubated with or without 5 x 10^4 CTLL-2 cells for 48 h, with the addition of 1 μCi of [3H]thymidine for the last 18 h. CTLL-2 proliferation was measured as: cpm incorporated by CTLL-2 - cpm incorporated by irradiated tumor cells.

**Lymphocyte Proliferation Assay.** Irradiated (20,000 rads) INT.Ov-2 and INT.Ov-3 ovarian carcinoma cells were incubated or not incubated with IL-2/MOV19 scFv as described above. After repeated washing with fresh medium, 10^6 treated and untreated tumor cells were plated in triplicate into round-bottomed 96-well plates, and 10^6 human lymphocyte antigen-matched PBLs were added to each well (E/T: 10:1); 100 IU/ml recombinant human IL-2

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5 C. Melani, unpublished observations.
or IL-2/MOV19 scFv or 1 μg/ml of phytohemagglutinin were added to control wells. Cell proliferation was measured with an 18-h \(^{3} \text{H}\)thymidine incorporation assay after 5 days of culture.

**Pharmacokinetics and Biodistribution.** The purified IL-2/MOV19 scFv was labeled with \(^{125}\text{I}\) as described previously (29) at a specific activity of 4 μCi/μg. Mice received Lugol solution (0.02% I, and KCIO\(_4\) (0.6 mg/ml) in their drinking water starting 3 days before radiolabeled fusion protein administration and throughout the experiments to block free iodine uptake by the thyroid gland and stomach mucosa. Groups of three BALB/c mice were injected i.p. with a single dose of \(^{125}\text{I}\)-labeled IL-2/MOV19 scFv diluted in 150 μl of 0.9% (w/v) saline (dose, 2.08 × 10\(^{-11}\) mol; activity, 4.9 μCi). Blood samples were drawn from the retroorbital sinus and allowed to clot for 30 min at 37°C, and the sera were collected by centrifugation. Pharmacokinetic analysis was done with serum samples as described previously (30). The pharmacokinetics of purified IL-2/MOV19 scFv were also assayed by injecting 10 μg of fusion protein i.p. into BALB/c mice and collecting blood at different time intervals. The amount of IL-2/MOV19 scFv was measured in mouse serum with an ELISA for human IL-2. Pharmacokinetic parameters were calculated by using PCNONLIN software (SCI Software, Lexington KY).

The biodistribution of IL-2/MOV19 scFv was similarly tested in BALB/c mice bearing 7-day lung metastases of C26α-FR or C26 obtained by i.v. injection of 5 × 10\(^{6}\) tumor cells. At 2, 6, 18, 24, and 30 h after the i.p. injection of radiolabeled IL-2/MOV19 scFv, groups of three mice were sacrificed by cervical dislocation, and their organs were weighed and counted for radioactivity. The %ID/g was calculated, and the fusion protein distribution was expressed as the ratio between the %ID/g of tissue and blood.

**In Vivo Treatment with IL-2/MOV19 scFv.** To obtain a continuous release of the fusion protein, groups of 10 BALB/c mice injected s.c. with 5 × 10\(^{4}\) C26α-FR or C26 obtained by i.v. injection of 5 × 10\(^{6}\) tumor cells. At 2, 6, 18, 24, and 30 h after the i.p. injection of radiolabeled IL-2/MOV19 scFv, groups of three mice were sacrificed by cervical dislocation, and their organs were weighed and counted for radioactivity. The %ID/g was calculated, and the fusion protein distribution was expressed as the ratio between the %ID/g of tissue and blood.

**RESULTS**

**Expression and Purification of IL-2/MOV19 scFv.** Our first attempt to express the fusion protein IL-2/MOV19 scFv in J558L cells by the pCDNA3 vector under the control of the cytomegalovirus promoter yielded a low but biologically active (data not shown) amount of secreted fusion protein (range, 0.1–0.12 μg/ml, as measured by an ELISA for human IL-2).

To improve the yield, the pH PCR\(_\gamma\) III vector that contains the immunoglobulin \(\kappa\) promoter, both \(\kappa\) and H enhancers, and the murine Ck sequence was modified to give pHIL2/MOV19SD (Fig. 1A). The Ck tail allows easy purification of the fusion protein by an immunoadfinity column charged with anti-Ck antibody. Two clones of transfected J558L cells secreted a high amount of IL-2/MOV19 scFv (up to 3.5 μg/ml/48 h) as assayed by the IL-2 ELISA. Western blot analysis performed with anti-IL-2 and anti-Ck Ab revealed a monomeric protein of \(M_t\) 52,500 that partially dimerized under nonreducing conditions (Fig. 1, B and C). The dimerization of the fusion protein may occur due to free SH present on Ck and also on IL-2 (Cys-125). The secreted fusion protein was shown to bind the α-FR antigen expressed on IGROV-1 cells in the immunofluorescence assay and displayed IL-2 activity in a CTLL-2 proliferation assay (data not shown).

IL-2/MOV19 scFv was purified from the supernatants of the J558-EF6.1 producer clone with an immunoaffinity column using an anti-Ck antibody, and the yield ranged from 2–3.5 mg of protein/liter.
CHARACTERIZATION AND FUNCTION OF IL-2/MOV19 scFv.

We found that 1 pg of purified IL-2/MOV19 scFv was comparable to human IL-2 in the CTLL-2 proliferation assay and compared with that of recombinant human IL-2. The bioactivity of the purified fusion protein was tested several times in a CTLL-2 proliferation assay, depending on the different batches of purified fusion protein. The mean bioactivity of the purified fusion protein corresponded to 7,500-12,500 IU of recombinant human IL-2 (Proleukin), depending on the different batches of purified fusion protein. However, the addition of 3.4 mM EDTA to PBS during dialysis and storage resulted in the best yield of monomeric fusion protein, as shown by the HPLC profile in Fig. 1D. The percentage of positivity remained unchanged, although the mean intensity of fluorescence was reduced by 1 log, because the transgene expression was extremely high in these cells.

Moreover, the binding of IL-2/MOV19 scFv but not that of MOV19 to the cell surface was detectable by an antihuman IL-2 Ab used as a secondary Ab (Fig. 2C).

LYMPHOCYTE PROLIFERATION ASSAY. The IL-2-dependent CTLL-2 cell line was cocultured with antigen-positive or -negative cells that were previously incubated with purified IL-2/MOV19 scFv. On antigen binding and repeated washing, IL-2/MOV19 scFv remained bound to the surface of α-FR-expressing cells and efficiently stimulated CTLL-2 cell proliferation (Fig. 3A), proving that fusion protein bound to the surface of antigen-positive cells could stimulate the high-affinity IL-2 receptor on CTLL-2 cells.

We also tested whether fusion protein bound to tumor cells could stimulate human PBLs through the binding of its IL-2 portion. Lymphocyte proliferation was induced by the antigen-positive INT.Ov-2 cells coated with IL-2/MOV19 scFv, but neither untreated INT.Ov-2 nor antigen-negative INT.Ov-3 preincubated with the fusion protein was able to sustain lymphocyte proliferation (Fig. 3B). Positive controls of lymphocyte proliferation were the fusion protein in solution, recombinant IL-2, or phytohemagglutinin (data not shown).

PHARMACOKINETICS AND BIODISTRIBUTION. 125I-labeled IL-2/MOV19 scFv was used for pharmacokinetic studies. The fusion protein was injected into mice, and the i.p. route was chosen in view of the prospective treatment of ovarian carcinoma patients by i.p. administration. The iodinated IL-2/MOV19 scFv reached the bloodstream rapidly (5 min), peaked at 1 h, and was completely secreted by 24 h. The clearance curve of radiolabeled IL-2/MOV19 scFv was biphasic, fitting a two-compartment model [a very rapid absorption phase (t1/2α = 5 min) and a slower elimination phase (t1/2β = 105.45 min)]. Similar results were obtained by measuring the amount of human IL-2 in the serum after i.p. injection of 10 µg of purified IL-2/MOV19 scFv (data not shown).

In tumor-bearing animals, the fusion protein did not accumulate in the tumor, and the antibody could be detected in the serum, liver, lungs, kidneys, and ovaries. Moreover, the antibody could be detected in the serum, liver, lungs, kidneys, and ovaries. Moreover, the antibody could be detected in the serum, liver, lungs, kidneys, and ovaries. Moreover, the antibody could be detected in the serum, liver, lungs, kidneys, and ovaries. Moreover, the antibody could be detected in the serum, liver, lungs, kidneys, and ovaries.

**Table 1** Comparison between IL-2/MOV19 scFv and MOV19 mAb for antigen recognition

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MOV19 mAb</th>
<th>IL-2/MOV19 scFv</th>
<th>IL-2/MOV19 scFv + chimeric MOV19</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGROV-1</td>
<td>99.14%</td>
<td>99.5 (112.6)</td>
<td>21.85 (16.3)</td>
</tr>
<tr>
<td>INT.Ov-1</td>
<td>99.97%</td>
<td>100 (225.5)</td>
<td>13.17 (18.06)</td>
</tr>
<tr>
<td>INT.Ov-2</td>
<td>85.9 (258.7)</td>
<td>87.2 (280.66)</td>
<td>6.7 (29.6)</td>
</tr>
<tr>
<td>INT.Ov-3</td>
<td>0.96 (21.3)</td>
<td>0.6 (41.4)</td>
<td>ND</td>
</tr>
<tr>
<td>INT.Ov-7</td>
<td>2.9 (71.6)</td>
<td>2.6 (71.4)</td>
<td>2.4 (70.4)</td>
</tr>
<tr>
<td>HT-29</td>
<td>67.2 (45.2)</td>
<td>67.4 (47.6)</td>
<td>8.4 (36.8)</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>63.6 (40.7)</td>
<td>64.3 (50.7)</td>
<td>10.2 (30.6)</td>
</tr>
<tr>
<td>C26</td>
<td>0.45 (25.8)</td>
<td>1.2 (14.1)</td>
<td>ND</td>
</tr>
<tr>
<td>C26/α-FR</td>
<td>99.4 (437.6)</td>
<td>99.9 (352.7)</td>
<td>93.8 (36.04)</td>
</tr>
</tbody>
</table>

*Percentage of cell positivity.

*Mean intensity of fluorescence.

*ND, not done.
Fig. 3. Tumor binding activity and lymphocyte stimulation by IL-2/MOV19 scFv. A, IL-2-dependent CTLL-2 cell proliferation stimulated by the fusion protein bound on the cell surface of antigen-positive tumor cells (see also Table 1), but not by untreated or by treated but antigen-negative tumor cells. ■, pretreatment with fusion protein (5 μg/ml); ●, no treatment. B, human ovarian carcinoma cells targeted with IL-2/MOV19 scFv induce or do not induce the proliferation of human resting PBLs according to their α-FR expression (inset). Tumor cell lines pretreated or not treated with the fusion protein and freshly isolated human lymphocyte antigen-matched PBLs were mixed at a 1:10 ratio and cocultured for 5 days. ■, pretreatment with fusion protein (5 μg/ml); ●, no treatment.

normal organs or in lungs containing micrometastases of parental non-transduced C26, whereas a specific accumulation of IL-2/MOV19 scFv was detectable in lungs bearing C26/α-FR micrometastases (Fig. 4).

**In Vivo Treatment with IL-2/MOV19 scFv.** The C26/α-FR clone was characterized in both immunocompetent and immunocompromised hosts and was found to behave like the parental cell line in terms of growth rate, tumor take, and metastatic potential.

Using the producer J558EF6.1 cells as a living pump to obtain a prolonged release of the IL-2/MOV19 scFv in vivo, we analyzed whether targeting IL-2 to C26/α-FR would affect tumor growth. Indeed, producer J558EF6.1 cells released detectable levels of human IL-2 in mouse serum for up to 4 days without forming tumors when injected into syngeneic BALB/c or nu/nu mice (Fig. 5A), thus behaving like J558L transduced with the IL-2 gene (31). BALB/c mice were injected s.c. with either C26 or C26/α-FR and injected with J558EF6.1 cells in the opposite flank. Growth of C26/α-FR was completely inhibited in 22% of mice, whereas C26 tumor growth was unaffected by IL-2/MOV19 scFv (Fig. 5B). The same treatment protected 60% of mice injected i.v. with C26/α-FR but did not protect mice injected with C26 cells (P = 0.011; Fig. 5C).

Because data from the pharmacokinetic experiments showed that the fusion protein had a fast clearance from the blood, we tested the effect of repeated i.p. injection of purified IL-2/MOV19 scFv in mice inoculated s.c. with C26/α-FR. In different experiments, treatment with the fusion protein significantly reduced the tumor volume (P = 0.03-0.003) in comparison with those of both control and
ANTIFOLATE RECEPTOR scFv/IL-2 FUSION PROTEIN

LIVER  MUSCLE  METASTATIC LUNG

Fig. 4. Biodistribution of 125I-labeled IL-2/MOV19 scFv in BALB/c mice bearing experimental lung metastases of C26 (filled symbols) or C26/α-FR (open symbols). The ratio between tissue and blood %ID/g was measured in three animals at each time point (error bars, SE).

IL-2-treated mice (Fig. 6, A and B), whereas tumor onset was not significantly affected by treatment with either recombinant IL-2 or IL-2/MOV19 scFv.

DISCUSSION

Here we report the construction of a fusion protein between IL-2 and the scFv of anti-α-FR Ab MOV19 to provide a new tool for combinatorial immunotherapy of human ovarian carcinoma. IL-2/MOV19 scFv was designed to be used first in a preclinical murine model, in which it was shown to specifically target antigen-positive tumor cells and to cooperate with the antitumor immune response. To our knowledge, this is the first demonstration of biologically active fusion proteins with IL-2 and scFv directed against a tumor antigen whose therapeutic effect has been tested in murine syngeneic models (6, 32).

In designing the IL-2/MOV19 scFv fusion protein, we considered several requirements: (a) the Ab must recognize an antigenic epitope different from that of the MOV18 mAb, which is part of the bsAb OC/TR that is used to retarget T lymphocytes for adoptive immunotherapy of ovarian carcinoma patients (18), thus allowing the combination of fusion protein and OC/TR treatments; (b) the molecule should remain on the tumor cell surface on antigen binding and should not be internalized to allow IL-2 to bind its receptor on effector lymphocytes; and (c) the fusion protein should have a small size to easily penetrate into the tumor masses and should have a fast blood clearance, thereby reducing the possibility of systemic toxicity. Therefore, we chose the MOV19 mAb, which does not compete with MOV18 for binding sites and is not internalized on binding (20), to fuse its scFv sequence with IL-2. In fact, the scFv fragment displays many of the required characteristics including a better tumor microdistribution in comparison with the intact IgG, which accumulates into the perivascular regions of the tumor (33).

Different cloning strategies have been used to express fusion proteins between cytokines and antibodies or their fragments (5, 34–36). Bacterial expression has been obtained for some fusion proteins, but although it is very convenient, it can result in low yields, especially when the protein is not released into the periplasmic space but accumulates in the inclusion bodies; moreover, bacterial expression can generate endotoxins that may be difficult to remove (32, 34). Attempts to express IL-2/MOV19 scFv in bacteria failed, because no fusion protein could be rescued from the cell supernatant or periplasmic extract, suggesting that the protein was confined to inclusion bodies. Refolding of the antibodies from inclusion bodies could be obtained, but this is a cumbersome procedure whose yield can vary greatly for distinct Fv regions (32, 34, 35). Therefore, we turned to eukaryotic expression, which may offer advantages, especially in terms of posttranslational modification; moreover, the use of myeloma cells for protein production allows for a continuous manufacturing of soluble protein with a high yield (35). We found that cloning the IL-2
open reading frame with its leader sequence at the 5' end and introducing the 13-amino acid spacer GSTSGSGKSSGK to separate IL-2 from scFv represented the best configuration for a functional fusion protein, as far as antigen recognition and IL-2 bioactivity are concerned. However, the expression of such a construct driven by the cytomegalovirus promoter in J558L cells resulted in an active fusion protein but a low yield. To optimize fusion protein production, we took advantage of the pH PCRγ III vector containing both immunoglobulin H and κ enhancers and the immunoglobulin κ promoter. Moreover, by linking the fusion protein to the κ chain, we could easily purify it by affinity chromatography without introducing exogenous, potentially immunogenic sequences.

Aggregation often occurs in scFv due to the coupling of VH and VL belonging to different molecules, and this can affect the pharmacokinetics and biodistribution of the molecule, reducing its bioavailability and function. We were able to drastically reduce aggregation by the addition of 3.4 mM EDTA to both the dialyzing and storage buffers, thereby conserving the functional properties of the fusion protein.

When compared with fusion proteins with a similar structure, such as L6 F(ab')/IL-2 described by Fell et al. (5), IL-2/MOV19 scFv displayed a higher IL-2 bioactivity that was equivalent on a molar basis with that of recombinant human IL-2. This feature is presumably related to the characteristic structure of IL-2/MOV19 scFv, with the IL-2 cDNA at the 5' end of the coding sequence, and the scFv spaced by a "rigid" linker that allows a better interaction between the cytokine at the amino terminus of the fusion protein and its receptor (5).

Purified IL-2/MOV19 scFv retained the specificity of the MOV19 mAb, because it competed with MOV19 mAb for antigen binding and targeted tumor cells expressing α-FR. Whereas other fusion proteins between IL-2 and mAb or their fragments were shown to bind to the IL-2 high-affinity receptor (5, 36), thus requiring the preactivation of lymphocytes, IL-2/MOV19 scFv, once bound to the cell surface of tumors such as INT-Ov-2, stimulated the proliferation of resting lymphocytes from human donor PBLs.

The biodistribution of 125I-IL-2/MOV19 scFv injected i.p. into naive BALB/c mice or BALB/c mice bearing experimental lung micrometastases of C26 showed no specific accumulation of the fusion protein in the lungs, spleen, liver, kidneys, stomach, large and small bowel, blood, and muscle. Conversely, mice bearing lung metastases of C26/α-FR tumor cells revealed an accumulation of IL-2/MOV19 in the lungs, as evaluated by the progressive increase of the %ID/g ratio between lung and blood, thus underlining the binding specificity of IL-2/MOV19 scFv. Because only part of the lung tissue is affected by the metastatic process, the ratio of labeled fusion protein localizing to the tumor can be expected to be even higher than that indicated by the experimental results.

The very rapid pharmacokinetics of IL-2/MOV19 scFv resemble that of other scFv (33), but the decay seems to be more accelerated, probably due to the IL-2 moiety, which, thanks to its short half-life and fast clearance, reduces the t1/2 of the fusion protein, a phenomenon that has also been described for IL-2 fused with an entire immunoglobulin molecule (37, 38).

We designed an initial set of experiments in which IL-2/MOV19 scFv was directly secreted in vivo by J558EF6.1 cells injected s.c. The treatment protected 22% of mice from the development of C26/α-FR tumors inoculated in the opposite flank and, as expected, did not affect the growth of C26 tumor cells. The same treatment was shown to significantly prolong the survival of mice bearing experimental lung metastases of C26/α-FR but not that of mice bearing parental C26 (Fig. 5C). These results prompted us to use purified IL-2/MOV19 scFv for repeated i.p. injections. Although the tumor volume at different time points was significantly reduced in treated versus untreated mice (Fig. 6A) or versus IL-2-treated mice (Fig. 6B), neither of the treatments significantly affected tumor onset and progression, suggesting that IL-2 targeting by the fusion protein, but not systemic IL-2, impairs the growth of C26/α-FR without being able to cure such an aggressive tumor as a single therapeutic agent.

Reisfeld et al. (4), Xiang et al. (7), Becker et al. (39, 40), and Lode et al. (8) have extensively analyzed the therapeutic activity of fusion proteins between IL-2 and the chimeric anti-GD2 ganglioside Ab ch14.18 that recognizes melanoma and neuroblastoma tumor cells or the huKS1/4 Ab that recognizes the colon carcinoma-associated antigen Ep-CAM; using syngeneic murine models, they demonstrated that these fusion proteins were able to eradicate established TAA-transduced tumors and their experimental metastases through the activation of CD8+ T lymphocytes (4, 7, 39, 40) or natural killer cells (8). These investigators also demonstrated that the ch14.18-IL-2 fusion protein induced a CD8+ T-cell-mediated, long-lived, transferrable tumor-protective immunity in mice after the eradication of established melanoma metastases (40). In comparison with the ch14.18-IL-2 or huKS1/4-IL-2 fusion proteins, the therapeutic efficacy of IL-2/MOV19 scFv alone seems to be limited, because our reagent was not able to cure mice bearing the C26/α-FR tumor. The differences between the therapeutic efficacy of the two types of fusion proteins can be attributed mainly to the molecular structure, because ch14.18-IL-2 and huKS1/4-IL-2 are immunoglobulin fusion proteins, and IL-2/MOV19
scFv uses the scFv, thus resulting in a smaller molecule with different pharmacokinetics and biological activity. Moreover, the tumour models are different, and the different release of various immunomodulatory factors by the neoplastic cells may affect the type of antitumor effector cells activated by the IL-2/Ab fusion protein (8, 39, 40).

The reduced size of the IL-2/MOV19 scFv molecule is expected to increase its tissue penetration and reduce its immunogenicity; moreover, the lack of the Fc tail avoids the activation of nonspecific effector cells through antibody-dependent cell-mediated cytoxicity, thus favoring the IL-2-mediated activation of T lymphocytes, a feature that could be helpful when the fusion protein is administered in combination with adoptive transfer of T cell redirected by the bsAb OC/CTR.

Although successful immunotherapy in preclinical models has often been achieved with single cytokines either given systemically or released by transduced cells, in humans, treatment with IL-2 that was either systemic, secreted by ex vivo engineered cells, or secreted by in vivo gene transduction resulted in limited therapeutic effects (41–44). Combination of different immunotherapeutic approaches should therefore be considered for the treatment of established tumors.

The pharmacokinetic characteristics of the murine IL-2/MOV19 scFv fusion protein call for different approaches to overcome its short serum half-life and to prolong its therapeutic efficacy. In this regard, minipumps implanted into animals may be helpful, because the constant release of the fusion protein would mimic the continuous i.v. administration achievable in patients. Finally, to be successful in a human setting, the molecule will have to be modified to reduce its immunogenicity by using human MOV19 F(ab), whose variable regions have recently been isolated by guided selection from an antibody phage library (23).

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