Elimination of Established Murine Colon Carcinoma Metastases by Antibody-Interleukin 2 Fusion Protein Therapy

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ABSTRACT

A recombinant humanized antibody-interleukin 2 fusion protein (huKS1/4-IL-2) was used to direct IL-2 to the tumor microenvironment and elicit a T cell-mediated eradication of established pulmonary and hepatic CT26-KSA colon carcinoma metastases in syngeneic BALB/c mice. This antitumor effect was specific because a fusion protein, which was nonreactive with these tumor cells, failed to exert any such effect. The efficacy of the huKS1/4-IL-2 fusion protein in eliminating metastases was documented because mixtures of monoclonal antibody huKS1/4 with recombinant human IL-2 were ineffective and, at best, only partially reduced tumor load. Two lines of evidence indicated the eradication of metastases and the absence of minimal residual disease in animals treated with the fusion protein: first, the lack of detection of CT26-KSA cells by reverse transcription-PCR, which can detect one tumor cell in 10^6 liver cells; and second, the tripling of life span. The effector mechanism involved in this tumor eradication is dependent on T cells because the IL-2-directed therapy is ineffective in T cell-deficient SCID mice. The essential effector cells were further characterized as CD8^+ T cells by in vivo depletion studies. Such T cells, isolated from tumor-bearing mice after fusion protein therapy, elicited MHC class I-restricted cytotoxicity in vitro against colon carcinoma target cells. Taken together, these data indicate that fusion protein-directed IL-2 therapy induces a T cell-dependent host immune response capable of eradicating established colon cancer metastases in an animal tumor model.

INTRODUCTION

Colon carcinoma is the third most common cancer in both men and women in the United States and kills almost 55,000 people each year (1). Although there has been recent progress in chemotherapy and radiotherapy of this disease, the outlook for patients with metastatic disease is still dismal. Efforts were made to improve this situation by immunotherapy with murine mAb^3^ 17-1A, directed against an Ep-CAM GA733-2, overexpressed on the surface of colon carcinoma cells. The therapeutic potential of mAb 17-1A was clearly established in a randomized adjuvant therapy trial of 189 patients with resected Dukes' C colon carcinoma. A median follow-up of 5 years indicated that antibody treatment reduced the death rate by 30% and decreased the recurrence rate by 27% (2). These encouraging data were confirmed by 7-year median follow-up data, suggesting that treatment with mAb 17-1A may have actually cured those colon cancer patients with no recurrence after 7 years. Probable mechanisms proposed for this antibody-induced antitumor effect were antibody-dependent or complement-dependent cytotoxicity and phagocytosis, although more indirect mechanisms like the idiotype-anti-idiotype cascade were not ruled out (2).

The induction of a T cell-mediated antitumor response is a major goal of cancer immunotherapy. Cytokines, particularly IL-2, have been used in such efforts based on initial findings from 2 decades ago that defective T-cell activation could be restored by cytokines (3). Since becoming available in recombinant form, IL-2 has been successfully applied in high-dose immunotherapy or in combination with IL-2-activated lymphocytes in the treatment of patients with advanced melanoma or renal cell carcinoma (4). Serious dose-limiting side effects of the systemic administration of IL-2 were avoided by direct intratumoral injection of physiological doses of IL-2 that caused growth suppression of melanoma (5). More recently, another form of in situ cytokine therapy has been extended by transducing cytokine genes with viral vectors into tumor cells. Designated gene therapy, this in vivo injection of cytokine-producing tumor cells, obtained from individual patients, achieved effective local concentration of the cytokine to generate an immune response by the host's immune system and induced a local inflammatory response resulting in the elimination of the injected tumor cells. In animal models, systemic immune responses were generated in some cases against challenge with the wild-type parental tumor (6, 7).

We recently established the proof of this concept for an alternative approach with recombinant antibody-cytokine fusion proteins that combine the unique targeting ability of antibodies with the multifunctional activities of cytokines. This approach achieves sufficient local concentration of cytokines, such as IL-2, to induce a T cell-mediated eradication of established metastases of murine melanoma in syngeneic mice (8). On the basis of the encouraging clinical results obtained in the treatment of colon carcinoma with mAb 17-1A, we attempted to further extend these results by inducing a T cell-mediated antitumor response with a different recombinant fusion protein consisting of a humanized antibody, huKS1/4, recognizing an Ep-CAM identical in amino acid sequence to that detected by mAb 17-1A (9, 10).

Here, we demonstrate that the huKS1/4-IL-2 fusion protein specifically directs IL-2 to the tumor microenvironment in concentrations that are sufficient to elicit a specific, CD8^+ T cell-mediated eradication of established experimental hepatic and pulmonary metastases of KSA-expressing CT26 colon carcinoma cells in syngeneic BALB/c mice. This therapy essentially cured these mice because it more than tripled their life span, compared to animals treated with PBS, a nonspecific antibody-IL-2 fusion protein, or mixtures of rhIL-2 and huKS1/4.

MATERIALS AND METHODS

Animals, Cell Lines, and Reagents. Female BALB/c mice and BALB/c scid/scid mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained under specific pathogen-free conditions and used for experiments when they were 7 weeks old. All experiments were performed according to the NIH Guidelines for Care and Use of Laboratory Animals.

CT26, a colon epithelial tumor cell line, derived by inbreed injection of N-nitroso-N-methylurethane in BALB/c mice, was kindly provided by Dr. I. J. Fidler (M. D. Anderson Cancer Center, Houston, TX). The KS antigen (KSA...
or Ep-CAM) was cloned by PCR and expressed in CT26 colon carcinoma cells using a retroviral vector. CT26 and CT26-KSA cells were maintained in DMEM, supplemented with 5% fetal bovine serum, vitamins, L-glutamine, sodium pyruvate, and nonessential amino acids, at 37°C and 7.5% CO₂.Geneticin (1 mg/ml) was added to the transfected CT26-KSA cells. rh-IL-2 was obtained from Chiron Corp. (Emeryville, CA) and had a specific activity of 16 × 10⁶ units/mg. All reagents were free of endotoxin, as determined by the limulus amoebocyte lysate assay, purchased from BioWhittaker, Inc. (Walkersville, MD).

**Antibody and Fusion Protein.** The murine KSI/4 antibody was described previously (11). A recombinant KSI1/4-IL-2 fusion protein was humanized and re-expressed in transfected myeloma cells. Briefly, the humanized V region cDNAs were inserted into an expression vector in which the cytomegalovirus promoter drives the expression of each antibody chain. Plasmid DNA was electroporated into NS/0 murine myeloma cells, and transfectants were selected in medium containing 0.1 μM methotrexate. Expressing clones were identified by ELISA, and high producers were expanded for protein purification on protein-A-Sepharose (Repligen Corp., Needham, MA).

**Experimental Hepatic Metastases.** A single cell suspension of 3 × 10⁴ CT26-KSA tumor cells in 100 μl of DMEM was injected with a 27-gauge needle beneath the splenic capsule over a period of 60 s. This was followed by ligation of the splenic pedicle with a 4.0 silk suture and the removal of the spleen 2 min later. After 4 days, disseminated metastases were present throughout the liver tissues. All animals were sacrificed and examined for metastases 28 days after tumor cell inoculation. Metastatic scores were established based on the percentage of liver surface covered with metastases because these were fused and could not be counted as individual foci.

**Experimental Pulmonary Metastases.** CT26-KSA cells (5 × 10⁵) in 100 μl of PBS were injected slowly into the lateral tail vein over a period of 60 s. After 4 days, disseminated metastases were present throughout the lung tissues. Grossly visible metastases were detectable on the surface of the organs 28 days after tumor cell injection, at which time the animals were sacrificed. Similar to hepatic metastases, pulmonary metastases also appeared fused and were thus scored in the same manner as hepatic metastases. Livers and lungs were fixed in Bouin fixative and examined under a low-magnification microscope for tumor foci. Sections from each of these organs were stained with H&E and examined histologically. Some of the samples were snap frozen in −70°C for subsequent RNA isolation.

**Biodistribution and Binding Assay.** BALB/c mice were injected with [125I]-labeled KSI1/4-IL-2 fusion protein, as described previously (12). Experimental pulmonary and hepatic metastases were induced by i.v. and intrasplenic injection, respectively, as described above. Twelve days after tumor cell inoculation, the mice received one i.v. injection of 5 μCi of [125I]-labeled huKSI1/4-IL-2 fusion protein. Groups of four animals each were sacrificed at 4, 8, and 24 h after injection. Naïve organs and those bearing tumor metastases were removed, weighed, and assayed in a γ counter for 125I radioactivity, after which radiolocalization values were determined.

**RNA Isolation and RT-PCR Assay.** Total cellular or tissue RNA was extracted by the guanidine, phenol, and chloroform method. RNA content and purity were determined by A(260/280) readings. Synthesis of cDNA was done with 1 μg of mRNA in the presence of Moloney murine leukemia virus reverse transcriptase as follows: reverse transcription for 15 min at 42°C; denaturation for 5 min at 99°C, and cooling for 5 min at 5°C for one cycle only. A 10-μl sample of the cDNA mixture was used in a 50-μl PCR mixture for amplification of the KSA antigen. Optimal performance of the PCR process is achieved by using a 60°C annealing temperature with 35 cycles. The primers used for detection of human KSA and to create a 471-bp fragment were: sense, 5′-CAA TGC CAG TGT ACT TCA GTF-3′; and antisense, 5′-ATA AGC TAT GTA TAT GTC-3′. If amplification did not reveal any KSA signals, the cDNA integrity was tested by amplification of glyceral-3-phosphate-dehydrogenase for control. The specificity of the KSA fragment was determined by analysis with restriction enzymes ApaI and BsrXI.

**Cytotoxicity Assay.** Cytotoxic activity was measured in a standard 51Cr-release assay. CT26-KSA and CT26 target cells (3 × 10⁶) were each labeled with 0.5 mCi of ⁵¹Cr for 2 h at 37°C. The ⁵¹Cr-labeled tumor target cells (5 × 10⁴) were washed three times in DMEM, suspended in the same medium and added to each well of a U-bottomed microtiter plate. Effector cells were isolated from spleens of BALB/c mice bearing pulmonary metastases, treated 4 days after i.v. inoculation of 5 × 10⁴ CT26-KSA colon carcinoma cells with seven daily i.v. injections of 15 μg of huKSI1/4-IL-2 fusion protein. Splenocytes were incubated for 3 days at 37°C in 25 ml of complete DMEM culture medium containing 1 ml of T-STEM culture supplement (Becton Dickinson, Bedford, MA). Subsets of CD4⁺ and CD8⁺ T cells were purified by magnetic-activated cell sorting (Miltenyi, Auburn, CA). Briefly, splenocytes were labeled with paramagnetic anti-CD4 or anti-CD8 Micro Beads and separated with the Mini MACS Separation unit, according to the manufacturer’s guidelines. Purities of CD4⁺ and CD8⁺ T cells were >95%, as determined by FACS analyses. Incubation with effector cells was done at different E:T ratios at 37°C for 4 h, with the addition of 50 units of rh-IL-2 per ml. Percent specific lysis was calculated by using the formula: [(E — S)/(T — S)] × 100%, where E is the average experimental release, S is the average spontaneous release, and T is the average total release.

**In Vivo Depletion with mAb.** Rat IgG₄, antimouse CD4 (clone GK1.5) and rat IgG₄, antimouse CD8 (clone 2.43) were used for in vivo depletion of T-cell subsets. BALB/c mice were injected i.p. with 0.5 mg of anti-CD4⁺ and anti-CD8⁺-specific T-cell mAbs, respectively, on days −4, −1, 4, 9, 14, 19, and 24. FACS analyses confirmed >95% T-cell depletion before the treatment was initiated. In vivo depletion of asialo-GM1⁺ NK cells was performed by i.p. injection of 0.2 ml anti-asialo GM1 antibody (Wako Pure Chemical Industries, Japan). The experimental conditions were the same as those used for T-cell depletion studies.

**Statistical Analysis.** The statistical significance of differential findings between experimental groups of animals was determined by Student’s t test. The nonparametric Wilcoxon rank sum test was chosen when the data for life span, and metastatic scores were not amenable for parametric tests. Findings were regarded as significant if two-tailed Ps were ≤0.01.

**RESULTS**

**Characterization of the Antibody-IL-2 Fusion Protein and the Syngeneic Tumor Model.** We previously demonstrated that the fusion of IL-2 to the COOH terminus of an antibody heavy chain did not change the biological activity of IL-2 or the binding affinity of the antibody (13). We also found this to be the case for the huKSI1/4-IL-2 fusion protein. The concentration of IL-2 in the huKSI1/4-IL-2 fusion protein was calculated as two molar equivalents of IL-2 per mole of fusion protein.

The expression of KSA on CT26-KSA cells remained uniform and stable in culture for at least 40 generations and was also stable on CT26-KSA cells that were propagated as tumors in BALB/c mice (data not shown). In addition, both CT26 and CT26-KSA cells stably expressed MHC class I antigens H-2Kᵇ and H-2Dᵈ, as determined by FACS analyses (data not shown).

A comparison of mAb huKSI1/4 and its fusion protein with IL-2 by direct binding assays revealed essentially identical binding patterns with the CT26-KSA cell line. Calculation of dissociation constants (KD) and number of binding sites from saturation binding curves and Scatchard plot analyses indicated an average KD of 1.15 nM and 3.8 × 10⁸ binding sites/cell for both mAb huKSI1/4 and the huKSI1/4-IL-2 fusion protein. We established that intranslational injection of 3 × 10⁴ CT26-KSA cells and i.v. injections of 5 × 10⁴ of these cells into BALB/c mice resulted in uniformly established hepatic and pulmonary metastases, respectively, each within 4 days after tumor cell inoculation (Fig. 1).

**Therapeutic Efficacy of huKSI1/4-IL-2 against Hepatic and Pulmonary Metastases.** To establish an optimal dose of fusion protein, initial tests were done at 5-, 10-, and 15-μg daily dose levels for 7 days. These experiments indicated 15 μg as the optimal biological dose of fusion protein that completely eradicated both hepatic and pulmonary metastases of CT26-KSA cells in BALB/c mice. Thus, there were no macroscopically detectable metastases in all six mice tested and organ weights were normal, i.e., 0.99 ± 0.1 g for livers and 0.21 ± 0.05 g for lungs. A dose response was observed because a 10-μg daily dose of fusion protein eradicated both pulmonary and
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15-µg dose level, these animals could still suffer from minimal residual disease. To rule out this possibility, we demonstrated the complete eradication of hepatic and pulmonary metastases by means of two different strategies: first, detection of murine CT26-KSA cells by RT-PCR; and second, determination of the treatment’s effect on survival time.

The sensitivity of tracing murine CT26-KSA cells by detecting mRNA encoding human KSA antigen by RT-PCR was approximately 10 tumor cells in 10³ murine liver cells (Fig. 2A). When this technique was applied, tumor-bearing mice treated with either PBS or a mixture of huKS1/4 and rhIL-2 showed CT26-KSA cells to be present in the liver throughout the entire experimental period of 24 days (Fig. 2, B and D). In contrast, KSA antigen could not be detected by RT-PCR in the livers of all mice treated with the tumor-specific huKS1/4-IL-2 fusion protein 28 days after tumor cell inoculation, indicating complete eradication of metastases (Fig. 2C).

Survival studies were done to further evaluate the extent of residual disease after treatment with the antibody-IL-2 fusion protein. The mean survival time of BALB/c mice after induction of experimental hepatic metastases of CT26-KSA colon carcinoma cells was 30 days. This survival time was not significantly altered by the addition of huKS1/4 plus rhIL-2 (mean = 32 days) but was more than tripled by treatment with the huKS1/4-IL-2 fusion protein (Fig. 3). Only one of eight animals treated with the fusion protein died, on day 32, but it showed no tumor upon autopsy. After 95 days, the remaining mice still showed no overt signs of any malignant disease.

Histological Evidence of Cellular Infiltrates in Regressing Tumors. To analyze effector mechanisms involved in the observed antitumor effects, groups of mice bearing experimental hepatic metastases, induced by intrasplenic injection of CT26-KSA colon carcinoma cells, were treated 4 days thereafter with either: 1) daily i.v. injections of 15 µg of huKS1/4-IL-2 fusion protein for 7 days, a combination of huKS1/4 plus rhIL-2 at the same dose level, or PBS.

On day 15 after tumor cell inoculation, i.e., 4 days after termination of treatment, sections of tumor specimens were prepared and subjected to staining with H&E. Biopsies of livers from animals treated with the fusion protein revealed a local inflammatory response with an extensive lymphocytic infiltrate (Fig. 4C). In contrast, biopsies obtained from mice treated with PBS (Fig. 4A) or a mixture of mAb huKS1/4 with rhIL-2 (Fig. 4B) revealed only minimal lymphocytic infiltrates.

Table 1 Eradication of murine colorectal carcinoma liver and lung metastases by the huKS1/4-IL-2 fusion protein

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metastatic scores</th>
<th>Organ mass (g)</th>
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<tr>
<td>Liver metastases</td>
<td></td>
<td></td>
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<tr>
<td>PBS</td>
<td>3 3 3 3 3 3 3</td>
<td>3.78 ± 0.71</td>
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<tr>
<td>huKS1/4 + IL-2</td>
<td>2 2 2 2 2 2 3 3</td>
<td>3.74 ± 0.78</td>
</tr>
<tr>
<td>ch14.18-IL-2</td>
<td>2 2 2 2 2 2 3 3</td>
<td>3.11 ± 0.55</td>
</tr>
<tr>
<td>huKS1/4-IL-2</td>
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<td>1.07 ± 0.07</td>
</tr>
<tr>
<td>Lung metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>3 3 3 3 3 3 3</td>
<td>0.80 ± 0.07</td>
</tr>
<tr>
<td>huKS1/4 + IL-2</td>
<td>1 2 2 2 2 2 3 3</td>
<td>0.58 ± 0.18</td>
</tr>
<tr>
<td>huKS1/4 - IL-2</td>
<td>0 0 0 0 0 0 0 0</td>
<td>0.19 ± 0.02</td>
</tr>
</tbody>
</table>

* Experimental liver and lung metastases were induced by intrasplenic injection of 3 x 10⁶ or i.v. injection of 3 x 10⁶ CT26 KSA cells, respectively. Treatment was started 4 days thereafter and consisted of daily i.v. administration of PBS, 15 µg huKS1/4, and 45,000 units of rhIL-2 or 15 µg of either the nonspecific fusion protein, ch14.18-IL-2, or the tumor-specific fusion protein, huKS1/4-IL-2, as indicated for 7 consecutive days.

† Results are given as metastatic score: 0, no visible metastatic foci; 1, less than 5% of liver or lung surfaces covered with metastatic foci; 2, between 5 and 50% of liver and lung surfaces covered with metastatic foci; 3, more than 50% of liver and lung surfaces covered with metastatic foci.

‡ Differences in metastatic scores and organ weights between the fusion protein group and all control groups were statistically significant (P < 0.001).

Although metastatic lesions could not be detected by macroscopic examination of the mice following treatment with huKS1/4-IL-2 at the hepatic metastases in only four of six mice, with two animals still showing metastases at reduced levels, with >5% of lung and liver surfaces being involved. A daily dose level of 5 µg of fusion protein was even less effective because only three of six mice were tumor free and the others showed up to 50% of their lung surfaces covered with metastases (data not shown).

A series of experiments addressed the efficacy and specificity of the antibody-IL-2 fusion protein in eradicating disseminated, established hepatic metastases. Treatment of mice 4 days after tumor cell inoculation by daily i.v. administration of 15 µg of huKS1/4-IL-2 fusion protein for 7 days completely eliminated hepatic metastases in all eight animals tested (Table 1). In contrast, all control animals receiving either PBS or a mixture of huKS1/4 and rhIL-2 at equivalent dose levels suffered from extensive hepatic metastases, including >3-fold increases in liver weight. Similar results were obtained when mice bearing disseminated pulmonary metastases, established for 4 days, were treated with 15 µg of huKS1/4-IL-2 for 7 days (Table 1). The specificity of the antitumor effect of the huKS1/4-IL-2 fusion protein was established because a ch14.18-IL-2 fusion protein that was not reactive with CT26-KSA tumor cells failed to exert effective eradication of metastases (Table 1). Differences in the metastatic scores of hepatic or pulmonary metastases between mice receiving the specific fusion protein and those subjected to other treatments were statistically significant (P < 0.001).

Fig. 1. Hepatic and pulmonary metastases 4 days after inoculation of CT26-KSA colon carcinoma cells. Representative H&E-stained sections of liver (A) and lung (B), 4 days after tumor cell inoculation, are shown at a magnification of ×200.
Fig. 2. CT26-KSA tumor cell detection by KSA RT-PCR. A, detection of KSA mRNA by RT-PCR in a mixture of CT26-KSA tumor cells with murine hepatocytes at reciprocal ratios of 10^1-10^6 (Lanes 1-6). Negative controls are shown in Lane 7 (normal liver tissue) and Lane 8 (CT26 cells). Lane 9, positive control (CT26-KSA cells). Detection of KSA mRNA in liver specimens of BALB/c mice, injected intrasplenically with CT26-KSA cells, followed after 4 days by i.v. injection of 125I-labeled huKS1/4-IL-2 fusion protein (D). Lanes 1-6, liver samples; Lane 7, normal liver control; Lane 8, positive control (CT26-KSA cells). Glycerol-3-phosphate-aldehyde-dehydrogenase (GAPDH) is shown as a control, amplified with samples lacking a KSA signal.

Biodistribution of huKS1/4-IL-2. To assess whether the huKS1/4-IL-2 fusion protein can target metastases in affected organs of a syngeneic host, we determined the biodistribution of 125I-labeled fusion protein in BALB/c mice with either hepatic or pulmonary metastases of CT26-KSA colon cancer cells. Fourteen days after experimental induction of these metastases, the amount of radioactivity in livers and lungs of either naive or tumor-bearing mice was determined 4, 8, and 24 h after i.v. injection of 125I-labeled huKS1/4-IL-2 fusion protein (Fig. 5, A and B). This analysis indicated preferential localization of the tumor-specific fusion protein within the tumor-bearing organs. The actual ratio of 125I-huKS1/4-IL-2 fusion protein localizing to tumor or normal issue will be higher than observed in these experiments because only parts of these organs’ tissues are affected by metastases.

T-Cell Dependency of Fusion Protein-induced Tumor Rejection. To assess whether T cells are essential for the antitumor effect elicited by the antibody-IL-2 fusion protein, we induced experimental pulmonary metastases of CT26-KSA cells in BALB/c scid/scid mice. These mice lack functional T-cell activity, and consequently, cellular immune responses in such animals are independent of T cells. Treatment of these tumor-bearing, T cell-deficient mice with the huKS1/4-IL-2 fusion protein completely abolished its ability to eradicate pulmonary metastases (Table 2). The same results were obtained in BALB/c scid/scid mice bearing experimental hepatic metastases (data not shown). Although NK cells present in these T cell-deficient mice were not stimulated by the antibody-IL-2 fusion protein to produce an antitumor response, another experiment was done in immunocompetent BALB/c mice to assess whether NK cells are involved in the treatment effect produced by the fusion protein. In this case, BALB/c mice were depleted of NK cells in vivo by repeated treatment with antiasialo GM1 antibody. Nearly all NK cell activity was eliminated, as revealed by a 51Cr-release assay against YAC-1 target cells (data not shown). Treatment of NK cell-depleted BALB/c mice with the huKS1/4-IL-2 fusion protein still resulted in the elimination of pulmonary tumor metastases (Table 2). Together, these data strongly suggest that a T cell-dependent mechanism is responsible for the antitumor effect induced by the fusion protein. Consequently, the participation of T-cell subsets in the eradication of tumor metastases was further investigated by in vivo depletion of CD4+ and CD8+ T-cell subpopulations with specific antibodies. Such studies, done in BALB/c mice, revealed that only depletion of CD8+ T cells completely abrogated the antibody-IL-2-induced immune response. Depletion of CD4+ T cells had a partial effect (Table 2), suggesting a contributory role of CD4+ T cells in the immune response elicited by the fusion protein.

Another line of evidence indicating a major involvement of CD8+ T cells was provided by cytotoxicity studies. In this case, spleen cells isolated from BALB/c mice, following induction of pulmonary metastases and subsequent treatment with huKS1/4-IL-2, exhibited high cytolytic activity in a 4-h 51Cr-release assay against both CT26-KSA cells and their parental CT26 cells, lacking the KS antigen (Fig. 6). The major contributors to the observed cytolytic activity were enriched CD8+ T cells (Fig. 6); however, CD4+ T cells were ineffective in this regard. In addition, blocking studies with antibodies against H-2Kb/H-2Dβ antigens (clone SF1-1.1; 34-2-12 IgG2a, κ) proved that the killing of CT26 and CT26-KSA cells by either unselected primed lymphocytes or their CD8+ subset is MHC class I restricted. This cytotoxicity is specific because spleen cells from CT26-KSA tumor-bearing mice treated with the same amount of nonspecific fusion protein ch14.18-IL-2, nonreactive with this tumor, failed to elicit any specific tumor cell lysis (Fig. 6). Taken together, these data suggest that CD8+ T cells isolated from the spleens of huKS1/4-IL-2-treated mice recognize a tumor antigen(s) common to both CT26-KSA and CT26 colon carcinoma cells and independent of the transfected KSA protein.
Fig. 4. Lymphocytic infiltrate in CT26-KSA hepatic metastases of BALB/c mice treated with the huKS1/4-IL-2 fusion protein. Hepatic metastases of BALB/c mice 15 days after intrasplenic inoculation of $3 \times 10^5$ CT26-KSA cells. Biopsies were obtained from mice treated with PBS (A), a mixture of mAb huKS1/4 with rhIL-2 (B), and huKS1/4-IL-2 fusion protein. Representative H&E-stained sections are shown at a magnification of $\times 200$. 
This approach allows the maintenance of effective IL-2 concentration of rhIL-2 were injected iv. on day 4 after tumor cell inoculum, daily for 7 days, when lung metastases were fully established. Animals were sacrificed on day 28.

\[ \text{T cell-mediated immune response with an antiganglioside GD2-IL-2 concept in a syngeneic murine melanoma model by inducing a CD8@} \]

\[ \text{established metastases (8).} \]

\[ \text{DISCUSSION} \]

A key feature of our approach to cancer therapy is the use of recombinant antibody-cytokine fusion proteins to direct cytokines, like IL-2, to the tumor microenvironment and to induce a tumor-specific cellular immune response capable of tumor cell destruction. This approach allows the maintenance of effective IL-2 concentrations at the tumor site. We recently established the initial proof of this concept in a syngeneic murine melanoma model by inducing a CD8@ T cell-mediated immune response with an antiganglioside GD2-IL-2 fusion protein, ch14.18-IL-2, that was effective in eliminating established metastases (8).

Knowledge of the effect of IL-2 that is present in the tumor microenvironment on the induction of an antitumor T cell response was initially gained from in vivo experiments with tumor cells that were genetically engineered to produce IL-2 (14–17). Several reports indicated that IL-2-producing tumor cells induce an antitumor response by stimulating an MHC-class I-restricted T-lymphocyte response and that the elimination of tumor cells is critically dependent on the presence of tumor reactive T cells (14–17). In contrast to this type of therapy involving IL-2-transduced tumor cells, immunocytokine therapy directs IL-2 to the microenvironment of already established and disseminated metastases. This may change the sequence of immune reactions because the host immune system has likely confronted the tumor prior to initiation of immunomodulations by IL-2. Thus, during this time interval, T cells may have already been primed by APCs or may have migrated to the draining lymph nodes for this purpose.

In view of these considerations, fusion protein-directed IL-2 therapy may influence a successful antitumor T cell response via two different mechanisms. First, tumor cells might interact directly with naive T cells, with IL-2 acting as the second costimulatory signal in the activation of cytotoxic T cells. Activation of naive T cells may occur by a mechanism, according to a model proposed by Sprent (18), based on studies with CD8@ T cells (19–21). Accordingly, high-avidity interactions between peptide-MHC class I complexes and the TCR promote strong cross-linking of TCR-CD3 complexes on the T-cell surface. This, in turn, leads to strong signaling that stimulates secretion of IL-2 and expression of its receptors. Costimulation by IL-2 is required for boosting these TCR-mediated signals; however, if the intensity of this signaling is too low, the responding T cells express IL-2 receptors but not IL-2 and fail to proliferate unless exposed to exogenous IL-2. In our model system, exogenous IL-2 is provided for this purpose by the antibody-IL-2 fusion protein.

A second mechanism for T cell activation is based on the processing of tumor antigens by APCs. In this regard, Maass et al. (15) reported that IL-2-transduced tumor cells are eliminated from the inoculation site by macrophages and granulocytes within 48 h. These investigators proposed that APCs, which invade the tumor cell inoculation site, process tumor-derived antigens and subsequently initiate priming of tumor-specific T cells in lymphoid organs. This contention was based on the absence of T cells at the inoculation site and the finding of transcripts indicative of T cell activation in the draining lymph nodes of immunized mice. However, preactivated APCs, such as dendritic cells or macrophages, express IL-2 receptors. Thus, these cells may be activated by IL-2 provided by the fusion protein, either directly in the tumor microenvironment and/or in secondary lymphoid organs, to subsequently present tumor antigens to T cells. This hy-

Table 2 Effect of antibody-IL-2 fusion protein therapy on micrometastases in immunodeficient, NK cell-depleted, and T cell-depleted mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>In vivo depletionsa</th>
<th>Treatmentb</th>
<th>Metastatic scoresc</th>
<th>Organ mass (g)</th>
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</thead>
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<td>BALB/c scid/scid</td>
<td>None</td>
<td>PBS</td>
<td>3 3 3 3 3 3</td>
<td>0.79 ± 0.09</td>
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<td>BALB/c beige/beige</td>
<td>None</td>
<td>KS1/4 + IL-2</td>
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<td>0.65 ± 0.2</td>
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<td>BALB/c beige/beige</td>
<td>CD4+</td>
<td>KS1/4 + IL-2</td>
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<td>0.61 ± 0.09</td>
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<td>BALB/c beige/beige</td>
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<td>BALB/c</td>
<td>None</td>
<td>KS1/4 + IL-2</td>
<td>3 3 3 3 3</td>
<td>0.62 ± 0.04</td>
</tr>
<tr>
<td>BALB/c</td>
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<td>KS1/4 + IL-2</td>
<td>0 0 0 0 0</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>BALB/c</td>
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<td>PBS</td>
<td>3 3 3 3 3</td>
<td>0.63 ± 0.68</td>
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<td>NK</td>
<td>KS1/4 + IL-2</td>
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<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>BALB/c</td>
<td>CD4+</td>
<td>KS1/4 + IL-2</td>
<td>0 1 1 3 3</td>
<td>0.34 ± 0.16</td>
</tr>
<tr>
<td>BALB/c</td>
<td>CD8+</td>
<td>KS1/4 + IL-2</td>
<td>2 3 3 3 3</td>
<td>0.50 ± 0.08</td>
</tr>
<tr>
<td>BALB/c</td>
<td>CD4+ + CD8+</td>
<td>KS1/4 + IL-2</td>
<td>3 3 3 3 3</td>
<td>0.68 ± 0.11</td>
</tr>
<tr>
<td>BALB/c</td>
<td>None</td>
<td>KS1/4 + IL-2</td>
<td>0 0 0 0 0</td>
<td>0.19 ± 0.01</td>
</tr>
</tbody>
</table>

\[ a\) BALB/c mice were depleted of NK, CD4@, and CD8@ T cells or both T cell subsets by seven i.v. injections of anti-asialo GM1, anti-CD4@, or anti-CD8@ mAbs on days —4 and —1 prior to therapy and then at 4-day intervals. BALB/c beige/beige mice were also depleted of CD4@ and CD8@ T cells, respectively.

\[ b\) Mice were injected i.v. with 5 × 10^6 CT26KSA murine colorectal carcinoma cells. huKS1/4-IL-2 fusion protein (15 μg) and a mixture of 15 μCi of huKS1/4 Ab + 45,000 units of rhIL-2 were injected i.v. on day 4 after tumor cell inoculum, daily for 7 days, when lung metastases were fully established. Animals were sacrificed on day 28.

\[ c\) No visible metastatic focus; 1, <5% of lung surface covered with metastatic foci; 2, 5–50% of lung surface covered with metastatic foci; 3, >50% of lung surface covered with metastatic foci. Differences in metastatic scores and organ weights between the fusion protein group and all control groups were statistically significant (P = ≤0.001).

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tropic murine leukemia provirus (23). However, this same immunodominant peptide is apparently not recognized by CD8+ T cells.

Nonamer from the gp70 envelope protein of an endogenous ectopic gene (24). This particular antigen was derived as a nonmutated murine-associated, MHC class I-restricted peptide antigen (SPSYVYHQF). Specific CTL lines that recognized a single immunodominant tumor cell line were reported to generate several tumor-secreted GM-CSF, were found to be effective against fragment (25). On the basis of this finding, it is likely that the CD8+ T cells because their depletion markedly decreased the efficacy of immunocytokine therapy, with only two of five mice showing complete eradication of pulmonary metastases. However, the specific role of CD4+ T cells induced by huKSI/4-IL-2 fusion protein therapy in our colon carcinoma model still remains to be elucidated.

The specificity of this fusion protein-mediated eradication of established colon cancer metastases was clearly indicated by the failure of the nonspecific ch14.18-IL-2 fusion protein to produce this antitumor effect. In addition, in vitro cytotoxicity tests demonstrated that spleen cells from BALB/c mice with pulmonary metastases that were treated with the nonspecific ch14.18-IL-2 fusion protein failed to elicit any specific tumor cell lysis in vitro. In contrast, specific MHC class I-restricted lysis of both CT26-KSA and their parental CT26 cells was achieved by splenocytes and enriched CD8+ but not CD4+ T cells from tumor-bearing BALB/c mice after treatment with the huKSI/4-IL-2 fusion protein. It is of considerable interest that CT26 colon carcinoma cells lacking the transduced KSA antigen, which serves simply as a docking site for the huKSI/4-IL-2 fusion protein, were lysed in vitro by our antibody-IL-2 fusion protein. It is of interest that, with the absence (open symbols) or presence (closed symbols) of 50 μg/ml antibodies directed against H-2K"/H-2D" MHC class I antigens (clone SF1—lI: 34-2-12 IgG2a, α), CD8+ T cells isolated from tumor-bearing mice treated with ch14.18-IL-2 were also used as effector cells (×). Experiments were performed in the absence (open symbols) or presence (closed symbols) of 25 μg/ml antibodies directed against H-2K"/H-2D" MHC class I antigens (clone SF1—lI: 34-2-12 IgG2a, α), B, same experiments were performed using CT26 parental cells, lacking the KS antigen, as target cells for splenocytes and CD4+ and CD8+ T cells from animals treated with the specific huKSI/4-IL-2 fusion protein. Splenocytes from animals treated only with PBS were also used as effector cells (×). Percentage of lysis is plotted on the Y axis for various E:T ratios. Data points, mean for three mice.

Fig. 6. T cell-mediated cytotoxicity induced by antibody-IL-2 fusion proteins against CT26-KSA and CT26 tumor cells. BALB/c mice bearing pulmonary metastases of CT26-KSA colon carcinoma cells were treated 4 days after tumor cell inoculation with 15 μg of huKSI/4-IL-2 or a nonspecific fusion protein, ch14.18-IL-2, for 7 days. Splenocytes, CD4+ T cells and CD8+ T cells were isolated 4 days after cessation of therapy and analyzed for their lytic activity in a 4 h 51Cr-release assay. A, CT26-KSA cells were used as target cells for splenocytes (○), CD4+ T cells (□), or CD8+ T cells (△). Splenocytes from mice treated with ch14.18-IL-2 were also used as effector cells (×). Experiments were performed in the absence (open symbols) or presence (closed symbols) of 50 μg/ml antibodies directed against H-2K"/H-2D" MHC class I antigens (clone SF1—lI: 34-2-12 IgG2a, α), B, same experiments were performed using CT26 parental cells, lacking the KS antigen, as target cells for splenocytes and CD4+ and CD8+ T cells from animals treated with the specific huKSI/4-IL-2 fusion protein. Splenocytes from animals treated only with PBS were also used as effector cells (×). Percentage of lysis is plotted on the Y axis for various E:T ratios. Data points, mean for three mice.

ELIMINATION OF COLON CARCINOMA BY IL-2 FUSION PROTEIN

REFERENCES


Elimination of Established Murine Colon Carcinoma Metastases by Antibody-Interleukin 2 Fusion Protein Therapy

Rong Xiang, Holger N. Lode, Carrie S. Dolman, et al.


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