Augmentation of Antitumor Activity of an Antibody-Interleukin 2 Immunocytokine with Chemotherapeutic Agents

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ABSTRACT

Purpose: Immune-based therapies, such as the immunocytokine huKS-IL2, exert potent antitumor responses in some animal models by targeting cytokine activity to the tumor microenvironment. We found that certain chemotherapy agents in the appropriate dose and schedule can augment the antitumor activity of huKS-IL2.

Experimental Design: Chemotherapy agents were given in a single dose followed 1 day (paclitaxel) or 3 days (cyclophosphamide) later with five daily doses of huKS-IL2 in mice bearing established s.c. tumors, liver metastases, or lung metastases. Tumor models used were CT26/KSA colon, 4T1/KSA mammary, or LLCKSA Lewis lung carcinomas. To measure huKS-IL2 distribution, radiolabeled protein was given to CT26/KSA tumor-bearing mice 1 or 24 h after paclitaxel. huKS-IL2 levels in the tumors were evaluated.

Results: Both paclitaxel and cyclophosphamide followed by huKS-IL2 resulted in enhanced antitumor responses compared with either of the treatments alone in the three different tumor models. Results from studies to determine whether the role of the cytotoxic agents in antitumor activity enhancement was related to tumor uptake indicated that a larger fraction of the radiolabeled huKS-IL2 penetrated the tumors when it was administered 24 h after cytotoxic drug "sensitization."

Conclusion: These data support the idea that prior drug therapy serves to decompress the tumor and lower the diffusion barrier for macromolecules, thus allowing for increased uptake of the huKS-IL2 immunocytokine into the tumor microenvironment. Because the toxicity of the immunocytokine is relatively low at optimal doses, the therapeutic index would likely be greater with the combination treatments.

INTRODUCTION

Antibody-cytokine fusion proteins ("immunocytokines") exert potent antitumor responses in animal models by targeting cytokine activity to the tumor microenvironment (1, 2). One example is huKS-IL2, which is composed of an antibody that binds to the epithelial cell adhesion molecule (EpCAM, or KS) antigen, fused to IL-2.[2] EpCAM is present on human tumor cells of epithelial origin, *i.e.,* carcinomas, which comprise approximately 80% of all solid tumors (3). *In vitro* analyses have shown that huKS-IL2 retains the ability to bind to tumor cells, mediate antibody-dependent cellular cytotoxicity, and stimulate proliferation of IL-2 receptor-bearing cells. Furthermore, treatment of animals bearing colon carcinoma tumors with a single 5–6-day course of huKS-IL2 induces long-lived protective immunity (4, 5).

Subsequently, we have found that large established s.c. tumors are more difficult than disseminated metastases to treat with a single cycle of huKS-IL2. Unfortunately, multiple courses of treatment in mice are precluded by antibody responses against the human immunocytokine (6). Given these limitations in animal tumor models, we have tested other ways to enhance the immunotherapeutic effects of single-cycle therapy, including combination with established chemotherapeutic drugs. Standard chemotherapy regimens using many different cytotoxic agents, such as alkylating agents, taxanes, purine and pyridine analogues, or *Vinca* alkaloids, are generally assumed to be immunosuppressive by killing or inhibiting cell proliferation in dividing cells including lymphocytes (7, 8). In fact, cyclophosphamide is commonly used as an immunosuppressive agent in allogeneic organ transplantation and for treatment of certain autoimmune diseases. Despite clinical use as an immunosuppressive agent, a large body of evidence has demonstrated that some chemotherapeutic agents have immunopotentiating properties; however, the drug dose, route, and time of administration are critical parameters that can influence the outcome (9). We will demonstrate that certain chemotherapeutic agents can act as "sensitizers," when given in the appropriate dose and schedule, further enhancing the antitumor activity of single-cycle huKS-IL2 therapy.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. 4T1, a murine mammary carcinoma cell line, was kindly provided by Dr. Paul Sondel (University of Wisconsin, Madison, WI). LL/2 (LLC), a murine Lewis lung carcinoma cell line, was purchased from the American Type Culture Collection (Rockville, MD). CT26, a murine colon epithelial cell line derived by intrarectal injection of *N*-nitroso-*N*-methylurethane in BALB/C mice, was kindly provided by Dr. Isaiah J. Fidler (M. D. Anderson Cancer Center, Houston, TX). 4T1, LLC, and CT26 cells were transfected with

[2] The abbreviations used are: IL, interleukin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium.

the human KS antigen (KSA or EpCAM), which was cloned by PCR and expressed in parental cells using a retroviral vector as described earlier (2). 4T1/KSA cells were maintained in RPMI, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, penicillin/streptomycin, and Geneticin (Life Technologies, Inc.) at 37°C and 7% CO_2. LLC/KSA cells were maintained in DMEM, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, penicillin/streptomycin, and Geneticin (Life Technologies, Inc.) at 37°C and 7% CO_2. CT26/KSA cells were maintained in DMEM, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, vitamins, sodium pyruvate, nonessential amino acids, penicillin/streptomycin, and Geneticin (Life Technologies, Inc.) at 37°C and 7% CO_2. Geneticin was added to the transfected cells to maintain KSA expression.

Drugs. huKS-IL2 was manufactured by DSM Biologicals (Montreal, Quebec, Canada). Formulated material was stored at −20°C. For animal studies, huKS-IL2 was thawed and stored at 4°C for less than 1 month. For dosing, an aliquot of formulated material was removed from stock vials, diluted with saline, and injected into animals within 1 h. Unused diluted material was discarded. Paclitaxel and cyclophosphamide were obtained from Sigma Chemical Co. (St. Louis, MO). Paclitaxel was dissolved in 50% ethanol:50% Cremophor RH40 (BASF, Parsippany, NJ). Immediately before injection, the paclitaxel stock solution was diluted 1:5 in saline. Cyclophosphamide was dissolved in saline.

Animal Studies. LLC/KSA tumors were implanted in C57BL6 mice. 4T1/KSA tumors and CT26/KSA tumors were implanted in BALB/C mice. All of the mice were obtained from Taconic (Germantown, NY).

Tumor Growth Assay. For tumor growth studies, either LLC/KSA or CT26/KSA tumors were implanted s.c. on the backs of mice. For LLC/KSA studies, tumors were transplanted from several stock tumors that had been injected with a single cell suspension of 1×10^6 cells in 100 μl of PBS. After about 2 weeks, tumors were aseptically collected and passed through a sieve fitted with a 150-μm screen. Cells were then passed through a syringe and 23-gauge needle, washed twice, and resuspended in PBS. A single-cell suspension of 1×10^6 LLC/KSA cells in 100 μl of PBS was injected s.c. using a 30½-gauge needle. For CT26/KSA studies, cells growing exponentially in culture were injected as a single cell suspension of 1×10^6 cells in 100 μl of PBS. After tumors had become established, about 2 weeks after implantation, dosing was initiated on day 0. Tumors were measured with calipers in three dimensions twice weekly. Tumor volumes were calculated using the equation:

$$Volume = ½ \times 4/3 \ \pi \ (L/2 \times W/2 \times H)$$

where L = length, W = width, and H = height of the tumor.

Animals were weighed, and general health was monitored during the course of the assay. If tumors became necrotic or if animals became moribund, they were euthanized by CO_2 asphyxiation. Data are presented in graphic form. Graphs depict the average tumor volumes during and after dosing. Data are also expressed as the percentage of control reduction of average tumor volumes from treated mice relative to vehicle-treated mice. Student's two-tailed t test was performed on the individual

tumor volumes to determine significant differences between treatment groups.

Experimental Lung Metastases. For experimental lung metastases studies, a single cell suspension of 2.5×10^5 4T1/KSA cells in 100 μl of PBS was slowly injected using a 27½-gauge needle into the lateral tail vein on day 0. Approximately 3 weeks after inoculation, the animals were sacrificed, and their lungs were removed and weighed. The lungs were then fixed and stained in Bouin's solution (Sigma Chemical Co.). Data are presented in graphic form depicting average tumor burdens at the time of sacrifice. Tumor burden was determined by subtracting the weight of a normal lung from the weight of the experimental lungs. Data are also expressed as the percentage of control reduction of average tumor burden from treated mice relative to vehicle-treated mice. Student's two-tailed t test was performed on the individual tumor burdens to determine significant differences between treatment groups. If animals became moribund, the animals were euthanized by CO_2 asphyxiation.

Experimental Hepatic Metastases. For experimental hepatic metastases studies, mice were anesthetized using 80 mg/kg ketamine hydrochloride (Fort Dodge Animal Health, Fort Dodge, IA) and 5 mg/kg xylazine (Bayer, Shawnee Mission, KS). A single cell suspension of 1×10^5 CT26/KSA cells in 100 μl of DMEM containing 25 mM HEPES (Life Technologies, Inc.) was injected using a 27½-gauge needle beneath the splenic capsule over a period of 60 s on day 0. After another 2 min, the splenic vessels were cauterized with a cautery unit (Roboz, Rockville, MD) and the spleen was removed. Animals were sutured using autoclips. Approximately 3 weeks after inoculation, the animals were sacrificed, and their livers were removed and weighed. The livers were then fixed and stained in Bouin's solution (Sigma Chemical Co.). Data are presented in graphic form depicting average tumor burdens at time of sacrifice. Tumor burden was determined by subtracting the weight of a normal liver from the weight of the experimental livers. Data are also expressed as the percentage of control reduction of average tumor burden from treated mice relative to vehicle-treated mice. Student's two-tailed t test was performed on the individual tumor burdens to determine significant differences between treatment groups. If animals became moribund, the animals were euthanized by CO_2 asphyxiation.

Tissue Distribution Assay. huKS-IL2 was labeled with ^{125}I by standard procedures through contract to a commercial vendor (New England Nuclear, Boston, MA). Skin tumors of CT26/KSA were implanted s.c. as described above and allowed to grow until they reached 100–200 mm^3. Two groups of four mice received injections with either paclitaxel (50 mg/kg) in vehicle or vehicle alone, followed in 1 h (experiment 1) or 24 h (experiment 2) by 10 μg of ^{125}I-labeled KS-IL2 (95 μCi). Six h after injecting the radiolabeled immunocytokine, the mice were sacrificed and their tumors were surgically removed. As a control, livers of the animals were also collected, and all of the tissues were weighed and then counted in a gamma counter. Results are expressed as the cpm/g of tissue by dividing the total cpm in the tissue by the weight.

MTS Cytotoxicity Assay. For determining *in vitro* cytotoxicity, cells were seeded at 1000 cells/well in 96-well flat-bottomed plates and incubated for 24 h at 37°C, 7% CO_2.

Paclitaxel (at 2-fold dilutions ranging from 3.125 ng/ml to 200 ng/ml), huKS-IL2 (at 200 ng/ml), and IL-2 (at 33.3 ng/ml; the equivalent amount of IL-2 in huKS-IL2) were added in duplicate to the cell plates and incubated for 6 days at 37°C, 7% CO_2. The MTS colorimetric assay (Promega, Madison, WI), a measure of cell viability based on the cellular conversion of a tetrazolium salt, was performed directly in the 96-well plates. After plates were read and recorded, viable adherent cells were stained with Crystal violet (Sigma Chemical Co.). Crystal violet-stained plates were used to verify MTS assay results. Results are expressed in tabular form. The IC_{50} is the concentration of drug that produced viability at a level of 50% of control.

RESULTS

Combination Therapy. The effects of the widely used chemotherapeutic agents, paclitaxel or cyclophosphamide, on huKS-IL2 activity were determined by administering both agents to mice in tumor growth assays, experimental lung metastasis assays, or liver metastasis assays. The chemotherapeutic agent was administered first, with a delay of either 1 or 3 days depending on the drug, followed by five daily doses of huKS-IL2. Our experience has shown that CT26/KSA tumors are quite sensitive to huKS-IL2, so only 7–10 μg/dose was used, whereas 4T1/KSA and LLC/KSA are less sensitive to huKS-IL2; therefore, higher doses of 15–30 μg were used. Furthermore, small metastatic tumor nodules growing in a well-vascularized organ in a lung or liver metastasis assay tend to be more sensitive to therapy than large tumors implanted s.c. in a tumor growth assay; therefore, for the same tumor type, a higher dose of huKS-IL2 was used in a tumor growth assay than in a lung or liver metastasis assay; *e.g.,* for 4T1/KSA, 30 μg/dose was used in a tumor growth assay, whereas only 15 μg/dose was used in a lung metastasis assay. The goal was to moderately reduce tumor burden by <50% with huKS-IL2 alone to observe additive or greater than additive interactions between huKS-IL2 and chemotherapy agents.

Paclitaxel and huKS-IL2. The interaction of paclitaxel, a microtubule stabilizer, with huKS-IL2 was examined in a tumor growth assay. Mice with established s.c. CT26/KSA colon carcinoma tumors received injections with paclitaxel (75 mg/kg) 1 day before a single 5-day course of huKS-IL2 (10 μg/mouse/day). Under these conditions, paclitaxel had no activity, and huKS-IL2 alone decreased the average tumor volume by only 29% on day 21 (Fig. 1). However, the combination treatment significantly reduced tumor volume by 92% compared with paclitaxel or huKS-IL2 alone ($P \leq 0.01$).

Next, we tested the same dosing strategy in a more relevant liver metastasis model to see whether we could achieve a similar effect against disseminated disease. Mice with established CT26/KSA metastases were treated with 25, 50, or 75 mg/kg of paclitaxel alone or in combination with 7 μg of huKS-IL2 for 5 days 1 day after paclitaxel. huKS-IL2 alone reduced the tumor burden by 66%. A dose response was observed for paclitaxel alone, in which 25, 50, or 75 mg/kg resulted in reductions of tumor burden by 51%, 77%, or 90%, respectively (Fig. 2). Combining paclitaxel with huKS-IL2 further reduced lung metastases by 88%, 91%, or 94% for the same respective doses of
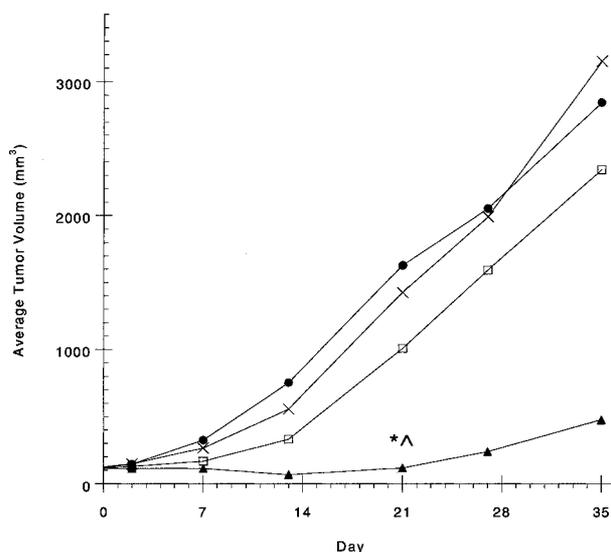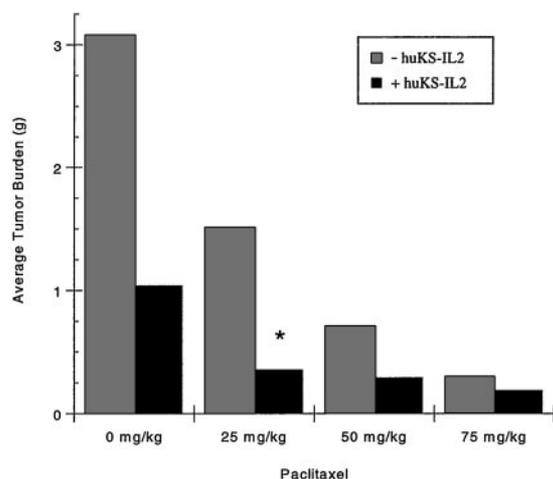

Fig. 1 Antitumor activity after combination therapy of huKS-IL2 with paclitaxel in a CT26/KSA murine colon carcinoma tumor growth assay. Mice bearing established s.c. tumors were treated with paclitaxel (75 mg/kg, i.v.) on day 0 alone (●), huKS-IL2 (10 μg/mouse, i.v.) on days 1–5 alone (□), both paclitaxel and huKS-IL2 (▲), or vehicle (×). On day 21, the average tumor volume ±SD for the vehicle-treated group was 1428 ± 540 mm³. The chart depicts the average tumor volumes of seven mice/group from one representative experiment. ∗∧, significant differences in tumor volumes of the combination treatment group compared with the paclitaxel-alone group ($P = 0.0012$) and the huKS-IL2-alone group ($P = 0.010$).

paclitaxel. Paclitaxel at the lowest dose of 25 mg/kg in combination with huKS-IL2 resulted in significant ($P < 0.01$) and the greatest reduction in tumor burden compared to the higher doses of paclitaxel with huKS-IL2. However, the higher doses of paclitaxel alone were rather efficacious in this model, so additional significant increases may be difficult to measure when added to huKS-IL2. The lowest dose of paclitaxel in combination with huKS-IL2 resulted in similar antitumor efficacy as the highest dose of paclitaxel alone; hence, good efficacy with lower toxicity can be achieved with the combination treatment.

Cyclophosphamide and huKS-IL2. The effect of cyclophosphamide, an alkylating agent that is commonly used for the treatment of breast cancer, was tested in a breast tumor growth assay in combination with huKS-IL2. Mice with established tumors of 4T1/KSA mammary carcinoma were treated with a single dose of 80 mg/kg cyclophosphamide, either alone or in combination with five daily doses of huKS-IL2 (30 μg) 3 days after the cyclophosphamide treatment. The increased interval (3 days *versus* 1 day) between cyclophosphamide chemotherapy and huKS-IL2 treatment was based on previous combination studies (10) with IL-2 in various animal models. Average tumor volumes for huKS-IL2 and 80 mg/kg of cyclophosphamide alone were reduced by 31% and 69%, respectively (Fig. 3). The combination treatment reduced average tumor volumes by 100% on day 25, which was significantly different from either huKS-IL2 alone or cyclophosphamide alone ($P < 0.05$) and completely eliminated tumors in six of eight mice up to 12 weeks after the initial treatment. Animals tolerated these treat-
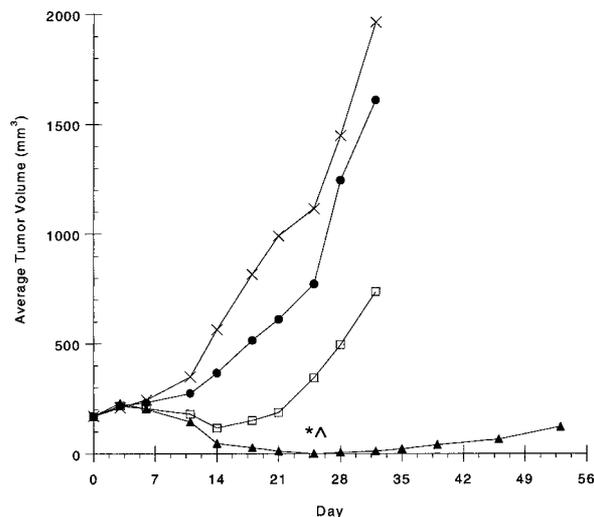
Fig. 2 Antitumor activity after combination therapy of huKS-IL2 with paclitaxel in a CT26/KSA murine carcinoma tumor liver metastasis assay. Mice that received injections with CT26/KSA tumor cells on day 0 were treated either with paclitaxel (25, 50, or 75 mg/kg, i.v.) on day 5 alone (*light gray bars*) or in combination with huKS-IL2 (7 μg/mouse, i.v.) on days 6–10 (*dark gray bars*). On day 20, the average tumor burden ±SD was 3.1 ± 1.3 g. The chart depicts the average tumor burdens of seven mice/group from one representative experiment. ∗, a significant difference ($P = 0.0017$) in the average tumor burdens of the combination treatment group compared with the paclitaxel alone group. The P for the combination group compared with the huKS-IL2 group is 0.16.

Fig. 3 Antitumor activity after combination therapy of huKS-IL2 with cyclophosphamide in a 4T1/KSA murine mammary carcinoma tumor growth assay. Mice bearing established s.c. tumors were treated with cyclophosphamide (80 mg/kg, i.p.) on day 0 alone (□), huKS-IL2 (30 μg/mouse, i.v.) on days 3–7 alone (●), both cyclophosphamide and huKS-IL2 (▲), or vehicle (×). On day 25, the average tumor volume ± SD for the vehicle-treated group was 1294 ± 618 mm³. The chart depicts the average tumor volumes of eight mice/group from one experiment. ∗∧, a significant difference in the average tumor burdens of the combination treatment group compared with the cyclophosphamide-alone group ($P = 0.033$) and the huKS-IL2-alone group ($P = 0.0048$).

ments well with less than 10% weight loss observed in all of the groups (data not shown).

The interaction of cyclophosphamide with huKS-IL2 on antitumor activity was also tested in a lung metastasis assay. Mice were implanted with 4T1/KSA mammary carcinoma cells followed with a single dose of 15, 40, or 100 mg/kg cyclophosphamide, either alone or in combination with five daily doses of huKS-IL2 (15 μg) 3 days after the cyclophosphamide treatment. Average tumor burdens for 15, 40, or 100 mg/kg of cyclophosphamide alone were reduced by 14%, 30%, or 76%, respectively, and for the same doses of cyclophosphamide in combination with huKS-IL2 were reduced by 63%, 94%, or 84%, respectively (Fig. 4, *A* and *B*). The low dose of huKS-IL2 alone had little to no effect in these experiments (0% to 19% reduction of tumor volume). Treatment with huKS-IL2 significantly reduced the tumor burden in combination with 40 mg/kg cyclophosphamide compared to either treatment alone ($P < 0.001$). Animals tolerated these relatively low doses of cyclophosphamide well, with less than 10% weight loss observed in all of the groups (data not shown).

Because cyclophosphamide has been used for several years in the treatment of non-small lung carcinoma, another EpCAM-positive tumor type, we examined its possible combination effect with huKS-IL2 in a syngeneic tumor model. The effect of a moderate dose of cyclophosphamide on the antitumor activity of huKS-IL2 was tested in a tumor growth assay in which LLC/KSA lung carcinoma tumor-bearing mice received injections with 100 mg/kg of cyclophosphamide on day 0, followed 3 days later by 20 μg of huKS-IL2 for 5 days. Treatment with huKS-IL2 alone significantly reduced the average tumor volume

by 48% ($P < 0.05$) on day 21 (Fig. 5). Combining huKS-IL2 with cyclophosphamide further reduced the average tumor volume by 88%, which was significantly different from huKS-IL2 ($P < 0.01$) but not for cyclophosphamide ($P = 0.055$) treatment alone. In this case, none of the treatments was curative. However, two of seven mice from the combination treatment of huKS-IL2 and cyclophosphamide remained alive at day 50. The mice tolerated all of the treatments well with no drug-related deaths and no average weight loss during treatments (data not shown).

Thus, a single low to moderate dose of cyclophosphamide followed 3 days later by huKS-IL2 treatment further reduced tumor growth beyond that achieved with cyclophosphamide treatment alone in both 4T1 murine mammary models and Lewis lung tumor models. We did not try longer time intervals between the two therapies that might allow for immune recovery before huKS-IL2 treatment.

Mechanism of Action. Administering chemotherapeutic agents of different classes, such as cyclophosphamide or paclitaxel, in combination with the immunocytokine huKS-IL2 was shown to increase the antitumor activity compared with either agent alone *in vivo*. We hypothesized that these results might be explained by either additive or synergistic toxicity of the two agents or increased delivery and uptake of huKS-IL2 in tumors. These possible mechanisms were tested by: (*a*) examining whether there was a direct cytotoxic effect of the combination treatments of paclitaxel and huKS-IL2 *in vitro* and (*b*) examining whether paclitaxel increased uptake of radiolabeled huKS-IL2 into the tumor relative to other tissues.
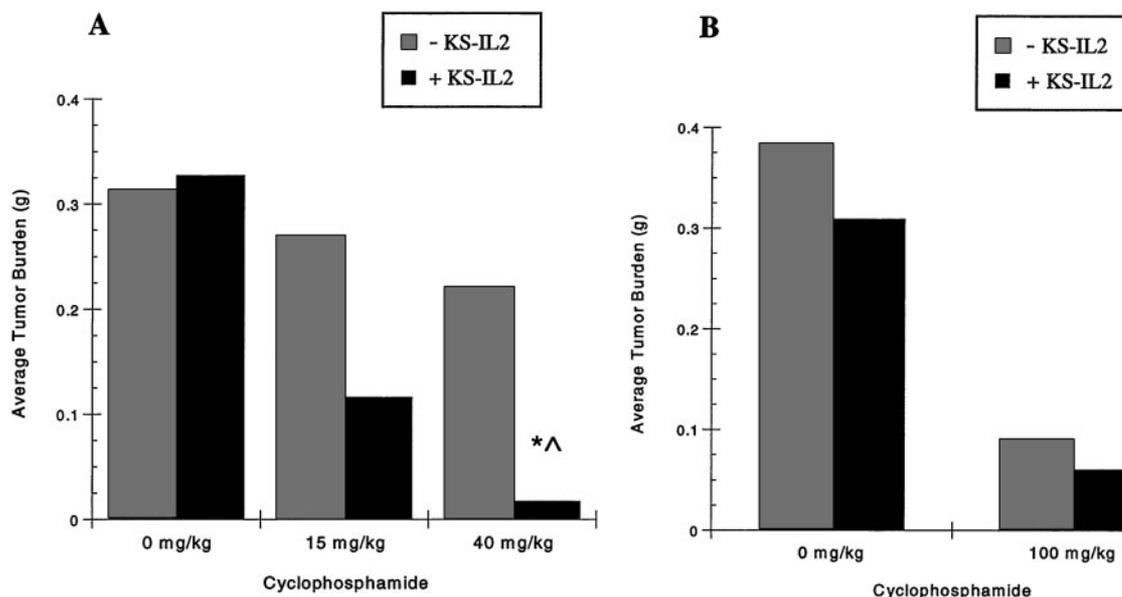
Fig. 4 Antitumor activity after combination therapy of huKS-IL2 with cyclophosphamide in a 4T1/KSA murine mammary tumor lung metastasis assay. Mice that received injections with 4T1/KSA tumor cells on day 0 were treated either with (*A*) cyclophosphamide (15 or 40 mg/kg, i.p.) on day 3 alone (*light gray bars*) or in combination with huKS-IL2 (15 μg/mouse, i.v.) on days 6–10 (*dark gray bars*) or with (*B*) cyclophosphamide (100 mg/kg, i.p.) on day 3 alone (*light gray bars*) or in combination with huKS-IL2 (15 μg/mouse, i.v.) on days 6–10 (*dark gray bars*). On day 21, the average tumor burden ±SD was 0.31 ± 0.22 g in *A* and 0.38 ± 0.21 in *B*. Charts depict the average tumor burdens of five mice/group from one representative experiment. ∗∧, a significant difference in the average tumor burdens of the combination treatment group compared with the cyclophosphamide-alone group ($P = 0.00040$) and the huKS-IL2-alone group ($P = 0.000001$).

In Vitro **Cytotoxicity.** A direct cytotoxic effect of the combination of paclitaxel and huKS-IL2 was tested using a cytotoxicity assay in which paclitaxel (3–200 ng/ml) alone or with huKS-IL2 (200 ng/ml) or IL-2 (33.3 ng/ml; the equivalent amount of IL-2 in huKS-IL2) was incubated with CT26/KSA, LLC/KSA, and 4T1/KSA cells. An MTS assay was performed to determine cell viability. There was little to no cytotoxicity of huKS-IL2 or IL-2 alone on any of the three cells lines tested (Table 1). There was also little to no effect of paclitaxel with the addition of either huKS-IL2 or IL-2 on the cytotoxicity of the three cell lines. Therefore, because neither huKS-IL2 nor IL-2 affected the cytotoxicity of paclitaxel directly, any augmentation in antitumor activity in mice by the combined treatments was because of physiological or immune mechanisms.

Tissue Uptake of huKS-IL2. When radiolabeled huKS-IL2 was injected 1 h after paclitaxel treatment (Fig. 6*A*), only a small increase in the amount of radioactivity was seen in the excised tumors from animals receiving the drug. In contrast, when labeled huKS-IL2 was injected 24 h after paclitaxel treatment, a dramatic increase in uptake was seen (>350%) relative to the vehicle control (Fig. 6*B*). This great difference in tumor uptake between the 1-h and 24-h time points is in agreement with the data on taxane-induced changes in interstitial pressure (11) and is consistent with the data in our tumor models showing that treatment beginning 24 h after paclitaxel was effective in enhancing antitumor activity. We have also found that treatment with cyclophosphamide 3 days before injection of radiolabeled huKS-IL2 similarly enhanced tumor uptake (data not shown). In both cases, the percentage increase was far greater than what was seen in the liver (data not shown), eliminating the possibil-
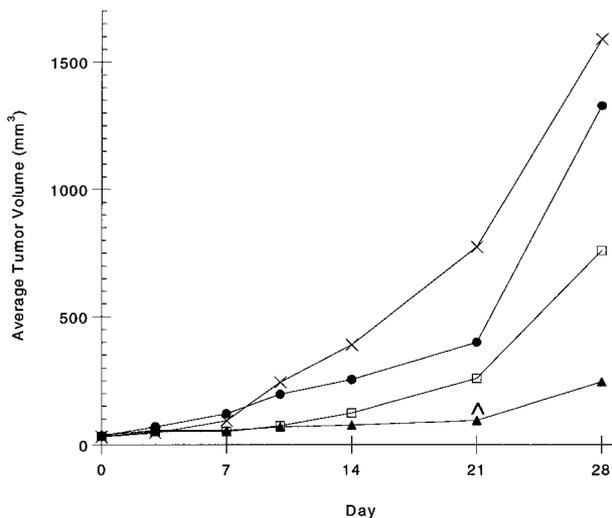

Fig. 5 Antitumor activity after combination therapy of huKS-IL2 with cyclophosphamide in a LLC/KSA murine lung carcinoma tumor growth assay. Mice bearing established s.c. tumors were treated with cyclophosphamide (100 mg/kg, i.p.) on day 0 alone (□), huKS-IL2 (20 μg/mouse, i.v.) on days 3–7 alone (●), both cyclophosphamide and huKS-IL2 (▲), or vehicle (×). On day 21, the average tumor volume ±SD for the vehicle-treated group was $802 ± 207$ mm^3. The chart depicts the average tumor volumes of seven to eight mice/group from one representative experiment. ∧, a significant difference ($P = 0.0020$) in the average tumor burdens of the combination treatment group compared with the huKS-IL2-alone group. *P* for the combination group compared with the paclitaxel group is 0.055.

Table 1 In vitro cytotoxicity of paclitaxel ± IL-2 or huKS-IL2

	IC$_{50}$ (ng/ml)^a		
	CT26/KSA	LLC/KSA	4T1/KSA
Paclitaxel	20	6	16
Paclitaxel + IL-2^b	20	7.5	20
Paclitaxel + huKS-IL2^c	15	5	19
	Percentage of control		
IL-2	99	101	81
huKS-IL2	99	100	95

^{a} IC$_{50}$ is the concentration of paclitaxel where viability is 50% of that observed in the control using the MTS assay.

^{b} IL-2 concentration was 33.3 ng/ml, which is equivalent to the amount of IL-2 in 200 ng/ml of huKS-IL2.

^{c} huKS-IL2 concentration was 200 ng/ml.

ity that the effect of the drugs was to increase the level of radioactivity in the blood pool, including that of the tumor.

DISCUSSION

We propose that certain cytotoxic chemotherapy agents, such as paclitaxel and cyclophosphamide, "sensitize" the tumor to the tumoricidal effects of the immunocytokine in the tumor microenvironment. Data from the current studies demonstrated that the antitumor activity of huKS-IL2 was augmented with the administration of either paclitaxel or cyclophosphamide before huKS-IL2; therefore, the augmentation may be drug-independent. Because the enhanced activity of the combination treatments was observed in murine non-small cell lung, mammary, and colon carcinoma tumor models, it may be tumor nonspecific.

One possible mechanism for the enhancement of antitumor activity is that pretreatment with paclitaxel increases the uptake of huKS-IL2 into tumors as shown by the tissue distribution studies. As has been reported in the literature, administering a taxane improves tumor vascular parameters such as decreased interstitial fluid and microvascular pressure via tumor cell apoptosis, increasing mean tumor vessel diameter within several days after taxane treatment (11). This supports the idea that prior drug therapy serves to decompress the tumor and lowers the diffusion barrier for macromolecules, thus allowing for increased uptake of the huKS-IL2 immunocytokine into the tumor microenvironment. Therefore, paclitaxel may improve delivery of huKS-IL2 because of improved vascular parameters in addition to its microtubule stabilization activity in tumor-bearing animals, thus explaining the augmented antitumor activity observed in our experimental tumor models. The idea that increased access of huKS-IL2 to tumors should result in improved activity is somewhat intuitive but also supported by our earlier studies using an isotypic variant of huKS-IL2 containing a γ 4 heavy chain constant region. This study demonstrated both improved tumor targeting and improved efficacy using this longer-lived form of the same immunocytokine (12).

Other investigators have demonstrated augmentation of antitumor activity by combining cyclophosphamide with IL-2 therapy using various schedules and doses; however, ours is the

first report demonstrating this effect using a tumor-targeted form of a cytokine. A decrease in tumor burden was observed when cyclophosphamide was followed by IL-2 treatment (cyclophosphamide, 100–150 mg/kg; and IL-2, 6,000–24,000 IU) in murine 109 lung, MCA-105 mammary, and 3LL lung carcinoma tumor models (13–15). An increase in survival time was also noted when IL-2 treatment followed cyclophosphamide (cyclophosphamide, 75–200 mg/kg; and IL-2, 7,000–24,000 IU) in MCA-105, H-2b sarcoma, M109, H-2d carcinoma, and P185 mastocytoma tumor models (10, 14, 16, 17).

Another possible explanation for the enhancement of antitumor activity in the studies mentioned above and our study described herein is that certain chemotherapeutic agents can act as immunomodulators. For example, taxanes augment macrophage activity by inducing IL-12, induce cytokine production, and enhance T-cell proliferative responses; low doses of cyclophosphamide improve cell-mediated immunity; cisplatin augments macrophage activity; doxorubicin stimulates cytokines, complement-dependent antibody cell killing, and can augment macrophage and CTL activity (7, 8, 9, 18). Additional studies will be needed to examine what effect of the combination of chemotherapy with immunocytokine treatment has on immune effector cells *in vivo*. Although the authors of the studies, described above, generally attribute their results to enhanced immune stimulation by inhibition or elimination of immune suppressive T cells, our studies provide a novel enhancing effect because of improved delivery of cytokine to the tumor itself.

It is possible that both mechanisms are acting in our system: first, increased delivery of huKS-IL2 into the tumor, and, second, enhanced huKS-IL2 activity attributable to the increased presence of immune effector cells. A careful balance would be needed between reducing tumor interstitial pressure with moderate or low dose chemotherapy and maintaining the (enhanced) immune functions required for huKS-IL2-mediated tumor destruction. Consequences of enhanced uptake of IL-2 in the tumor microenvironment have not yet been studied mechanistically in the context of immunocytokine therapy. However, previous work (1) in which effective huKS-IL2 treatment was associated with increased lymphocytic infiltration in CT26/KSA hepatic metastases, compared with a noneffective combination of huKS antibody and rhIL-2, suggests the importance of cytokine localization as the key to the successful initiation of an antitumor response. The longer residence time of IL-2 as a result of enhanced antibody targeting would be expected to provide a sustained signal for T-cell costimulation at the same time as tumor antigens are being presented, either directly by the tumor cells or indirectly by tumor-associated macrophage and dendritic cells.

One can only imagine how much more difficult a barrier exists in large bulky tumors growing in the skin. As we have shown in this report, even large tumors could be effectively treated using chemotherapeutic combinations that significantly increased uptake of huKS-IL2 into the tumor. Fortunately, this effect was achieved at chemotherapeutic doses that were not immunosuppressive and perhaps even immunostimulatory. Thus far, we have not been able to dissociate the relative contributions of the two enhancements, immune potentiation and increased targeting. Studies are currently underway to address this.
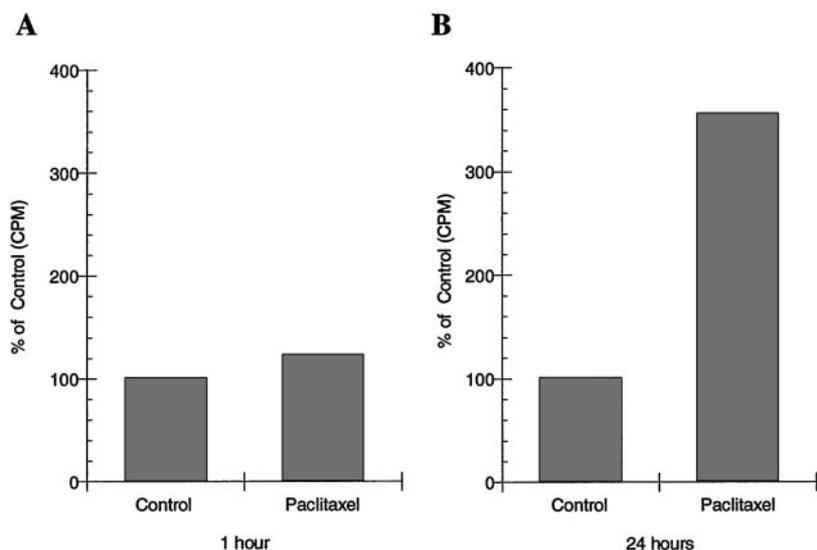
Fig. 6 Uptake of ^{125}I-labeled huKS-IL2 in CT26/KSA tumors after treatment with paclitaxel. Mice bearing established s.c. tumors were treated either with huKS-IL2 (10 μg/mouse, i.v.) alone or with paclitaxel (50 mg/kg, i.v.) followed by huKS-IL2. *A*, ^{125}I-labeled huKS-IL2 uptake in the tumor 1 h after paclitaxel treatment; *B*, ^{125}I-labeled huKS-IL2 uptake in the tumor 24 h after paclitaxel treatment. Each chart depicts the percentage relative to controls of the average cpm/g of tissue from four to five mice from one experiment.

Clinical studies with huKS-IL2 combined with either cyclophosphamide or paclitaxel are being planned for several tumor indications. It remains to be determined whether the same additive or synergistic effects observed in our preclinical models will be observed. The combination of low-dose cyclophosphamide (350 mg/m^2) followed by IL-2 (21.6 × 10^6 IU/m^2/day × 5 days/week × 2 week/course by IV bolus) has been tested in the clinic in melanoma, renal cancer, and breast cancer. Responses were observed for melanoma and breast cancers, but the necessity of the addition of low-dose cyclophosphamide to IL-2 therapy has not yet been established (7). Perhaps the use of targeted huKS-IL2 in combination protocols will more clearly differentiate between the treatment arms in such trials as it has in our studies.

In summary, both paclitaxel and cyclophosphamide were able to amplify the antitumor activity of huKS-IL2 in murine lung, mammary, and colon carcinoma tumor models. Similar results obtained with two different chemotherapy agents and three different tumor models suggest that the augmentation of antitumor activity may be drug-independent and tumor nonspecific. The targeting of huKS-IL2 to established tumors was increased after paclitaxel treatment. Our data support the hypothesis that prior drug therapy serves to decompress the tumor and lowers the diffusion barrier for macromolecules, thus allowing for increased uptake of the huKS-IL2 immunocytokine into the tumor microenvironment. An even greater augmentation of antitumor activity may be achieved if optimal doses of huKS-IL2 are used. Because the toxicity of the immunocytokine is relatively low at optimal doses, the therapeutic index would likely be greater with the combination treatments. On the basis of these data, clinical trials using these promising therapies are currently being planned.

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