Elimination of Established Liver Metastases by Human Interleukin 2-activated Natural Killer Cells after Locoregional or Systemic Adoptive Transfer

Kazuhiko Okada, Ulf Nannmark, Nikola L. Vujanovic, Simon Watkins, Per Basse, Ronald B. Herberman, and Theresa L. Whiteside


ABSTRACT

An in vivo model of liver metastasis induced by human gastric carcinoma was established in nude mice and used for locoregional or systemic immunotherapy with a subset of human A-natural killer (NK) cells defined previously. A single intrasplenic (i.s.) delivery of A-NK cells (1 x 10^7) and interleukin 2 (IL-2; 60,000 international units, twice a day for 5 days, i.p.) to animals with 3-day established liver metastases, but not IL-2 alone, resulted in rapid (within 24 h) elimination of the majority of metastases and significantly improved survival. A single i.s. or i.v. transfer of these effector cells and IL-2 significantly prolonged survival of the mice with 3-day established metastases (P < 0.03 and P < 0.02, respectively) compared with untreated mice. Using 51Cr-labeled A-NK cells, it was found previously. A single intrasplenic (i.s.) delivery of A-NK cells that we have established lends itself well to these studies.

INTRODUCTION

AIT of cancer with activated lymphoid cells and cytokines has been evaluated extensively in recent years (1, 2). To gain insights into mechanisms responsible for in vivo antitumor effects, both syngeneic and xenogeneic tumor models of AIT and a variety of effector cells and cytokines have been used (3, 4). We have developed a xenograft model of liver metastasis using human gastric carcinoma cells and a subset of human IL-2-activated NK cells called A-NK cells (5). In this model, locoregional (i.s.) AIT with A-NK cells and IL-2 for mice bearing established liver metastases resulted in a rapid elimination of metastases and prolonged survival of the treated animals (5). The promising therapeutic results consistently observed with 3- or 7-day established liver metastases of gastric carcinoma point to the potential clinical application of A-NK cells for elimination of liver metastases.

To gain insights into the antitumor effects of these cells, we have performed more detailed investigations in this tumor model, with a focus on the distribution of the adoptively transferred effector cells and their interactions with microvascular and tumor cells.

A number of factors may be involved in determining a favorable outcome of AIT in a liver metastasis model. Among the parameters most frequently considered are the nature and characteristics of effector cells, their ability to localize to tumor metastases and to eliminate tumor cells in vivo, and the extent to which regression of metastases leads to prolongation of survival. Human A-NK cells have been shown to possess several attributes that make them a particularly attractive population of effector cells for AIT compared with CTLs, widely considered to be the most desirable antitumor effector cells (6, 7). In contrast to CTLs, A-NK cells are non-MHC-restricted effectors and recognize tumor cells without the need for prior sensitization; they can be generated readily from peripheral blood of most individuals, including cancer patients (8, 9); they proliferate extensively in vitro in the presence of IL-2, yielding potent antitumor effector cells, which seem not to harm normal tissue cells (9); and, by virtue of being able to produce and release a wide spectrum of cytokines and growth factors (10), A-NK cells may not only modify the tumor microenvironment but also ultimately contribute to the generation of more effective, long-lasting antitumor responses. In a syngeneic murine model of pulmonary metastases B16 melanoma, A-NK cells have been shown to selectively localize to the sites of metastasis (3). Finally, the significant prolongation of survival observed with human A-NK cells, as a result of elimination of the vast majority of liver metastases within 24 h of AIT in our experimental model, suggests that locoregional effects of the therapy with A-NK cells may translate into a clinically meaningful benefit (5).
account for elimination of metastases and, ultimately, prolonged survival of the animals. Our data indicate that modest and not necessarily selective localization of A-NK cells to liver metastases seem to be sufficient for the therapeutic efficacy of these effector cells delivered either locoregionally or systemically to animals with established liver metastases.

MATERIALS AND METHODS

Cell Lines. The HR cell line was established in our laboratory from a hepatic metastasis of gastric cancer, as described (11). The HRLZ cell line was established by introducing the lacZ gene construct pRCmCMLZII into HR cells as described previously (3) and maintained in DMEM (GIBCO, Grand Island, NY) supplemented with G418 (200 µg/ml) and containing 10% (v/v) fetal bovine serum (GIBCO). The line was periodically screened for β-Gal activity, using X-Gal staining and flow cytometry (3). K562 and Daudi cell lines were maintained and passaged as described previously (12). All cell lines were periodically tested for mycoplasma, using a Gene Probe (San Diego, CA) kit, and found to be negative.

Nude Mice. Six-week-old BALB/c female nude mice were obtained from Taconic Farms (Germantown, NY) and were produced under contract from the National Cancer Institute. The mice were maintained under pathogen-free conditions in a laminar air flow room.

Generation of Hepatic Metastases. To abolish NK activity of nude mice and to facilitate establishment of metastases, the animals were treated with cyclophosphamide (200 mg/kg; Sigma Chemical Co., St. Louis, MO) and antiasialo GM1 antibody (0.2 mg/mouse; WAKO, Dallas, TX) by i.p. injections 1 day prior to tumor injection. The injections of antiasialo GM1 antibody were continued twice weekly throughout the course of each experiment. These injections were intended to eliminate endogenous murine NK cells, and they did not have adverse effects on adoptively transferred human A-NK cells. A persistent lack of detectable NK activity was observed when the splenocytes obtained from these mice were tested in 4-h 51Cr-release assays. Under general anesthesia, using Methoxyflurane (Pitman-Moore, Mundelein, IL), mice were positioned on the right side, and a 0.5-cm-long incision was made in the left subcostal region. The spleen was exposed, and a 0.2-ml aliquot of HR or HRLZ tumor cells (1 X 10^7) suspended in HBSS (GIBCO) was slowly injected into the spleen with a 27-gauge needle.

Culture of IL-2-activated NK (A-NK) Cells. Human NK cells were purified from peripheral blood mononuclear cells by negative selection on antibody-coated magnetic beads as described by us previously (9). Purified NK cells at 1 X 10^6 cells/ml were incubated in RPMI 1640 (GIBCO) tissue culture medium containing 10% (v/v) human AB serum and 6000 IU/ml recombinant IL-2 (Chiron Corp., Emeryville, CA). A-NK cells were selected by adherence to plastic (9) and then cultured in tissue culture medium in the presence of mitogen-primed stimulator, irradiated, allogeneic PBL feeder cells as described earlier (9). The cultures were harvested on day 14 of growth, checked for cytotoxic activity against K562, Daudi, and HR cells, and used for in vivo AIT experiments.

Flow Cytometry. The phenotype of fresh or cultured NK cells was determined by two-color flow cytometry as described by us previously (9). Briefly, cells were adjusted to a concentration of 1 X 10^6/ml in 0.1% (v/v) sodium azide-PBS solution, and 0.2 ml of this cell suspension was incubated with 5 µl of various fluorescein- or phycoerythrin-labeled monoclonal antibodies at 4°C for 30 min. The cells were then washed three times in PBS-sodium azide and fixed in 2% (w/v) paraformaldehyde solution in PBS. Two-color analysis was performed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The monoclonal antibodies used were purchased from Becton Dickinson and included the following specificities: Leu4 (anti-CD3); Leu19 (anti-CD56); and Leu16 (anti-CD11a). As controls, mouse IgG1 and IgG2 isotypes were used in all experiments.

Cytotoxicity Assays. The cytotoxicity of human effector cells was determined in 4-h miniaturized 51Cr-release assays as described earlier (12). Briefly, 1 X 10^6 target cells labeled with 51Cr (5 µCi/ml; New England Nuclear, Boston, MA) were plated in triplicate in wells of a 96-well V-bottom plate (Costar, Cambridge, MA) and mixed with effector cells at E:T ratios ranging from 12:1 to 1:5:1. Cells were centrifuged at 1000 rpm for 5 min and incubated for 4 h at 37°C in a CO2 incubator. The amount of 51Cr released into the supernatant (20 µl) was measured using a beta counter (LKB Instruments, Gaithersburg, MD). Spontaneous and maximal radioisotope release were determined in wells containing target cells in culture medium alone or after the addition of 5% (v/v) Triton X-100 (Sigma), respectively. The percentage of specific lysis was determined as:

\[
\% \text{ specific lysis} = \frac{\text{mean experimental cpm} - \text{mean spontaneous cpm}}{\text{mean maximal cpm} - \text{mean spontaneous cpm}} \times 100
\]
overnight. The specimens were postfixed in 1% OsO₄ in 0.05 M sodium cacodylate (1 h), dehydrated, and infiltrated with Agar 100 epoxy resin. Thin sections (1 μm) for light microscopy were cut, stained, and examined in a Nikon FXA microscope. Light microscopic sections were photographed and mapped for transmission electron microscopic sectioning. Ultrathin sections of specific areas were cut in a Reichert Ultracut E microtome and stained with lead citrate and uranyl acetate. Examinations of the sections were performed in a Zeiss (Oberkochen, Germany) CEM 902 electron microscope.

**Locoregional AIT with A-NK Cells.** To establish liver metastases, mice were injected i.s. with HR or HRLZ tumor cells as described above. Three or 7 days later, mice were anesthetized, and the spleens were exposed. Aliquots of A-NK cells (1 × 10⁷ in 0.2 ml) plus IL-2 (or HBSS in control animals) were injected i.p. Groups of animals were sacrificed at various times after AIT, depending on the experiment, as described in “Results.” Livers were recovered for visual examination and image analysis following staining with X-Gal or anti-human CD45 antibody or for histological and electron microscopic examinations. In experiments with ⁵¹Cr-labeled effector cells, various organs were recovered and weighed, and the number of cpm/organ was determined in a gamma counter.

In survival experiments, IL-2 or HBSS (in controls) was administered i.p. to groups of mice receiving AIT or control animals. The latter received i.p. injections of 0.1 ml HBSS twice daily for 5 days, whereas experimental groups were treated with IL-2 at 60,000 IU in 0.5 ml HBSS twice daily for 5 days. During the period of therapy, all mice were treated with antiasialo GM1 antibody (0.2 μg/mouse; WAKO) by i.p. injections twice a week to eliminate endogenous NK activity. To determine survival, the animals were maintained until death or sacrificed on day 62 after therapy. Each survival experiment was performed with groups of three to five animals, depending on the availability of tumor cells or A-NK cells at the time of the experiment. The mortality rate due to surgical procedures was <2%.

**Systemic AIT with A-NK Cells.** Groups of 4 animals with established 3-day liver metastases were treated i.v. with A-NK cells (1 × 10⁷) and IL-2. A-NK cells were resuspended in 0.2 ml RPMI medium containing IL-2 (6000 IU/ml) and injected through the tail veins into the animals. Subsequently, IL-2 was administered i.p. at 60,000 IU twice daily for 5 days, as described above. Survival of groups of mice treated systemically was compared with that of mice treated i.s.

**Detection and Quantitation of Liver Metastases by X-Gal Staining.** To visualize metastases of HRLZ cells in liver tissue, nude mice bearing 3-day established liver metastases were sacrificed at 4, 8, 16, or 24 h after AIT with A-NK cells and IL-2. Livers were recovered, cut into individual lobes, embedded in the OCT medium (Miles, Inc., Elkhart, IN) and stored frozen at −70° C. Cryostat sections (7 μm thick) were cut and fixed with 2% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde in PBS for 5 min at room temperature. Liver sections were then incubated for 24 h at 4° C in a staining solution containing 1 mg X-Gal/ml, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.02% (v/v) NP40, and 0.01% (w/v) sodium deoxycholate (15). The stained cryosections were washed in distilled water and coverslipped, using an aqueous mounting medium (Immuno-Mount; Shandon, Pittsburgh, PA), and the number of blue-stained metastases was determined by image analysis. Using a low-power objective (X2), an image of 14 mm² in a visual field was acquired by an Optimas image analysis system. The threshold was set so that all blue metastases could be recognized and any other structures present in the tissue could not. The number and mean area of blue-stained metastases in the image was measured, using an Optimas image analysis program. Three different sections, each of a different liver lobe, per animal were analyzed, and the mean number of metastases/liver section and the mean total area in mm² of metastases/liver section were determined, always using the same area (14 mm²) for these counts.

**Statistical Analysis.** The significance of differences between experimental and control groups was analyzed using Student’s t test, Wilcoxon’s signed rank test, or the Kruskal-Wallis test, as appropriate. The level of significance was set at P < 0.05.

**RESULTS**

**Cultures of A-NK Cells.** Human A-NK cells were cultured for AIT under conditions described previously (14). At the time of cell harvest for AIT, A-NK cells were routinely examined by phenotypic and functional characteristics. At the minimum, flow cytometry was performed to determine the purity of cells, as were ⁴-h ⁵¹Cr-release assays against HR, K562, and Daudi targets. A-NK cell preparations were highly enriched in CD3⁻CD56⁺ cells (>90%) and mediated vigorous in vitro antitumor cytotoxicity ranging from 2800 to 3000 LU against HR and from 3100 to 3700 LU against HRLZ targets in the series of experiments reported here.

**Kinetics of the Elimination of 3-Day Liver Metastases by A-NK Cells and IL-2.** In the 3-day metastasis model, A-NK cells delivered i.s. plus IL-2 given i.p. to tumor-bearing mice were able to significantly decrease the number of liver metastases (5). The process of metastasis elimination was very rapid, and by 24 h, when the liver was obtained for studies, few remaining metastases were detectable, using a sensitive X-Gal staining method for lacZ-labeled tumor cells (5). A series of additional experiments was performed to determine how soon after a single locoregional delivery of A-NK cells a significant reduction in the lacZ gene-labeled metastases could be demonstrated. As shown in Fig. 1, the i.s. delivery of 1 × 10⁷ A-NK cells and 60,000 IU IL-2 i.p. resulted in a decrease of the number of liver metastases within 16 h after therapy. Because not only the number but also the size of the metastases could be reduced as a result of A-NK cell transfer, quantitative image analysis was performed to determine both of these parameters at 4, 8, 16, and 24 h following AIT. Control animals were treated with HBSS or IL-2 alone, and because no significant differences in the numbers or sizes of the metastases were observed between these control groups (data not shown), they were eliminated overnight.
combined to give a single control group of eight animals. The results of this experiment indicated that relative to controls, AIT with A-NK cells and IL-2 resulted in the reduction in the number and in the size of liver metastases, which was first observed at 16 h and became statistically significant at 24 h after therapy (Fig. 1).

**Systemic Delivery of A-NK Cells in the 3-Day Liver Metastasis Model.** Previous results showed that i.s. AIT with A-NK cells and IL-2 for 7-day established metastases significantly prolonged survival of the treated mice (5). To determine whether systemic delivery of A-NK cells might be similarly effective in prolonging survival, mice bearing 3-day liver metastases were treated intravascularly (through the tail vein) with a single dose of $1 \times 10^7$ A-NK cells and a 5-day course of IL-2 given i.p. twice daily. As shown in a representative experiment (Fig. 2), the survival of mice treated either systemically or locoregionally with AIT was significantly prolonged ($P < 0.026$ and 0.034, respectively) in comparison to untreated mice, all of which died by day 35. On day 62, the surviving treated mice were sacrificed, and their livers were examined for evidence of macrometastases. In comparison to livers of mice that died, those from the sacrificed animals had fewer visible metastases, and areas free of metastases were detectable in some of the liver lobes (data not shown). These results indicate that systemic AIT with A-NK cells and IL-2 delivered i.p. has efficacy in prolonging survival of the mice comparable to that of i.s. AIT.

**Distribution of $^{51}$Cr-labeled A-NK Cells after i.s. or i.v. Transfer.** In the experiments described above, a relatively small number of transferred A-NK cells ($1 \times 10^7$) were found to have a significant impact on reducing the number and size of liver metastases and prolonging survival. To determine the proportions of A-NK cells that localized to the liver after locoregional or systemic adoptive transfer, short-term in vivo experiments with $^{51}$Cr-labeled A-NK cells and IL-2 were performed. As shown in Fig. 3, both in control (non-tumor-bearing) mice (Fig. 3A) and in mice with 7-day established metastases (Fig. 3B), ~70% of $^{51}$Cr label was recovered at 30–240 min in the livers of animals treated locoregionally with labeled A-NK cells. Only about 25% of the total counts injected were recovered in the spleen. A similar distribution was observed in the experiments with animals bearing 3-day metastases (data not shown). In contrast, the distribution of i.v.-administered A-NK cells seemed different in control and tumor-bearing animals (Fig. 3, C and D). At 30 min after i.v. injection, 65% of A-NK cells were found in the lungs, and only 25% were found in the livers in control mice, whereas in animals with 3-day metastases, these proportions were 80% and 15%, respectively. At 4 h after systemic delivery, close to 80% of radioactivity was recovered in the lungs, and only 5% was recovered in the lungs in controls, whereas only 50% of the total counts were recovered in the livers, and almost 40% were still present in the lungs of tumor-bearing animals (Fig. 3, C and D; $P < 0.05$), an indication that much slower redistribution between lungs and livers occurred in animals with 3-day established metastases than in controls. In these distribution experi-

![Fig. 2. Survival curves for nude mice with 3-day established liver metastases (groups of four animals) treated i.s. or i.v. with single injections of $1 \times 10^7$ human A-NK cells and IL-2 given i.p. at 60,000 IU twice daily for 5 days. The Kruskal-Wallis test was used to calculate significance of the data. $P$ values are for comparisons of control and experimental groups.](cancerres.aacrjournals.org)
mements, we routinely recovered 80-90% of total cpm injected, and the percentage of radioactivity recovered in the spleen never exceeded 10% of the total. These results allowed us to conclude that in animals with established liver metastases, 50-75% of total counts are recoverable from the liver 4 h after systemic or locoregional transfer of radioactively labeled A-NK cells. Thus, even after intravascular injection of effector cells, up to 50% of the cells seem to reach the liver 4 h later.

**Numbers of Transferred A-NK Cells in the Liver.** Radioactivity counts performed with $^{35}$Cr-labeled A-NK cells injected i.s. or i.v. into mice provided data on their distribution among various organs, including the liver. To determine the number of effector cells present in the liver at 30 min and 24 h after transfer, Di-O-labeled A-NK cells were injected systemically or locoregionally into mice with 3-day metastases. Using image analysis, doubly labeled A-NK cells were enumerated (green fluorescence and blue Hoechst nuclear dye) in liver tissue sections (Fig. 4A). Double labeling was necessary to be able to distinguish with confidence A-NK cells from fluorescent cell fragments or nonspecific background staining, which is common with liver tissues. As shown in Table 1, the number of cells present in the liver at 24 h after transfer approached that of injected cells ($10^6$ A-NK cells), regardless of the route of their delivery.

**Histology of 3-Day Liver.** In sections of fixed liver tissues obtained from animals with 3-day liver metastases established by i.s. injection of $1 \times 10^7$ tumor cells, intravascular accumulations of tumor cells were common (Fig. 5A). Nevertheless, in most cases, these intravascular metastases were beginning to spread into the liver tissue on day 3 after i.s. injection (Fig. 5B). Aggregates of tumor cells in the liver parenchyma near blood vessels (Fig. 5B) and single tumor cells among the hepatocytes (Fig. 5C) were detectable throughout the liver. Within 12-24 h after AIT of these 3-day established metastases with A-NK cells and IL-2, by light microscopy of liver tissue, it was possible to observe the process of "dissolution" of intravascular tumor cell occlusions and of resumption of the normal flow of blood elements in blood vessels of the liver (Fig. 5, D and E). However, at the level of light microscopy, it was not possible to identify with certainty A-NK cells in these vessels.

**Localization of Rhodamine- or Di-O-labeled A-NK Cells in the Liver with Established Metastases.** To confirm the presence of A-NK cells in regressing liver metastases, rhodamine-labeled A-NK cells were used for AIT, as described previously (16). After $1 \times 10^7$ rhodamine-labeled A-NK cells were injected i.s. into the livers of mice bearing 3-day metastases, the distribution of these cells in liver tissue between 12 and 24 h after transfer was studied by confocal microscopy. This time window was selected because it corresponded to the optimal time for metastasis reduction, as determined in experiments with the lacZ gene-labeled tumor cells (Fig. 1). Using confocal microscopy, labeled A-NK cells were detectable in the liver 12-24 h after their locoregional adoptive transfer. However, no selective accumulations of A-NK cells in association with intravascular or extravascular metastases were observed. Instead, single-labeled A-NK cells scattered in liver tissue, and especially evident in the close proximity to the blood vessels (Fig. 4E) and among tumor cells in "dissolving" intravascular tumor cell aggregates (Fig. 4F), were consistently observed by confocal microscopy throughout the liver tissue at 12-24 h after locoregional AIT. A-NK cells were detectable in most but not all intravascular tumor cell aggregates, and at 12-24 h after AIT, only few labeled cells (often one or two) were in direct contact with tumor cells. These experiments demonstrate that some A-NK cells are able to interact with tumor cells in situ, supporting the hypothesis that such interactions might be important for therapeutic effects.

Confocal microscopy with rhodamine-labeled A-NK cells also permitted us to more closely examine the spatial orientation and shape of A-NK cells, which entered into tissue and were interacting with liver tissue cells. Examining these cells by fluorescence microscopy 12 h after transfer, we were impressed by the characteristic appearance of cells in motion (see Fig. 4B). Three-dimensional reconstruction of optical sections from confocal microscopy confirmed this impression, at the same time providing an image of A-NK cells in tissue with several long appendages extending into intercellular spaces and making contact with surrounding tissue cells (Fig. 4C). These observations are consistent with our previous in vitro data of A-NK cells being able to enter tumor spheroids and to migrate in between the tumor cells, with the concomitant destruction of the spheroid structure and death of tumor cells (14).

It has been reported by Basse et al. (3) that differential distribution of adoptively transferred murine A-NK cells between normal tissue and pulmonary metastases and selective accumulation of these cells to metastases requires a period of about 24 h. We, therefore, considered a possibility that an equally long or longer time period may be necessary for human A-NK cells transferred to the liver to begin to accumulate in metastases. To be able to follow localization and tissue distribution of A-NK cells in the liver 24-48 h after locoregional or intravascular adoptive transfer, we resorted to a lipophilic dye, Di-O, known to be more stable than rhodamine and applicable to studies of lymphoid cell trafficking in tissues (17). Preliminary labeling experiments of human A-NK cells with Di-O indicated that a brief (5-min) labeling period at room temperature allows for preservation, lasting for up to 3-4 days, of most of their functional characteristics, including antitumor toxicity, proliferation in response to IL-2, and migration into tumor spheroids (data not shown). When injected i.s. or i.v. into animals with 3- or 14-day established liver metastases and examined 24 h later, Di-O-labeled effector cells were found to be scattered throughout liver tissue, without any evidence for selective accumulation to metastases. Nevertheless, as shown in Fig. 4D, at least one or two effector cells were generally detectable even in the very large metastases. The same results were obtained by immunostaining, using CD45 staining to detect human A-NK cells in murine liver tissue (data not shown). These observations again indicate that A-NK cells are capable of entering metastases and that the presence of even a few of these cells in direct contact with tumor cells seems to be associated with beneficial therapeutic effects observed in this animal model.

**EM of A-NK Cells in Liver Tissue.** To be able to examine in a greater detail interactions between A-NK cells and tumor cells in situ, EM was performed. Twelve to 24 h following locoregional delivery of A-NK cells to liver tissues with 3-day established metastases, tissue samples were harvested and prepared for transmission EM. The liver specimens examined by EM were the same as those used for studies of rhodamine-labeled A-NK cells and for light microscopy. As shown in Fig. 6, A-D, A-NK cells were seen at various locations in liver tissue as well as metastases. For example, in Fig. 6A, an A-NK cell is located between tumor cells in a microvessel, presumably as a result of migration along the luminal surface of endothelial cells. A number of tumor cells in contact with A-NK cells seemed to be disorganized in their ultrastructure and showed morphological changes consistent with both apoptotic and necrotic cell death (Fig. 6B). A-NK cells were also capable of leaving the blood vessels, migrating through the basal membrane, and entering the surrounding tissues (Fig. 6C). At 24 h after AIT, A-NK cells could still be seen inside the blood vessels, probably in those from which tumor cells had been mostly eliminated (Fig. 6D). The A-NK cell shown in Fig. 6D is in mitosis, which indicates that by 24 h after transfer, these cells are able to replicate in situ.

In addition, tumor cells adjacent to or in contact with A-NK cells seemed to be undergoing apoptosis, as evidenced by EM, which
Fig. 4. Confocal microscopy of liver sections harvested 12-24 h after AIT with A-NK cells and IL-2. A, distribution of human A-NK cells doubly labeled with Di-O (green) and Hoechst dye (blue) in liver tissue 24 h after adoptive transfer by i.v. injection. The mice had established 3-day liver metastases. Fluorescence microscopy; ×300. B, rhodamine-labeled A-NK cell migrating through liver tissue. Fluorescence microscopy; ×200. C, confocal microscopic reconstruction of three rhodamine-labeled A-NK cells in the liver with 3-day metastases obtained 12 h after AIT with A-NK cells and IL-2. Note the bizarre shape and long cellular appendages. Arrows point to two of three A-NK cells. ×200. D, immunofluorescence microscopy showing adoptively transferred human A-NK cells labeled with a lipophilic dye, Di-O, around or inside a 14-day liver metastasis (arrows). The animals were sacrificed at 24 h after intravascular AIT with A-NK cells and i.p. IL-2. ×400. E, rhodamine-labeled human A-NK cells (1 x 10^7) were injected i.s. in nude mice bearing 3-day established metastases. The animals were sacrificed 12 h later. A-NK cells are seen scattered throughout liver tissue and around a blood vessel (arrows) ×500. F. A-NK cells (arrows) are seen among tumor cells inside a blood vessel. The plug of tumor cells seems loose and in the process of destruction. ×500.
ELIMINATION OF LIVER METASTASES BY HUMAN NK CELLS

Table 1  Number of Di-O-labeled A-NK cells in the livers of mice with established 3-day liver metastases

<table>
<thead>
<tr>
<th>Time after injection of A-NK cells</th>
<th>Route</th>
<th>Cell no./0.113 mm²</th>
<th>Estimated total cell no./liver (x10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>i.v.</td>
<td>3.0 ± 0.5</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>24 h</td>
<td>i.s.</td>
<td>4.8 ± 0.8</td>
<td>6.1 ± 1.1</td>
</tr>
<tr>
<td>24 h</td>
<td>i.v.</td>
<td>8.2 ± 2.5</td>
<td>10.4 ± 3.1</td>
</tr>
</tbody>
</table>

identified numerous morphological changes (cell shrinking and apoptotic bodies) consistent with apoptosis in liver tissue of mice treated with A-NK cells and IL-2 (Fig. 7).

DISCUSSION

In this article, we examined several parameters that seem to be important for therapeutic efficacy of human A-NK cells following their administration to nude mice with established (3- or 7-day) liver metastases of human gastric carcinoma. On transfer to the sites of tumor metastasis and in the presence of IL-2, this subset of human NK cells has been observed to cause a rapid and dramatic decrease in the number of established liver metastases and significant improvement in survival of the treated animals (5). Although these results have been impressive, they provide no clues regarding possible mechanisms or factors, such as the route of delivery, number, or ability of effectors to localize to tumors or their functional attributes, that might determine the therapeutic efficacy of A-NK cells in this model. In view of the data obtained with A-NK cells by Basse and collaborators (3) in a syngeneic B16 melanoma metastasis model in mice, in which selective accumulations of these cells in metastases were observed, we expected that the therapeutic effects of transferred A-NK cells and IL-2 will be accompanied by selective localization and accumulation of the effector cells at the sites of liver metastases. In contrast to these studies with murine A-NK cells, however, neither locoregional (i.e., i.s.) nor systemic (i.e., i.v.) transfer of human effector cells to animals with established liver metastases resulted in accumulation of effector cells in metastases even at 24 h after AIT. We found that under conditions that were associated with nearly complete elimination of metastases and prolonged survival, only small numbers of transferred effector cells were found in metastases.

For years, it has been controversial whether adoptively transferred, activated lymphoid cells accumulate at the sites of tumor metastasis, and whether such accumulation is necessary for therapeutic effects (3, 18–21). The ability of effector cells to extravasate, migrate through tissue, and localize to metastases is thought to determine, to a large extent, their antitumor efficacy. However, it could be argued that i.v.-transferred effector cells must cross the endothelial cell barrier and migrate into the tissue to eradicate tumor cells or tumor metastases (22). Studies with various effector cells, lymphokine-activated killer cells, tumor-infiltrating lymphocytes, and A-NK cells, all indicated that only a small fraction, at best 5–10% of the i.v.-transferred effector cells, localize at the tumor site (3, 23, 24). The vast majority of infused cells is eliminated on reaching the first capillary bed, and only a few seem to reach more distant organs (23). Thus, the likelihood of these effector cells reaching tumor metastases in the downstream capillary beds seems to be limited. Based on this type of reasoning, it is not unexpected to observe that even CTLs, which are considered capable of specific recognition of the tumor or its metastases, have been variously reported to migrate and accumulate (24, 25) or not to selectively home to the tumor after i.v. transfers to humans or experimental animals (26, 27). Considering the therapeutic benefits of AIT, it seems that a small number of antitumor effector cells might be effective in eliminating established metastases, provided their functional antitumor attributes in situ are sustained or enhanced by, e.g., IL-2, secondary cytokines, or the presence of tumor cells (28). Clearly, the mere presence of an excess of effector cells in the tumor metastases does not always result in elimination of the tumor or improved survival, as observed in the syngeneic B16 melanoma model by Basse et al. (3). In contrast, in two different tumor xenograft models in nude mice, one a liver metastasis (as reported here) and the other squamous cell carcinoma of the head and neck (as reported previously), a small number of human A-NK cells transferred locoreg-
Fig. 6. Transmission EM of human A-NK cells in the liver with 3-day established metastases 12-24 h after i.s. therapy with A-NK cells and IL-2. A, intravascular A-NK cell is wedged in between two tumor cells (TU) at 12 h after i.s. delivery. The A-NK cell has extended processes, penetrating the sinusoidal lining (arrowhead). On both sides, the A-NK cell is in close contact with the tumor cells. ×3000. B, A-NK cell in direct contact with a tumor cell that is in the process of lysis 24 h after i.s. injection of effector cells and IL-2. Arrows, granules specific for A-NK cells. ×7000. C, A-NK cell actively migrating through the vascular endothelium into liver tissue. Arrowheads, endothelial opening. ×4400. D, an A-NK cell in mitosis. The A-NK cell is inside a blood vessel. *, probable remnants of a tumor cell 24 h after AIT. ×4400.

Regionally had a significant therapeutic impact, without substantially or selectively infiltrating the metastases or the tumors (5, 29). In these xenograft models, therapeutic benefits were manifested by either significantly prolonged survival or tumor regression (5, 29). Similarly, in a syngeneic rat model of MADB 106 breast carcinoma with 3-day established lung or liver metastases, systemically transferred IL-2-activated A-NK cells did not substantially infiltrate metastases, and yet they mediated antitumor effects (30).

Although it seem that delivery and localization of at least some transferred effector cells and IL-2 to the sites of metastasis may be necessary for control of tumor spread (3), it has been unclear whether the route of A-NK cell delivery influences their accumulation in the liver. Our study indicates that significantly fewer of the delivered A-NK cells were accounted for in the livers of tumor-bearing mice than in those of control mice 4 h after i.v. transfer. This was due to the tendency of these cells to remain in the lungs of tumor-bearing mice. Nevertheless, depending on the route of delivery, ~5 × 10⁶ A-NK cells, representing 50% of the total number injected i.v., to 10 × 10⁶ A-NK cells (Table 1) were present in the liver. Assuming that 3 days after tumor cell injection, there still are as many as 1 × 10⁷ tumor cells in the liver,
the E:T ratio per liver would be 1:2. But in the established metastases, tumor cells are likely to greatly outnumber effector cells, because A-NK cells do not seem to localize to metastases. In fact, based on our microscopic observations, the E:T ratio in HR metastases seems to be very low, and it is difficult to account for the observed rapid elimination of metastases and improved survival by direct tumor cell lysis. These results suggest that mechanisms other than direct cytolysis might be responsible for destruction of liver metastases in this model.

In addition to direct lysis of tumor cell targets, several different mechanisms may be responsible for antitumor efficacy of A-NK cells in situ. We have recently shown that A-NK cells can induce apoptosis, resulting in DNA damage (31), and that A-NK cells may preferentially use apoptosis to eliminate tumor cells in spheroids or monolayers. This process requires direct contact between effector and target cells, is mediated by membrane-bound cytokines of the tumor necrosis factor family (32), and might involve interactions of the death-transducing molecule Fas (APO-1) on the target with its ligand on the effector cells. Yet another mechanism, mediated by cytokines present in supernatants of A-NK cells, has also been shown to lead to the cell cycle arrest and DNA fragmentation in tumor cell targets (33). In addition, A-NK cells might alter the behavior of the tumor in situ by delivery and release of a variety of cytokines. We have shown that these effector cells express mRNA for and secrete a broad spectrum of cytokines, including tumor necrosis factor, IFN-γ, IL-1, granulocyte-macrophage colony-stimulating factor, and others (10). A-NK cells or their soluble products might have pronounced effects not only on the tumor cells but also on the vascular elements in the tumor or in the liver tissue. These and perhaps other indirect consequences of A-NK cell delivery to the liver might lead to magnified antitumor effects, resulting in rapid elimination of established metastases.

NK cells have been long considered to be particularly effective against intravascular metastases (34–36). In the 3-day liver metastasis model, many tumor cells were found in the intravascular location, obstructing the blood flow. Thus, at the early stages of AIT, these metastases could be particularly vulnerable to antitumor activities of intravasally transferred A-NK cells. Our EM data indicate that A-NK cells, having penetrated into metastases, are in contact with tumor cells in the vessels and may be responsible for “dissolving” tumor metastases occluding these vessels. A-NK cells might be able to contribute to the tumor cell demise by any one of the direct or indirect mechanisms listed above or most likely by using multiple ways of tumor cell destruction.

We have previously reported that A-NK cells are able to penetrate and enter tumor spheroids better than other IL-2-activated subsets of human NK cells (14). Preliminary results indicate that this ability to enter solid tissue formations is a consequence of the ability of A-NK cells to produce collagenases and other tissue-degrading enzymes. Our image analysis of A-NK cells transferred to the liver shows that these cells exhibit characteristics consistent with the process of active migration. They migrate out of the sinusoids, move through the extracellular matrix, and seem to be able to interact with liver tissue cells. Furthermore, A-NK cells were seen, albeit in small numbers, inside large liver metastases, confirming the in vitro data and providing further evidence for their excellent functional potential in vivo.

A single locoregional or systemic infusion of $1 \times 10^7$ A-NK cells/liver was found to result in rapid (within 24 h) elimination of most of the established metastases and significantly prolonged survival of the treated mice. This early destruction of metastases in the liver was not accompanied by selective localization of effector cells to liver metastases. Thus, in the liver, even a modest number of transferred A-NK cells, relative to the number of tumor cells, could successfully mediate regression of metastases. Although a single delivery of these cells prolonged survival, it did not cure the mice with established metastases. It is possible that repeated delivery of A-NK cells to the liver may improve the therapeutic efficacy of such transfers. It is also possible that heterogeneity of metastases, including substantial differences in their sensitivity to immunotherapy, is responsible for their escape. Studies are in progress to optimize the therapeutic effects observed in this model and to determine antitumor mechanisms used by these effector cells in situ. The development of a model in which limitations of cellular therapy in achieving complete eradication of micrometastases can be examined would be an important and useful contribution.

REFERENCES


N. L. Vujanovic, unpublished observations.
N. L. Vujanovic, unpublished data.
Elimination of Established Liver Metastases by Human Interleukin 2-activated Natural Killer Cells after Locoregional or Systemic Adoptive Transfer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/7/1599

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.