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ORIGINAL ARTICLE

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Observed localization of the long-term cultured rat adherent natural killer cells in mammary tumor tissues

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Abstract Adherent natural killer (A-NK) cells were isolated from splenic lymphocytes and treated with long-term culture in the presence of recombinant interleukin-2 (rIL-2). Immunocytochemical and flow-cytometric analysis revealed that most of the A-NK cells strongly expressed lymphocyte-function-associated antigen 1 (LFA-1α, and LFA-1β) throughout the incubation. All A-NK cells from 8- to 150-day cultures, particularly those cultured for 8 days, showed significant cytolytic activity against all targets. Analysis of the tissue distribution of the injected [^{3}H] uridine-labeled A-NK cells demonstrated that, in the first 3 h, most (over 60%) cells localized in the lungs, and that most cells remained temporally within the cavities of blood capillaries of the lungs and moved gradually into lymphoid and other tissues. Peritumoral injection of various kinds of adjuvant, particularly Freund's complete adjuvant (FCA) plus bacillus Calmetee-Guérin (BCG), resulted in a marked accumulation of [^{3}H]A-NK cells in mammary tumor tissues 24 h after injection, and simultaneously in the formation of vessels resembling high-endothelium venules (HEV), infiltration of LFA-1$^+$ lymphocytes and expression of the ICAM-1 molecule on the tumor cells in the sites of tumor tissues. When 30×10^6 A-NK cells were intravenously administered, significant retardation of tumor growth and prolongation of survival of tumor-bearing rats were observed in the groups that received the prior injection of adjuvants, especially FCA + BCG and Freund's incomplete adjuvant (FIA) + BCG. The suppressive effect of combination therapy on tumor growth was blocked effectively by the injection of anti-ICAM-1 mAb. These results indicate that the prior injection of proper adjuvant into the peritumoral region is effective for the selective accumulation or infiltration of A-NK cells into the sites of tumor tissues, and results in the marked retardation of tumor growth.

Key words A-NK cells · Long-term culture · Adjuvant · Accumulation · Mammary tumor · Immunotherapy

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Introduction

Adoptive immunotherapy using lymphokine-activated killer (LAK) cells or plastic-adherent natural killer (A-NK) cells [3], in combination with recombinant interleukin-2 (rIL-2), has proved to be useful in a variety of animal tumor models and in some cancer patients. The effects are usually transient and require the transfer of large numbers of LAK cells and a high dose of rIL-2 [21, 22]. The majority of patients treated, however, have not shown any definite response to such therapy [21, 33]. It is in general believed that, when transferred intravenously, LAK cells migrate selectively into tumor tissues and exert their antitumor effects though direct cellular attachment or firm adhesion to tumor cells.

Lymphocyte migration into the perivascular region occurs through the mechanism of lymphocyte homing though vessels resembling high-epithelium venules (HEV) during the course of a chronic inflammatory reaction [24, 25]. The first step in the migration of LAK cells into tumor tissue is the process of LAK cell adherence to the vascular endothelium. A number of studies have demonstrated that various kinds of adhesion molecule mediate the adherence of lymphocytes to the endothelium and the extravasation [24, 25]. The transmigration of NK cells though endothelial cells has been shown to be dependent on lymphocyte-function-associated antigen 1 (LFA-1) [5], and the vascular antigen VLA-4 mediates NK cell adhesion to the extracellular matrix component fibronectin [10]. The migration capacity of NK cells is found to be higher than that of other lymphocytes. NK cells also have the

ability to increase the migratory response rapidly [23]. Localization of A-NK cells in the microvasculature of growing tumors results from recognition of the intracellular and vascular cell-adhesion molecules ICAM-1 and VCAM-1 on the tumor endothelium, mediated by LFA-1 and vascular lymphocyte-function-associated protein VLA-1 [16]. It has therefore been hypothesized that the formation of HEV-like vessels in the peritumoral region prior the adoptive transfer of antitumor A-NK cells might be crucial for selective accumulation or infiltration of the effector cells into tumor tissue through the lymphocyte homing mechanism. Deeper insight into the selective accumulation of antitumor LAK cells transferred to tumor-bearing animal models may guide therapeutic approaches in further clinical trials.

Thus, in the present study, we attempted to clarify what kind of antigenic phenotypes or adhesion molecules and cytotoxic killer activity against tumor cells A-NK cells can express, when cultured for a long period in vitro. Furthermore, we tried experimentally to demonstrate what kind of experimental protocol is useful for the selective accumulation of A-NK cells in mammary tumor tissues, induced by a chemical carcinogen in rats, and for the effective suppression of tumor growth, when various kinds of adjuvant were injected into the peritumoral region. The data obtained indicate that peritumoral injection of some kinds of adjuvant prior to the adoptive cell transfer therapy can augment the accumulation of effector cells into the tumor tissue as well as the extent of tumor regression. The mechanisms by which the effector cells accumulate in the tumor tissue and exert antitumor effects are discussed.

Materials and methods

Animals

Specific-pathogen-free female DA/Slc rats, 8–10 weeks old and weighing 100–120 g, were purchased from Japan Slc Co.(Hamamatsu, Japan) and maintained in a specific-pathogen-free animal facility at the Hamamatsu University School of Medicine. The rats were used throughout the study.

Culture medium and interleukin-2

The culture medium consisted of RPMI-1640 medium (Nissui Co., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Nichirei Co., Tokyo, Japan), 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Human rIL-2 was kindly provided by Shionogi Co. (Osaka, Japan).

Preparation of A-NK cells

The preparation of A-NK cells has been described elsewhere [11, 31]. Briefly, single-cell suspensions from DA rat spleen were prepared in RPMI-1640 medium and the splenic lymphocytes were isolated by Ficoll-Paque (density = 1.077 g cm^{-3}; Pharmacia, N.J., USA) four-step Percoll gradients (48%, 52%, 56% and 60% Percoll). The gradients were centrifuged at 400 *g* for 30 min and the cells were collected from the 48%/52% interface [20]. After two washes, 10×10^6 cells were transferred into 25-cm^2 plastic flasks (Falcon Co., N.J., USA) with 5 ml complete medium and 2000 U/ml rIL-2. After 2–3 days of incubation at 37 °C 5% CO_2 in air, non-adherent spleen cells were removed, and the flasks were gently washed with preheated (37 °C) complete medium to remove cells not attached to the plastic. A 5-ml portion of fresh complete medium containing 1000 U/ml rIL-2 was added and the cells were cultured in vitro for more than 50 passages (approximately 150 days). The A-NK cells were harvested by scraping the flasks with a rubber policeman 0, 8, 30 and 150 days after incubation.

Antibodies

Three different monoclonal antibodies (mAb), Mar1, Mar2 and Mar3, recognizing rat macrophages, were produced in our laboratory [36]. The mouse mAb 1F4 (anti-CD3), W3/25 (anti-CD4), OX6 (anti-Ia), OX8 (anti-CD8), OX39 (anti-IL-2 receptor), AG-1 (anti-asialoGM-1) anti-(hamster Ig) second antibody and a fluorescein-isothiocyanate-conjugated rabbit $F(ab')_2$ fragment anti-(mouse IgG) were purchased from Seikagaku Co. (Tokyo, Japan). Anti-(adhesion molecule) mAb WT-1 (anti-LFA-1α), WT-3 (anti-LFA-1β), 1A29 (anti-ICAM-1), and HRL4 (anti-LECAM-1) were kindly provided by Dr. M. Miyasaka (Osaka University School of Medicine, Osaka, Japan). A second antibody, alkaline-phosphatase-conjugated anti-(mouse IgG), was purchased from Sigma Chemical Co. (Mo. USA).

Immunohistochemical staining

Cell smears were prepared by cytospin centrifugation (Shandon Ltd., Cheshire, UK). The unfixed tumor specimens were embedded in OCT (Miles Inc., Ind., USA) and frozen at −70 °C, and 5-μm cryostat sections were prepared. Tissues were stained by an indirect immunoalkaline phosphatase method and the Fast red technique [2, 17]. The slides were incubated with the primary antibodies for 60 min at room temperature. After washing with 0.01 M cold phosphate-buffered saline (PBS) pH 7.4, the slides were incubated with second antibodies for 45 min at room temperature. The slides were developed, after additional washes with PBS, using ABC alkaline phosphatase substrate kit 1 (Vector red; Funakoshi Co., Tokyo, Japan). Finally, the slides were slightly counterstained with hemotoxylin solution and observed by a light microscope.

Flow-cytometric analysis

A-NK cells (1×10^6) were treated with mAb for 30 min at 4 °C. After two washes with PBS, the fluorescein-isothiocyanate-conjugated rabbit anti-(mouse IgG) antibody at 1:50 dilution was added and incubated for a further 30 min at 4 °C. After two washes with PBS, the cells were analyzed on an EPICS Profile flow cytometer (Colt Co., Fla., USA).

Preparation of mammary-tumor-bearing rats

Female DA rats received an intravenous injection of a fat emulsion containing 0.5% 7,12-dimethylbenzanthracene (DMBA; Upjohn Co., Mich., USA) [15]. Between 50 and 80 days after DMBA administration, when the palpable tumors had reached about 10 mm in diameter, the tumors were removed and washed twice with cold RPMI-1640 medium. Small fragments (about 2×2 mm) of the freshly removed tumor were inoculated subcutaneously into the axillary region of syngeneic DA hosts. Within 2–3 weeks after the grafting, the tumor was well developed and had reached about 8–10 mm in diameter when it was used to study the in vivo treatment of mammary-tumor-bearing rats with A-NK cells.

Primary culture of DMBA-induced mammary tumor cells

DMBA-induced mammary tumor cells were incubated as described by Ethier [8].

In vitro cytotoxicity assay

Cytotoxicity was measured in a standard 4-h ^{51}Cr-release microcytotoxicity assay using 96-well V-bottomed microplates (Falcon Co., N.J., USA). As the target cells, YAC-1 and P815 cells, obtained from the Cancer Cell Repository of Tohoku University (Sendai, Japan), and primary cultured mammary tumor cells were used. Each sample of 10^6 target cells was labeled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham Co., Buckinghamshire, England), washed and seeded into triplicate wells to give various E/T ratios in final volume of 200 ml. After incubation at 37 °C for 4 h, 100 ml supernatant was removed from each well and counted in a gamma counter (Aloka Co., Tokyo, Japan) to determine the experimental release (ER). The spontaneous release (SR) was obtained from wells that contained target cells and medium only, and the total release (TR) was obtained from wells receiving 1% Triton X-100 solution. The percentage cytotoxicity was calculated as: cytotoxicity (%) = $100 \times [(ER - SR)/(TR - SR)]$.

Adoptive transfer of [^{3}H] uridine-labeled A-NK cells

Mammary-tumor-bearing rats were divided into six groups ($n = 3$). The control group received a single injection of 0.1 ml 0.9% saline solution into the peritumoral region. The other five groups served as experimental groups and received a 0.1-ml single peritumoral injection of Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA) (Funakoshi Co., Tokyo, Japan), bacillus Calmette-Guèrin (BCG) (BCG Co., Tokyo, Japan) (0.4 mg/ml), FCA + BCG, or FIA + BCG. Samples containing 10^8 A-NK cells in 10 ml complete medium were incubated for 1 h with 100 μCi [^{3}H] uridine (Amersham Co., Buckinghamshire, England) at 37 °C. After three washes, the cells were resuspended in RPMI-1640 medium at a concentration of 30×10^6/ml.

Labeled A-NK cells were administered to the female DA rats by intravenous injection [35]. The cell viability was over 90%. Recipient rats were injected intraperitoneally with 50 000 units rIL-2 2 h earlier and treated with two subsequent doses of rIL-2 separated by an 8-h interval. The abdominal aorta was flushed with 50 ml 0.9% saline solution 3, 12, 24, 48 and 72 h after injection, to minimize the background radioactivity. Various organs were then removed and weighed. The distribution of A-NK cells in each organ was evaluated by measuring the radioactivity with a sample oxidizer (Aloka Co., Tokyo, Japan) and by autoradiography. Aliquots of tissue samples from these organs were oxidized in a sample oxidizer, and the end-product ([^{3}H] water) was determined in a liquid scintillation counter (Beckman Co., Cal., USA). The radioactivity recovered in each experiment was expressed as cpm/mg wet tissue. Paraffin-embedded tissues for autoradiography were cut at 5 μm, and deparaffinized slides were dipped in autoradiographic emulsion (Konika Co., Tokyo, Japan). After exposure for 2 weeks at 4 °C the slides were developed, fixed and stained with hematoxylin.

In vivo treatment studies

The antitumor effect of the combination of peritumoral injection of adjuvant and A-NK cell adoptive transfer was studied in vivo, by determining the tumor size and the survival time of mammary-tumor-bearing rats after the in vivo treatment. Mammary-tumor-bearing rats were divided into six groups ($n = 6$), as described before: the control, FCA, FIA, BCG, FCA + BCG, and FIA + BCG groups. Ten days after the peritumoral injection of various kinds of adjuvant, 30×10^6 A-NK cells were administered in combination with multiple injections of rIL-2. To determine the blocking effect of anti ICAM-1 mAb on the ability of the combination of adjuvant and A-NK cells to suppress the growth of mammary tumors, 30×10^6 A-NK cells and anti-ICAM-1 mAb (1A29, 0.1 mg/kg body weight) were injected intravenously into the mammary-tumor-bearing rats that had received the peritumoral injection of various of adjuvants 10 days previously. The recipient rats in each group were injected intraperitoneally with 50 000 units rIL-2 2 h prior to the injection of A-NK cells and treated with two subsequent doses of rIL-2 with separated by an 8-h interval on 4 consecutive days. Tumor size was measured with a caliper every 5 days for 30 days.

Statistical analysis

Statistical analysis was performed using the two-tailed Student's *t*-test.

Results

Characteristics of A-NK cells after long-term culture

Expression of the surface marker phenotypes of A-NK cells was analyzed on an EPICS flow cytometer to quantify the degree of cell staining (Fig. 1). The pattern of surface marker expression on A-NK cells during long-term culture was classified into four types: the initial increase and late decrease type (type A), the gradual decrease type (type B), the gradual increase type (type C) and the initial decrease and late increase type (type D). Cells positive for ICAM-1 (1A29$^+$), IL-2R (OX39$^+$), CD8 (OX8$^+$), and Ia (OX6$^+$) belong to type A; those positive for LFA-1α (WT-1$^+$), LFA-1β (WT-3$^+$), CD3 (1F4$^+$), and CD4 (W3/25$^+$) to type B; macrophage-antigen-positive (Mar 1$^+$, Mar 3$^+$) and asialoGM-1-positive (AG-1$^+$) cells to type C; LECAM-1-positive (HRL-4$^+$) cells to type D.

Following the degree of expression of these surface markers revealed that early-stage A-NK cells (day 8) are LFA-1^{high}, ICAM-1^{high}, CD3^{high}, CD8^{high}, Ia^{high}, IL-2R^{high}, CD4^{low}, Mar 3^{low}, and asialoGM-1^{low}; middle-stage A-NK cells (day 30) are LFA-1^{high}, ICAM-1^{high}, CD3^{high}, Ia^{high}, IL-2R^{high}, Mar 3^{low}, CD4^{low}, CD8^{low}, and asialoGM-1^{low}; late-stage A-NK cells (day 150) are LFA-1^{high}, asialoGM-1^{high}, and others^{low}. Most A-NK cells strongly expressed LFA-1α, and LFA-1β molecules throughout the incubation.

Cytolytic activity against tumor cells

Because there were some differences in the intensity of surface marker expression among early-, middle-, and late-stage A-NK cells, it is pertinent to examine the relationship between the expression of these molecules and the in vitro cytolytic activity against tumor cells. The cytolytic activity of A-NK cells against fresh mammary tumor cells, YAC-1, and P815 targets was assessed with a ^{51}Cr-release microcytotoxicity assay. A-NK cells obtained after 8, 30, and 150 days of culture showed significant cytolytic activity against all targets, compared with normal spleen cells (Fig. 2). Among three kinds of effectors, day-8 A-NK cells showed twice the cytolytic activity of day-150 A-NK cells at various E/T ratios. Day-30 A-NK cells showed a degree of activity intermediate between that of day-8 and that of day-150 cells. More potent cytolytic activity at a low E/T ratio was

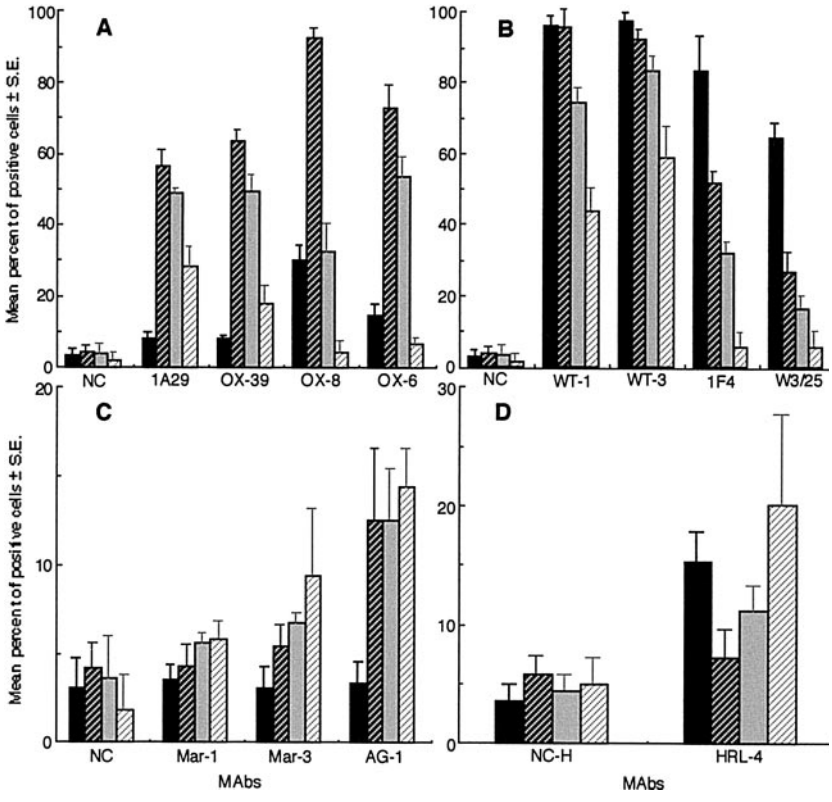

Fig. 1A–D Changes of antigenic phenotype expression of adherent natural killer (A-NK) cells during long-term culture. Four types: initial increase and late decrease type (**A**), gradual decrease type (**B**), gradual increase type (**C**) and initial decrease and late increase type (**D**), for the antigenic phenotype expression were observed in A-NK cells, 0 (■), 8 (▨), 30 (▦), and 150 (▨) days after incubation

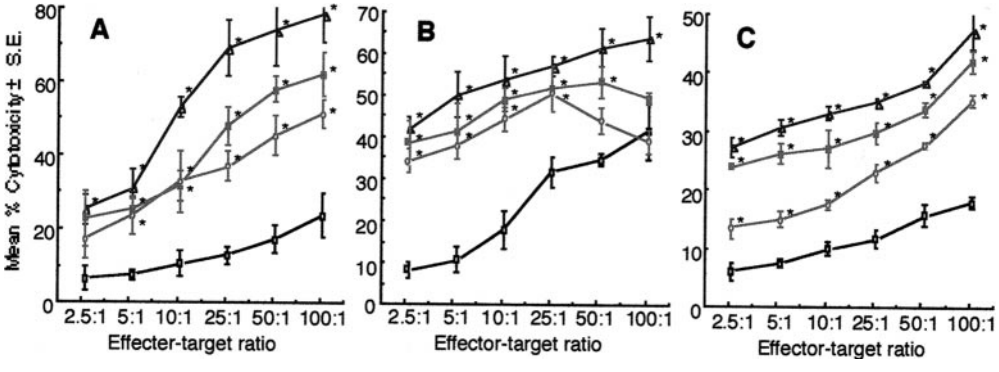

Fig. 2A–C Cytolytic activity of A-NK cells. The cytolytic activity of A-NK cells 8 (△), 30 (■), and 150 (○) days after incubation, and normal spleen cells (□) was tested with a 4-h ^{51}Cr-release assay using fresh mammary tumor cells (**A**), YAC-1 lymphoma cells (**B**), and P-815 mastocytoma cells (**C**), which were labeled with ^{51}Cr, as the target tumor cells. *$P < 0.05$ versus normal spleen cells

observed for all effector cells tested than for fresh mammary tumor cells and P-815 cells, when YAC-1 lymphoma cells were used as the targets.

Distribution of A-NK cells in vivo

[^{3}H]uridine-labeled A-NK cells after 8, 30, and 150 days culture were injected intravenously into normal DA rats or tumor-bearing DA rats, as described previously [4, 32, 35]. Figure 3 shows the distribution pattern of ^{3}H-labeled A-NK cells in various kinds of organ 3–72 h after a single intravenous injection. Immediately after injection (3 h), a high level of radioactivity (over 60% of the total) was found in the lungs, and 5%–10% of the radioactivity recovered was found in the liver and the spleen. However, autoradiographic observation revealed that most of the ^{3}H-labeled A-NK cells found in the lungs was adhering to the endothelia of small blood vessels, remaining within the vascular cavity without extravasating into the parenchyma (Fig. 4).

The radioactivity in the lungs was sharply decreased to 17% of the 3-h radioactivity 12 h after injection, implying the detachment of [^{3}H] A-NK cells from the pulmonary blood vessels. On the other hand, relatively high radioactivity was observed in the spleen (Fig. 3), whereas very low radioactivity was found in the kidney, the adrenal gland, the small intestine, the thymus and serum (data not shown). Between 24 and 72 h after injection, the radioactivity in all organs tested gradually decreased. No difference was observed in the distribution pattern among [^{3}H] A-NK cells obtained from 8, 30, and 150 days of culture, when these were injected into normal rats and mammary-tumor-bearing DA rats (data not shown).

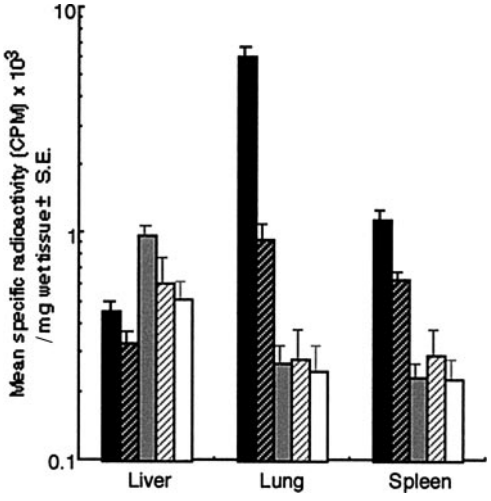

Fig. 3 Tissue-distribution pattern of [^{3}H]uridine-labeled A-NK cells after an intravenous injection. Radioactivity recovered in liver, lung, and spleen, 3 (■), 12 (▨), 24 (▩), 48 (▨), and 72 (□) h after single intravenous injection of [^{3}H] uridine-labeled A-NK cells (30×10^6 cells) was measured with sample oxidizer. Bars: standard error ($n = 3$)

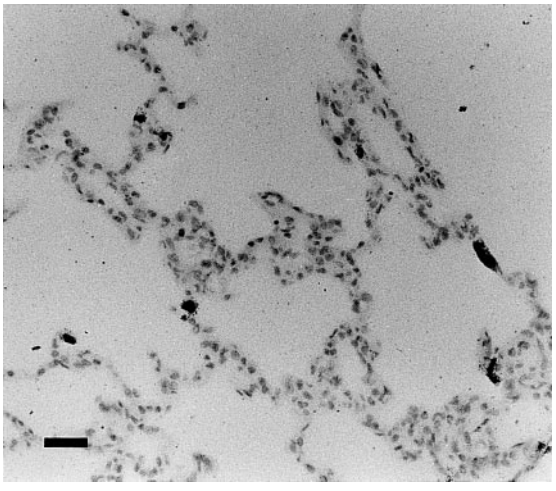

Fig. 4 Autoradiographic localization of [^{3}H]uridine-labeled A-NK cells in the lung. An autoradiograph of the lung was prepared 3 h after an intravenous injection of [^{3}H]uridine-labeled A-NK cells (30×10^6). Note that most of ^{3}H-labeled A-NK cells remain within the cavity of the blood capillaries of the lung. Hematoxylin stain; bar: 50 μm; 2 weeks exposure

Distribution of A-NK cells into mammary tumor tissues

In order to determine the pattern of distribution of [^{3}H] A-NK cells in the mammary tumor tissues, the radioactivity of the mammary tumor tissues receiving the peritumoral injection of various kinds of adjuvant 10 days previously was measured 3–72 h after a single intravenous injection of [^{3}H] A-NK cells obtained after 0, 8, 30, or 150 days of culture. As shown in Fig. 5, the radioactivity of the tumor tissues receiving the peritumoral injection of FIA or FCA with or without BCG was much higher than that of the saline controls. The

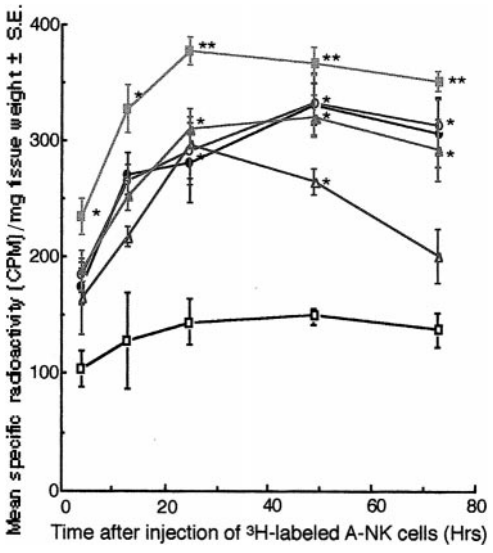

Fig. 5 Distribution of [^{3}H]uridine-labeled A-NK cells (day 150) in mammary tumor tissues. Radioactivity of mammary tumor tissues that had received a peritumoral injection of various kinds of adjuvant 10 days previously was measured with sample oxidizer 3–72 h after an intravenous injection of [^{3}H]uridine-labeled A-NK cells (30×10^6). Recipient rats were treated with recombinant interleukin (rIL-2) with an 8-h interval. ■ Freund's complete adjuvant (FCA) + bacillus Calmette-Guérin (BCG), ○ Freund's incomplete adjuvant (FIA) + BCG, ● FIA, ▲ BCG, △ FCA, □ 0.9% saline solution; $*P < 0.05$, $**P < 0.01$ versus saline control

highest radioactivity was observed in the FCA + BCG group compared with other groups 24 h after injection. The effect of the peritumoral injection of FCA + BCG, FIA + BCG, FIA or BCG on the accumulation of [^{3}H] A-NK cells was greater and longer than that of FCA injections.

The localization of [^{3}H]A-NK cells in the mammary tumor tissues after peritumoral injection of FCA + BCG was investigated with autoradiography (Fig. 6). When measured 24 h after the injection of [^{3}H]A-NK cells, the degree of distribution of [^{3}H]A-NK cells into the tumor tissues given the peritumor injection of FCA + BCG was much higher than that of the saline controls (Fig. 6). It was also observed histologically that the degree of tumor necrosis in the former was much higher than that of the latter.

The formation of HEV-like vessels that permit the extravasation or the infiltration of lymphocytes into the peritumoral region was investigated histologically. Figure 7 shows a representative collection of HEV-like vessels produced by the prior injection of FCA + BCG into the peritumoral region. Moreover, the infiltration of mononuclear cells consisting of lymphocytes, natural killer cells, and macrophages was often observed in the perivenular space of HEV-like vessels that were produced within the loose connective tissue of the peritumoral region and the peripheral region of necrotic foci of the tumor tissue.

Expression of adhesion molecules on the mammary tumor cells and on the tumor-infiltrating lymphocytes

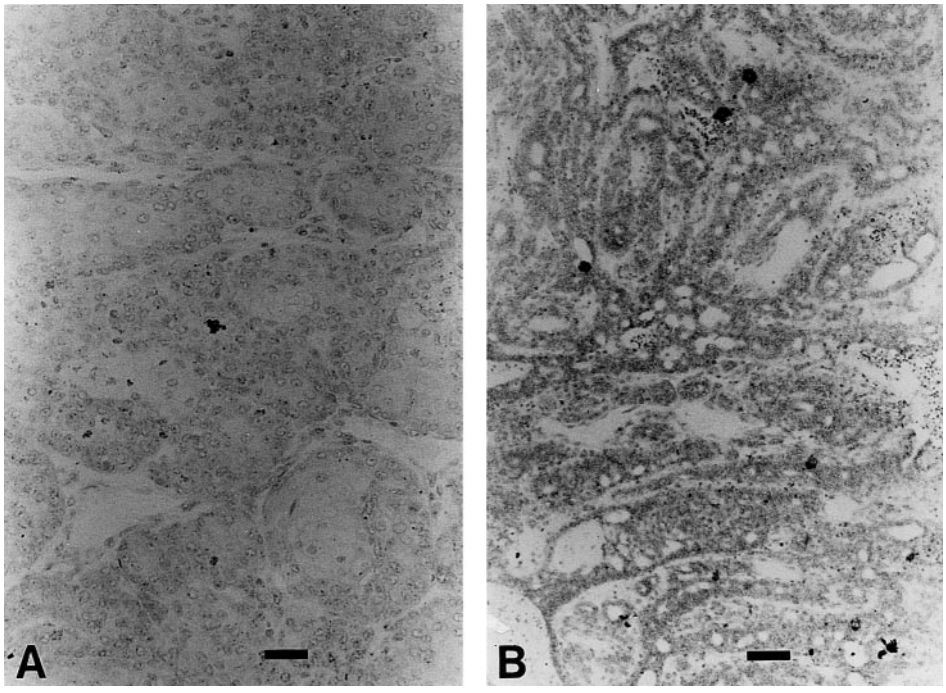

Fig. 6A, B Autoradiographic localization of [^{3}H]uridine-labeled A-NK cells (day 150) in mammary tumor tissues. Autoradiographic mammary tumor tissues that had received a prior injection of 0.9% saline (**A**) or FCA + BCG (**B**) into the peritumoral region were prepared 24 h after an intravenous injection of [^{3}H]uridine-labeled A-NK cells (30×10^6). Hematoxylin stain; bars: 30 μm; 2 weeks exposure

was investigated immunohistochemically. The degree of distribution of ICAM-1$^+$ mammary tumor cells in the tumor tissues given the peritumoral injection of FCA + BCG was much higher than that of the saline controls, indicating that the adjuvant injection augments the expression of ICAM-1 molecules on the tumor cells (Fig. 8). Moreover, it was found that the injection of adjuvant results in the selective accumulation of LFA-1$^+$ lymphocytes into the stroma surrounding the tumor foci.

In vivo treatment studies

Figure 9 shows the changes in the growth of mammary tumor tissue from rats receiving various adjuvants by peritumoral injection after adoptive transfer of A-NK cells and rIL-2 treatment. Fifteen days after the tumor grafting, mammary-tumor-bearing rats were given single peritumoral injections of various kinds of adjuvant and then, 10 days later, an intravenous injection of A-NK cells obtained from a 150-day culture. Prior injection of adjuvants resulted in significantly less tumor growth in all the groups tested, compared with that of the control group. A marked reduction was observed particularly in the FCA + BCG and FIA + BCG groups, when evaluated 30–50 days after tumor grafting. In the blocking experiment, simultaneous injection of anti-ICAM-1 mAb (1A29. 0.1 mg/kg) blocked or effectively cancelled the suppressive effect on tumor growth of previously injected adjuvant combined with A-NK cell adoptive transfer (Fig. 10). No effect was found in the groups treated simultaneously with adjuvants and adoptive A-NK cell transfer (Fig. 11). The longest mean survival times that were found in the groups pre-treated with FCA + BCG or with FIA + BCG were 48.6 and 45.9 days ($P < 0.05$) respectively. The survival rate of the two groups was significant higher than that of the control group (Table 1).

These findings indicate that the peritumoral injection of proper adjuvants augments the infiltration of effector cells and the cellular interaction with or adhesion to target tumor cells, not only by inducing the neovasculogenesis of HEV-like vessels, but also by augmenting the expression of relevant adhesion molecules on both target tumor cells and effector cells.

Discussion

The effect of adoptive immunotherapy by transfer of LAK cells, which is a novel cancer therapeutic modality, is still controversial and there are many difficulties in obtaining a large amount of effector cells and in generating sufficient quantities of potent effector cells to exert

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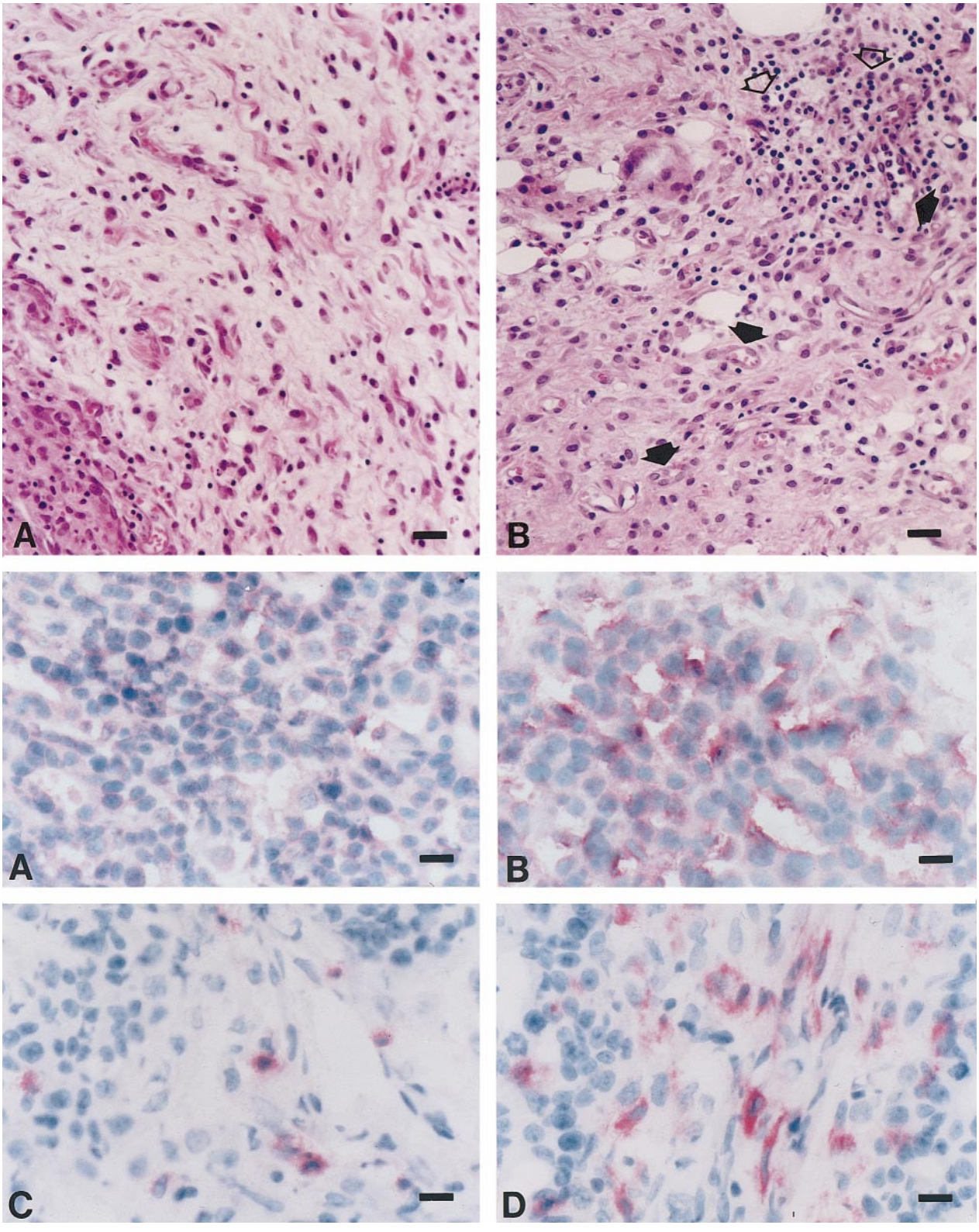
Fig. 7A, B Section of the peritumoral region of mammary tumor tissues that had received a peritumoral injection of 0.9% saline (**A**) and FCA + BCG adjuvants (**B**). Note the formation of high-epithelium-venule-like vessels (*black arrows*) and the infiltration of mononuclear cells (*white arrows*) in the peritumoral region (**B**). Hematoxylin/eosin stain; bar: 40 μm

Fig. 8A–D Distribution of adhesion-molecule-positive cells in the mammary tumor tissues. **A**, **B** ICAM-1 molecule expression on mammary tumor cells stained with 1A29. **C, D** LFA-1 molecule expression on tumor-infiltrating lymphocytes in the loose connective tissues of the peritumoral region stained with WT-3. Control groups (**A**, **C**) and adjuvant groups (**B**, **D**) had been injected with 0.9% saline solution or FCA + BCG adjuvants 10 days previously. Indirect immunostaining with alkaline-phosphatase-conjugated antibody. Bars: 12 μm

a broad spectrum of antitumor activity. In the present study, we successfully isolated A-NK cells more potent than standard LAK cells by incubating spleen cells from DA/Slc rats in the presence of rIL-2 for over 150 days. It was revealed that, when incubated in the presence of rIL-2, A-NK cells rapidly change both the surface structure and the expression of their antigenic phenotype, enabling them to adhere to plastic flasks and exert tumor cytotoxicity. The evidence is follows: (a) the cells showed the morphological characteristics of large



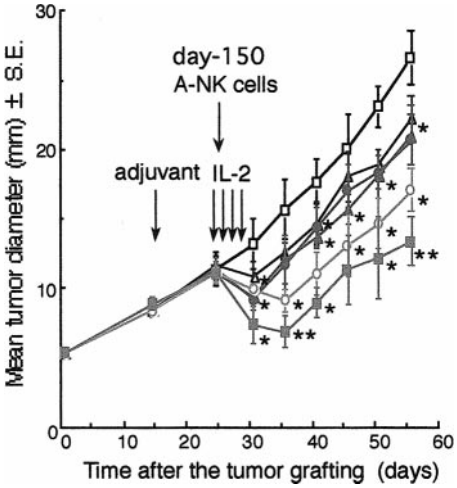


Fig. 9 Effect of the prior injection of adjuvants and adoptive transfer of A-NK cells on the growth of mammary tumor. Ten days after the injection of various kinds of adjuvant into the peritumoral region, A-NK cells (30×10^6) were injected i.v. together with multiple injections of rIL-2. □ 0.9% saline solution, △ FCA, ▲ BCG, ● FIA, ○ FIA + BCG, ■ FCA + BCG, $n = 6$; $^*P < 0.05$; $^{**}P < 0.01$ versus saline control

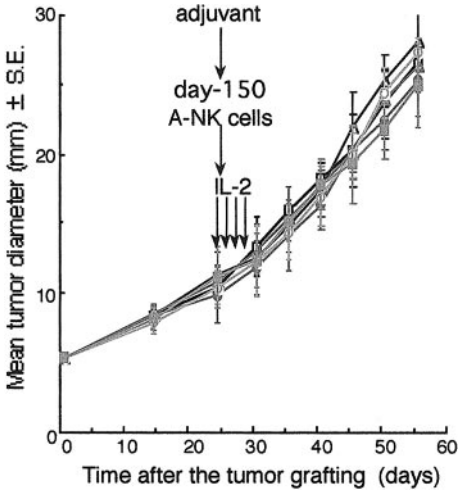


Fig. 11 Effect of the simultaneous injection of adjuvant and A-NK cell adoptive transfer on the growth of mammary tumor. Peritumoral injection of various kinds of adjuvant and A-NK cell (30×10^6) adoptive transfer in combination with multiple injections of rIL-2 were simultaneously given to the mammary-tumor-bearing rats. □ 0.9% saline solution, △ FCA, ▲ BCG, ● FIA, ○ FIA + BCG, ■ FCA + BCG; $n = 6$

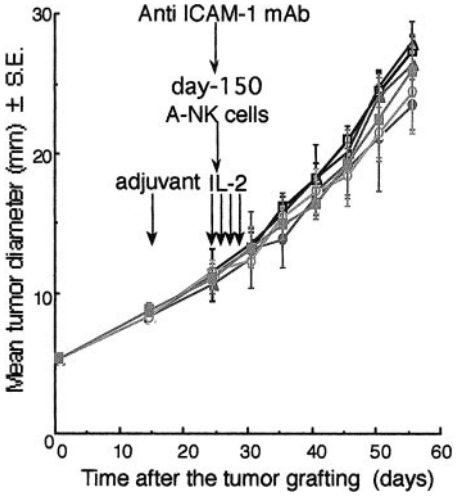


Fig. 10 Blocking effect of anti ICAM-1 mAb on the suppression of mammary tumor by the combination therapy of adjuvant and A-NK cell adoptive transfer. Ten days after the peritumoral injection of adjuvants, A-NK cells (30×10^6) and anti-ICAM-1 mAb were injected i.v. together with multiple injections of rIL-2. □ 0.9% saline solution, △ FCA, ▲ BCG, ● FIA, ○ FIA + BCG, ■ FCA + BCG; $n = 6$

Table 1 Survival time of mammary-tumor-bearing rats after the adjuvant plus adherent natural killer (A-NK) cell adoptive transfer treatment. *FCA* Freund's complete adjuvant, *FIA* Freund's incomplete adjuvant (*adj.*), *BCG* bacillus Calmette-Guérin, *NC* control

Groups (no. of rats)	Mean survival time (days) ± SE		
	Pre-treated with adj.	Co-treated with adj.	Blocked with adj.
FCA + BCG (6)	48.6(±2.8)*	34.9(±2.6)	37.2(±3.6)
FIA + BCG (6)	45.9(±3.6)*	35.1(±1.4)	33.0(±0.9)
FIA (6)	40.5(±3.2)	37.4(±3.1)	36.7(±1.6)
BCG (6)	41.2(±5.0)	34.5(±1.0)	35.4(±1.8)
FCA (6)	38.8(±2.8)	32.2(±0.7)	30.8(±2.1)
NC (6)	33.9(±1.0)	33.1(±2.1)	35.1(±0.8)

$^*P < 0.05$

granular lymphocytes (data not shown), (b) the cells expressed similar surface markers to NK cells and some adhesion molecules, and (c) the cells acquiring NK cytolytic activity in response to rIL-2 exerted high levels of cytolytic activity against fresh tumor cells, YAC-1 and P-815. These observations are consistent with previous reports of work on rats, humans and mice, in which precursor activity was found to be contained in the LGL/NK lymphocyte subset [29, 30]. After a 150-day incubation, the cytolytic activity of A-NK cells was still significantly higher than that of normal splenic lymphocytes (Fig. 2). It has been reported that CD8 and asialoGM1 are NK cell markers, that NK cells exhibit major-histocompatibility-complex-unrestricted cytolytic activity against NK-sensitive targets [38], and that the interaction of rIL-2 with the rIL-2 receptor on NK cells promotes the generation of LAK cells [28], which thus acquire the ability to recognize and lyse NK-insensitive targets [18]. In the present study, it was also found that some populations of A-NK cells obtained from 8- to 30-day cultures expressed CD8, asialoGM-1, Ia and IL-2 receptor on their surface, indicating that they had acquired the surface markers essential for cytolytic activity against both NK-sensitive and -nonsensitive targets during incubation. It could be assumed that the longer the adoptively transferred cells circulate in a viable and healthy condition in the vascular system, the better are

the chances of tumor infiltration and killing. The continuous supplementing of rIL-2 was essential for the maintenance of cytolytic A-NK cells, because more than 80% of the cells died during the following 24 h, when rIL-2 was removed from an A-NK cell culture plate. Thus, survival, cytolytic activity and tumor localization of A-NK cells depend strictly on the presence of an optimal dose of rIL-2. A-NK cells strongly expressed LFA-1α and LFA-1β molecules on their surface throughout the incubation, although these did the gradually decrease (Fig. 1). The LFA-1 molecule is likely to play a crucial role in NK cell function, since it was responsible not only for the cellular adhesion of NK cell to potential target cells, but also for transduction of the signal triggering the cytolytic machinery [19, 26]. In this context, one may speculate that the expression of the LFA-1 molecule on A-NK cells may represent a major pathway for the activation of A-NK cells. Moreover, the expression of ICAM-1 on target tumor cells appears to be an important factor for the accumulation of effector cells in tumor tissue, since previous studies have shown that the cellular interaction between ICAM-1 and its ligand LFA-1 is essential for the function of effector lymphocytes and NK cells, including tumor cell lysis [14, 24, 26].

It is generally assumed that A-NK cells can migrate into lymphoid tissues and into inflammatory sites through HEV, indicating that the signals and steps for A-NK cells migration are similar to those of normal lymphocyte recirculation at the molecular level [24–27]. Extravasation of A-NK cell through HEV is regulated by at least three distinct molecular signals; first, tethering to the vessel wall and rolling; second, adhesion strengthening and, third transendothelial migration [25, 26]. Our previous studies on the in vivo localization of A-NK cells in rats supported these observations, as described previously [4, 32]. As shown in Fig. 3, [^{3}H]uridine-labeled A-NK cells were first trapped in the lungs, and subsequently migrated into the liver and the spleen 24 h after injection. The radioactivity in the liver was still higher than that in other organs after 72 h. The autoradiographic observation of the lungs failed to detect significant numbers of A-NK cells in the lung parenchyma, indicating that the A-NK cells adhere to the vascular endothelium and remain within the cavity of the pulmonary capillaries before entering the general circulation 24 h after injection. The present findings indicated that, when [^{3}H]A-NK cells were adoptively transferred, the prior injection of a proper adjuvant results in their selective accumulation in tumor tissues both directly and indirectly (Figs. 5–7). No difference was observed in distribution pattern among [^{3}H]-A-NK cells obtained after 8, 30, and 150 days of culture, when injected into normal rats and mammary-tumor-bearing DA rats (data not shown). When incubated in the presence of rIL-2, the A-NK cells rapidly changed their expression of antigenic phenotype, but the changes in LFA-1α and LFA-1β were not marked. It has been suggested that LFA-1 plays an important role in A-NK cell migration and this supports previous observations in vitro [5, 16]. The same study showed that LFA-1 expression of A-NK cells was less clearly correlated to A-NK cell binding to and lysis of tumor target cells and their migration into the intercellular space of tumors [7]. Direct migration of A-NK cells from the blood stream to the tumor tissues is initiated by their selective binding to specialized endothelial cells [37], particularly to HEV-like vessels newly produced within the tumor tissues [34]. Tumor-infiltrating LAK cells as well as inflammatory cells are thought to circulate in the vascular system until they reach their target tissues [3]. A number of granulomatous foci that contain a number of inflammatory cells, necrotic foci of tumor tissues and HEV-like vessels were observed in the peripheral region of tumor tissues injected peritumorally with adjuvants, particularly the FCA + BCG mixture (Fig. 7). In vivo treatment studies used day-150 A-NK cells. Although earlier A-NK cells express higher levels of LFA-1 and are more cytolytic, it is difficult to get enough cells for treatment studies. Peritumoral injection of adjuvants 10 days previously caused marked retardation of tumor growth as compared with the control group, especially in the groups receiving FCA + BCG and FIA + BCG (Fig. 9). In the ICAM-1 blocking experiment (Fig. 10), no significant retardation of tumor growth was observed. Moreover, marked accumulation of LFA-1$^+$ lymphocytes in the perivenular space of newly formed HEV-like vessels and in the surrounding stroma of foci of ICAM-1$^+$ tumor cells was observed (Fig. 8). Once in the tumor area, interactions between adhesion molecules on effector cells and ligand ICAM-1 on tumor cells should result in the firm adhesion of each passing effector cell, and chemoattractive factors should provide strong signals that further accelerate the migration of cells across the endothelial lining and into the tumor.

The exact mechanism by which adjuvant injection augments the accumulation of A-NK cells in tumor tissues is poorly understood. Jackson et al. [12] have previously proposed an intriguing hypothesis for the mechanism of action of BCG when it is a major component of FCA. They emphasized the importance of cytokines including interferon γ and tumor necrosis factor α released after BCG treatment, which promote the expression of ICAM-1 on tumor cells. Moreover, it was reported that the expression of ICAM-1 on tumor cells is up-regulated by interferon γ and tumor necrosis factor α released after intravesical BCG treatment, and that ICAM-1 plays a role in the binding of LAK cells to bladder carcinoma cells, neovasculogenesis of HEV-like vessels, local release of cytokines and cellular interactions [6, 9]. Allavena et al. [1] and Kasahara et al. [13] have shown that LGL themselves possess the ability to secrete a variety of cytokines, including interferon γ, IL-1, IL-2, B cell growth factor, and colony-stimulating factor. There is a possibility that the LGL population may play an important immunoregulatory role by inducing self-regulation of cytolytic activity. Moreover, besides the functions of tumor-specific killer T cells, other types

of nonspecific effector, including A-NK cells and macrophages, may play a crucial role in resistance against tumor growth, by infiltrating effectively into the peritumoral region via adhesion and migration mechanisms between effector cells and the target.

It is clear that adjuvant treatment should be further investigated, including its effects on modulation of cell traffic, expression of adhesion molecules, the cytokine network and HEV formation, or its synergism with an immunopotentiator. A number of factors important for the localization of adoptively transferred A-NK cells in a tumor remain to be resolved; for example, (a) the difference in the activities of subpopulations of effector cells, (b) the difference in the expression of the antigenic phenotype and adhesion molecules between ''young'' and ''old'' A-NK cells during long-term culture, (c) the difference in the route of administration of A-NK cells, (d) the different doses of IL-2 administered, (e) the subsequent release of cytokines in vivo, and the effect of combination, or synergistic effects, with several kinds of immunopotentiator. These questions should be clarified to establish a much improved modality for adoptive immunotherapy using A-NK cells. We have demonstrated, using an in vivo model, that long-term cultured A-NK cells indeed possess the ability to infiltrate selectively into malignant tissues through ICAM-1/LFA-1 interaction on both HEV-like vessels and tumor cells, and that they exert a suppressive effect on tumor growth in when there has been a prior injection of proper adjuvants. It is, however, still an open question whether these rodent models reflect the tissue distribution and tumor cytotoxicity of A-NK cells in humans.

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