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Therapeutic effect of neuraminidase-treated LAK cells on liver metastasis of Colon 26.

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Abstract

To improve the lymphokine-activated killer (LAK) cell therapy for liver metastasis, two methods which enhance accumulation of LAK cells in the liver were examined for their effects on the liver metastasis of Colon 26 cancer cells in BALB/c mice. Distribution of LAK cells in the mice was examined by the 51Cr labeling method. Portal vein infusion of LAK cells or tail vein infusion of neuraminidase treated-LAK (N-LAK) cells showed an augmented accumulation of infused cells in the liver. In the first experiment, LAK cells (5 x 10(7) cells) were infused in the portal vein or tail vein at days 3 and 7 after the inoculation of 5 x 10(4) tumor cells and 1 x 10(4) units of IL-2 were given three times a day from day 3 to day 7. The portal infusion of LAK cells produced a greater reduction of liver metastases compared with the peripheral infusion. In the second experiment, 5 x 10(7) LAK cells or N-LAK cells were infused via the tail vein on days 1 and 3, and 1 x 10(4) units of IL-2 were given once a day from day 1 to day 5 after the inoculation of 1 x 10(4) tumor cells. The therapeutic effect of N-LAK cells was greater than non-treated LAK cells on the number of metastatic lesions and the survival time of mice. Since access to the human portal vein is difficult and risky in clinical situation, peripheral infusion of N-LAK cells is preferable.

KEYWORDS: lLAK cell, neuraminidase, liver metastasis

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Therapeutic Effect of Neuraminidase-Treated LAK Cells on Liver Metastasis of Colon 26

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To improve the lymphokine-activated killer (LAK) cell therapy for liver metastasis, two methods which enhance accumulation of LAK cells in the liver were examined for their effects on the liver metastasis of Colon 26 cancer cells in BALB/c mice. Distribution of LAK cells in the mice was examined by the ^{51}Cr labeling method. Portal vein infusion of LAK cells or tail vein infusion of neuraminidase treated-LAK (N-LAK) cells showed an augmented accumulation of infused cells in the liver. In the first experiment, LAK cells (5×10^7cells) were infused in the portal vein or tail vein at days 3 and 7 after the inoculation of 5×10^4 tumor cells and 1×10^4 units of IL-2 were given three times a day from day 3 to day 7. The portal infusion of LAK cells produced a greater reduction of liver metastases compared with the peripheral infusion. In the second experipent, 5×10^7 LAK cells or N-LAK cells were infused via the tail vein on days 1 and 3, and 1×10^4 units of IL-2 were given once a day from day 1 to day 5 after the inoculation of 1×10^4 tumor cells. The therapeutic effect of N-LAK cells was greater than non-treated LAK cells on the number of metastatic lesions and the survival time of mice. Since access to the human portal vein is difficult and risky in clinical situation, peripheral infusion of N-LAK cells is preferable.

Key words : LAK cell, neuraminidase, liver metastasis

Lymphokine-activated killer (LAK) cells are generated by cultures of normal lymphocytes with interleukin-2 (IL-2) without antigenic stimulation. They were first documented by Grimm *et al.* as activated lymphocytes having lytic activity for fresh, autologous and syngeneic cells, regardless of whether the tumor target cells were natural killer sensitive or resistant(1, 2). In animal models, transfer of LAK cells and IL-2 induced regression of pulmonary and hepatic metastasis (3). Large doses of IL-2 and LAK cells resulted in tumor regression in patients with certain advanced metastatic cancers, including renal cell carcinoma and melanoma. However, the therapeutic effects were limited to a minority of patients(4, 5), and considerable toxicity such as increase in capillary permeability accompanied the therapy (6).

Further enhancement of the therapeutic effects of LAK cells requires study of the following two problems a) improvement of the accessibility to tumor tissues, and b) enhancement of the anti-tumor cytotoxicity. Motility is important for lymphocytes to infiltrate tumors or other inflammatory lesions. However, tumoricidal activity and motility of LAK cells are independently and sometimes inversely regulated (7). Intercellular adhesion is also important for the killing activity of lymphocytes. Neuraminidase treatment of lymphocytes increases the binding efficiency of lymphocyte subsets with endothelial cells (8). However, this treatment interferes with the normal distribution of lymphocytes in organisms, so that the majority do not home into lymphoid organs but are trapped in the liver (9). In this study, we have examined the effects of neuraminidase-treated LAK (N-LAK) cells on liver metastasis.

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Materials and Methods

Animals. Specific pathogen-free BALB/c mice between 6 and 7 weeks old, were obtained from the Shizuoka Experimental Animal Farm (Hamamatsu, Japan). Mice were housed in groups of 10 or less per cage, and fed an Oriental solid diet (Oriental Yeast Co., Tokyo, Japan).

Tumors. The Colon 26 (10), an undifferentiated colon adenocarcinoma generated from BALB/c mice injected with N-methyl-N-nitrosourethane was cultured *in vitro* with RPMI 1640 containing 10% fetal calf serum (FCS, Grand Island Biological Co., Grand Island, NY, USA). A single cell suspension of these cells, obtained by trypsin treatment, was injected at concentrations of 1×10^4 or 5×10^4 cells per BALB/c mouse body via the mesenteric vein under light ether anesthesia.

Preparation of LAK cells. Spleens were removed, cut finely in the medium, passed through a 150 mesh, and placed in 0.83% NH_4Cl-Tris buffer to lyse erythrocytes, thus isolating lymphocytes. The lymphocytes were washed 3 times with Hanks balanced saline solution (HBSS) and resuspended in complete medium (CM), which was RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 25mM N-2-hydroxyethyl piperazine-N-2 ethane sulfonic acid (Sigma Chemical Co., St. Louis, MO, USA), 2mM L-glutamine (Wako pure Chemical Industries, Ltd., Osaka, Japan), 50μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), streptomycin (100μg/ml), Penicillin G (100 units) and 10% heat inactivated FCS. Lymphocytes were cultured at a concentration of 2.5×10^6 cells/ml in CM added with 1,000 units/ml of human recombinant IL-2 (Shionogi Pharmaceutical Co., Osaka, Japan) under 5% CO_2 in air at 37℃ for 3 days.

Neuraminidase treatment of LAK cells. LAK cells were incubated at 5×10^7 cells/ml with various amounts (0.02-0.5 unit/ml) of neuraminidase (Behring Institute, Germany) at 37℃ for 30 min, and washed three times with HBSS. Cell viability, determined by Trypan-blue dye exclusion test, was not affected by this treatment.

Assay for the distribution of infused lymphocytes. Distribution of LAK cells was examined using normal mice without tumor inoculation. LAK cell suspension (5×10^7 cells) was added with 200μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA, USA) and incubated at 37℃ for 1h. The cells were then washed three times. Approximately 2×10^7 viable cells in 0.2ml were transfused into the tail vein (peripheral infusion) or into the transposed spleen (portal infusion) of 3 mice under anesthesia with ether. Recipients were killed 24h after injection, and the liver, lungs and spleen were removed. The radioactivity of each organ was expressed as a percentage of the total radioactivity of injected cells.

Cytotoxicity assay. Cytotoxic function was examined by ^{51}Cr-release assay. Colon 26 undifferentiated carcinoma cells (10), YAC-cells from a Molony virus-induced lymphoma in A/St mice (11), and JTC-11 cells from Ehrlich ascites tumor (12) were labeled with radioactive chromium and used as target cells. Various numbers of effector cells were incubated with 1×10^4 ^{51}Cr -labeled target cells in 0.2ml of CM in 96-well round-bottomed microtiter plates. The plates were centrifuged at 300 *g* for 5 min and incubated at 37℃ in a 5% CO_2 for 12h. After incubation, 0.1ml of the supernatant was counted in a gamma counter. Cytotoxicity was calculated by the following formula:

$$\% \text{ specific lysis} = 100 \times \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}$$

Transposition of the spleen. Under general anesthesia by ether, each mouse was positioned in the right decubitus, and the skin was sterilized with chlorhexidine alcohol, and a 1cm lateral incision was made in the left subcostal arch region. The spleen was transposed to the subcutaneous region, and the peritoneum and skin were closed, leaving the spleen attached by its vascular pedicle.

Experimental procedure. Protocol 1 (4 groups of 5 mice each) was designed to examine the effects of the portal infusion of LAK cells via the transposed spleen. All mice were inoculated with 5×10^4 Colon 26 cells via a mesenteric vein (day 0). LAK cells were injected at 5×10^7 cells into the portal vein through the transposed spleen or into the tail vein on days 3 and 7. IL-2 (1×10^4 units in 1ml HBSS) was given intraperitoneally every 8h for 5 days after the first transfusion of LAK cells. All were killed 14 days later, and liver metastases were assessed macroscopically. Whitish tumor nodules were mostly localized on the surface of the liver.

In protocol 2 (4 groups of 5 mice each), the effects of N-LAK cells were examined. LAK cells or N-LAK cells were injected at 5×10^7 via the tail vein on days 1 and 3. After inoculation of 1×10^4 tumor cells, IL-2 (1×10^4 units in 1ml HBSS) was given intravenously once a day on days 1 through 5. One group of these mice was killed 21 days later, and the metastatic liver foci were examined. Another group of these mice was observed to measure their survival time.

Statistics. Statistical analysis was performed using Student's *t*-test.

Results

Lymphocyte distribution by portal and peripheral infusion. ^{51}Cr-labeled fresh lymphocytes and LAK cells were infused via the tail vein or portal vein, and the accumulation of radioactivity in the liver, spleen, and lungs was examined at 24h after infusion. As shown in Table 1, LAK cells infused via the portal vein accumlated more in the liver than LAK cells infused via the tail vein. Fresh lymphocytes showed no changes in their distribution by the route of infusion (Table 1).

Effect of neuraminidase treatment on the cytotoxicity

of LAK cells. After the neuraminidase treatment, LAK cells were observed to be morphologically intact and unagglutinated. The cells also showed no significant changes in the cytotoxic activity against JTC-11 and YAC-1 cells by the treatment at different concentrations between 0.01 and 0.5 units/ml of neuraminidase. The cytotoxicity of LAK cells against Colon 26 was not significantly affected by incubation with 0.5 units/ml of neuraminidase (Fig. 1)

Effect of neuraminidase treatment on the distribution of lymphocytes examined in normal mice. Fresh lymphocytes and LAK cells were incubated with 0.5 units/ml of neuraminidase, labeled with ^{51}Cr, and transfused intravenously into the normal mice in which tumor cells were not injected. The radioactivity examined at 24 h after infusion was mainly detected in the liver and spleen. The treatmen of lymphocytes with neuraminidase had so altered their distribution *in vivo* that there was less radioactivity in the spleen and substantially more in the liver. Similar changes were observed in the distribution of LAK cells (Table 2).

Augmentation of therapeutic effect on liver metastasis by portal infusion of LAK cells. This experiment used protocol 1, and metastatic nodules were counted on day 14 for each group. The first group was infused with physiological saline solution via the tail vein, the second

Table 1 Effect of administration route of ^{51}Cr-labeled lymphocytes on accumulation of the radioactivity in tissues of recipients

Infused lymphocytes	Route of administration	Trapping rate(%)[a]		
		Lung	Liver	Spleen
Spleen cells	Tail vein	1.5±0.1	28.2±0.5	30.1±1.8
Spleen cells	Portal vein	1.0±0.2	23.8±1.2	37.6±1.2
LAK cells	Tail vein	3.0±0.2	37.3±3.1 *	32.3±3.4
LAK cells	Portal vein	6.5±1	45.3±2.2 *	31.2±2.6

[a] : Radioactivity of each organ is presented by % of the total activity of injected cells, and values are the mean ± SD of three mice.
* $p < 0.01$ (student's t test)

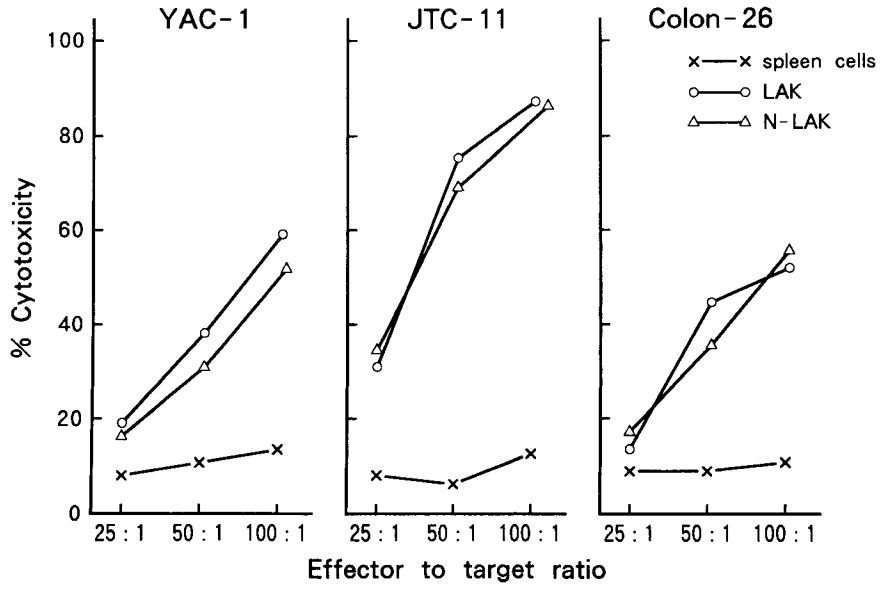

Fig. 1 Effect of neuraminidase on cytotoxic activity of LAK cells. LAK: lymphokine-activated killer; N-LAK: neuraminidase treated-LAK.

Table 2 Effect of neuraminidase treatment of ^{51}Cr-labeled lymphocytes on the distribution of the radioactivity in tissues of recipients

Infused lymphocytes	Neuraminidase treatment	Trapping rate(%)[a]		
		Lung	Liver	Spleen
Spleen cells	−	0.64±0.05	34.5±0.4 *	29.4±1.1 *
	+	0.70±0.02	53.2±2.2 *	13.5±0.6 *
LAK cells	−	0.66±0.01	29.1±3.2 *	36.0±0.3 *
	+	0.71±0.02	55.7±1.5 *	14.2±0.5 *

a : Values are mean ±SD of three mice. * $p < 0.01$ (Student's t test)

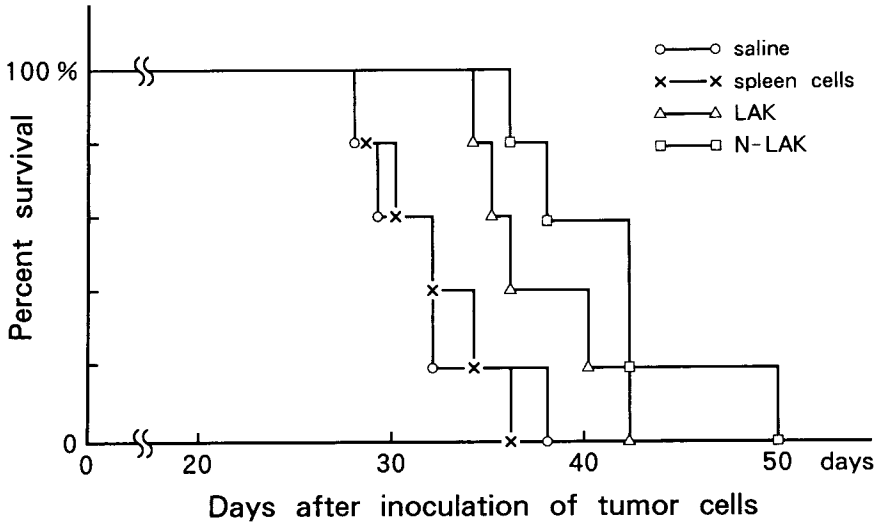

Fig. 2 Effect of transfusion of neuraminidase-treated LAK cells on the survival rate of tumor inoculated mice. LAK, N-LAK: See Fig. 1.

with fresh spleen cells via the portal vein, the third with LAK cells combined with IL-2 via the tail vain, and the fourth with LAK cells combined with IL-2 via portal vein. There were no significant differences between the first two groups. The experimental group infused with LAK cells via the tail vein showed significantly fewer metastases than the control group ($p < 0.01$). Furthermore, the portal infusion of LAK cells led to markedly fewer metastatic nodules than the peripheral infusion ($p < 0.05$)(Table 3).

Augmentation of the therapeutic effect of LAK cells on liver metastasis by in vitro neuraminidase treatment. The experiment was conducted according to protocol 2. There was no detectable difference in the number of metastatic foci between the two groups infused with either physiological saline solution or fresh spleen cells. Compared with the latter group, the group infused with LAK cells and IL-2 showed significant reduction in the number of metastases. Further, the group infused with N-LAK cells and IL-2 showed the greatest reduction of metastases in the three experimental groups, with 44 % fewer metastases than the control (Table 4).

Effect of LAK therapy on survival time of tumor bearing mice. Survival studies were carried out by protocol 2. The mean survival time of the group infused with fresh lymphocytes was 32.0 days. Compared with this group, the experimental group infused with LAK cells or N-LAK cells in combination with IL-2, showed a significantly longer survival time; 37.4 days for the former and 41.6 days for the latter. The significant difference ($p < 0.05$) between these two experimental groups indicat-

Table 3 Effect of transfusion route of LAK cells on liver metastasis

Group	Route	No. of surface tumor[a] nodules in livers	
Saline	Tail vein	> 230	
Spleen cells	Portal vein	225±43	*
LAK cells	Tail vein	98±23	*
LAK cells	Portal vein	24±26	

a : Values are mean±SD of 5 mice. LAK: Lymphokine activated killer. $*p < 0.01$; $**p < 0.05$

Table 4 Effect of transfusion of neuraminidase treated LAK cells on liver metastasis and survival time of tumor inoculated mice

Group	No. of surface tumor[a] nodules in livers		Survival times[b] (days)	
Saline	76.2±7.8		31.8±3.8	
Spleen cells	72.2±7.9	*	32.0±3.1	*
LAK cells	46.4±6.8	**	37.4±3.4	**
N-LAK cells	33.6±4.3		41.6±3.8	

a, *b* : Values are mean±SD of 5 mice. LAK: Lymphokine activated killer; N-LAK: neuraminidase treated LAK.
$*p < 0.01$; $**p < 0.05$

ed a therapeutic effect of N-LAK cells on liver metastasis (Fig. 2, Table 4).

Discussion

The efficacy of cancer therapy with LAK cells depends on their cytolytic potential against tumor cells and their localization in the tumor tissue. For the latter, lymphocytes infused into a tumor-bearing host must avoid being trapped in irrelevant capillary beds, extravasate at appropriate points, and penetrate into primary or metastatic tumor masses. The efficiency of adoptive immunotherapy depends in part upon the motility of infused lymphocytes (13). However, tumoricidal lymphocytes show low activity in spontaneous motility and in chemokinetic response to IFN-γ, showing that LAK cells may not be the best tumor infiltrators (7). The poor locomotion of tumoricidal cells is not simply due to their large size (14, 15). LAK cells are significantly less deformable than fresh NK cells and T cells (16). This increased rigidity combined with their large size may explain the high retention of LAK cells in the lungs immediately after intravenous injection. Therefore, LAK cells should be locally infused toward the target organ having tumor metastases. In the present study, the portal infusion of LAK cells through the transposed spleen was an effective method for delivering lymphocyte to the liver. However, in the case of peripheral infusion, accumlation of LAK cells in the tumor site must be augmented by other methods; augmentation of the motility of lymphocytes, or making a tumor attract the lymphocytes. Concerning the latter method, Hosokawa *et al.* reported that combined anti-cancer chemotherapy using cyclophosphamide enhanced the accumulation of LAK cells in the tumor (17).

Surface properties of lymphocytes control their traffic and distribution in the body. Treatment with a crude glycosidease preparation (18), trypsin (19), or neuraminidase, prevents redistribution of the lymphocytes in the body. Many treated lymphocytes do not home into lymphoid organs but are trapped in the liver until normal membrane properties are recovered (20, 21). We also observed in this study that N-LAK cells were trapped more in the liver and less in the spleen. The liver was the only organ in which increased trapping was found. Neuraminidase-treated rat lymphocytes have been reported to adhere strongly to rat hepatocytes *in vitro* (22). Treatment of lymphocytes with the proteolytic enzyme or neuraminidase has been known to increase cell mediated cytotoxicity, antibody-dependent cytotoxicity (23) and NK activity (24). In the present study, cytotoxic activities of LAK cells were not augmented by neuraminidase treatment, but the infusion of N-LAK cells had a good therapeutic effect on liver metastasis. Some other mechanism may be involved in the effect. Since isolated hepatocytes are mitogens for desialyated T cells (25), accumulated T cells in the liver may be activated to produce any cytokine which may further induce killer cells. In conclusion, N-LAK cells may effectively suppress liver metastses by their enhanced accumulation in the liver.

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