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Abstract

Lymphokine activated killer (LAK) cells can destroy not only tumor cells but also syngeneic liver cells. In this study, the effects of passive transfer of LAK cells on liver regeneration were examined by the 3H-thymidine uptake and bromodeoxyuridine (BrdU) labeling methods after resection of 70% of the volume of the liver. LAK cells were infused 12h after hepatectomy and the effects on regeneration of liver cells were examined 36 h later. The transfusion of LAK cells induced significant inhibition of liver regeneration at a dose of 5-10 x 10(7) cells. Neuraminidase treatment of lymphocytes is desirable to enhance the selective entrapment of LAK cells into the liver. When LAK cells were treated with neuraminidase (0.5 units/ml), and transfused into hepatectomized mice, more potent suppression of liver regeneration was induced in comparison with the same dose of LAK cells. The intraperitoneal injection of recombinant interleukin 2 (rIL-2) after partial hepatectomy also inhibited the regeneration of remnant liver. From these results, lymphocytes such as LAK cells appear to regulate liver regeneration.

KEYWORDS: liver regeneration, hepatectomy, lymphokine activated killer cell, interleukin-2, neuraminidase

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Lymphokine activated killer (LAK) cells can destroy not only tumor cells but also syngeneic liver cells. In this study, the effects of passive transfer of LAK cells on liver regeneration were examined by the ^{3}H-thymidine uptake and bromodeoxyuridine (BrdU) labeling methods after resection of 70 % of the volume of the liver. LAK cells were infused 12h after hepatectomy and the effects on regeneration of liver cells were examined 36h later. The transfusion of LAK cells induced significant inhibition of liver regeneration at a dose of $5-10\times10^7$ cells. Neuraminidase treatment of lymphocytes is desirable to enhance the selective entrapment of LAK cells into the liver. When LAK cells were treated with neuraminidase (0.5 units/ml), and transfused into hepatectomized mice, more potent suppression of liver regeneration was induced in comparison with the same dose of LAK cells. The intraperitoneal injection of recombinant interleukin 2 (rIL-2) after partial hepatectomy also inhibited the regeneration of remnant liver. From these results, lymphocytes such as LAK cells appear to regulate liver regeneration.

Key words : **liver regeneration, hepatectomy, lymphokine activated killer cell, interleukin-2, neuraminidase**

The factors necessary for stimulation and inhibition of liver regeneration after extended hepatectomy or diffuse hepatocellular injury are not fully understood despite many experimental works. Clinically, considerable reliance has been placed on the inherent capacity of hepatocytes to proliferate at the time of lobectomy of the liver and acute hepatic failure. However, many authors have pointed out the failure of adequate regeneration in fulminant hepatitis (1) or liver cirrhosis (2). Information on the stimulatory factors for liver cell growth including insulin and glucagon has greatly increased (3-5), but less is still known about mechanisms possibly involved in negative growth regulation (6,7). We have been interested in the relationship between liver and spleen. Splenectomy along with extended hepatectomy was found to lead to greater liver regeneration, as shown by liver weight measurement (8, 9). Plasma separated from blood of the splenic vein and cytosol from spleen cells are both inhibitors to ^{3}H-thymidine uptake in primary cultured hepatocytes (12,13). Lymphokine activated killer (LAK) cells generated from the spleen during liver regeneration which exhibit greater cytotoxic activity against various tumor cells lines, have been recently shown to be also cytotoxic against primary cultured liver cells (14-16). Spleen cells would thus appear to have inhibitory effect on liver regeneration.

The present investigation was carried out to assess the influence of LAK cells transferred intravenously on hepatic regeneration following extended hepatectomy.

Materials and Methods

Animals. Specific pathogen-free C_3H/He mice aged 6 to 10

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week, were obtained from the Shizuoka Experimental Animal Farm (Hamamatsu, Japan). Five mice were subjected to 70% hepatectomy under ether anesthesia according to the method described by Higgins-Anderson (8), and killed by cervical dislocation for the following examinations.

Measurement of radioactive thymidine in the liver. The rates of DNA synthesis of regenerating liver cells were determined by injection of ^{3}H-thymidine (50 μCi/mouse, spec. act. 20 mCi/mM, New England Nuclear, Boston, MA, USA) 1h before the mice were killed. The liver was removed and homogenized in saline solution, suspended in cooled 10% trichloroacetic acid, and centrifuged twice at 3,500 rpm for 10 min. The precipitate was extracted twice with ethanol, centrifuged at 3,500 rpm for 10 min, then suspended in 5% trichloroacetic acid, and finally heated at 90 ℃ for 15 min. Radioactivity of the acid soluble fraction containing DNA was measured by a liquid-scintillation spectrometer (15). Incorporation of ^{3}H-thymidine into the liver tissue was expressed as disintegration uptake per min per 17.7 mg of the liver (wet weight).

Measurement of bromodeoxyuridine (BrdU) labeling index in vivo. The mice were injected intravenously with 40 mg of BrdU (Sigma, St. Louis, MO, USA) per kg 36h after operation. The remaining livers were removed and their wet weight was measured. Blocks of liver were fixed in 70% ethanol, embedded in paraffin. Deparaffinized 3 μ-thick sections were mounted on glass slides. Each section was treated at 20℃ for 2h in PBS containing a 1:20-dilution of monoclonal anti-BrdU antibody (Becton Dickinson, Mountain View, CA, USA), and treated with 1:200 diluted biotinated anti-mouse antibody (Vector Laboratories, Inc., Burlingame, CA, USA) at 20℃ for 30min. The sections were then treated with Avidin-biotin-peroxidase complex (Vector) for 30min and stained with 33'-diamino benzidine-tetrahydrochloride as previously reported (16,17). For determination of hepatocyte labeling indices, the number of positive stained hepatocyte nuclei was counted with a light microscope using a high power objective. Labeling indices (LI) for a single liver was assumed as number of labeled cells in two thousand hepatocytes. Inhibition of liver regeneration was caluculated by the following:

$$\text{Inhibition rate} = 100 \times \frac{\text{control LI} - \text{experimental LI}}{\text{control LI}}$$

Preparation of spleen cells. The spleens were thoroughly cut in medium, passed through a mesh, and placed in 0.83% NH_4Cl-Tris buffer for erythrocytes lysis. The lymphocytes were isolated and washed 3 times, and then resuspended in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan). Complete Medium (CM) was supplemented with 25 mM N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (Sigma Chemical Company, St., Louis, MO, USA), 2 mM L-glutamine (Wako Pure Chemical Ind., Ltd., Osaka, Japan), 50 μM 2-Mercaptoethanol, streptomycin (100 μg/ml), penicillin G (100 units) and 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Gland Island, NY, USA).

Induction of LAK cells. Spleen cells were prepared at a concentration of 2.5×10^6 cells/ml, and cultured in CM with the addition of 10×10^3 units/ml of human recombinant IL-2 (rIL-2, Shionogi Pharmaceutical Company, Osaka, Japan) under 5% CO_2 in air at 37℃ for 3 days.

Neuraminidase treatment of LAK cells. 5×10^7 LAK cells in 1.0ml of CM were incubated with different amounts (0.02-0.5unit/ml) of neuraminidase (N-LAK cells)(Behring Ingelheim Institute, Germany) at 37℃ for 30min, and washed three times. Cell viability was not affected by this treatment.

Assay for distribution of infused lymphocytes. To cell suspension which includes 5×10^7 LAK cells/ml was added 200 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA, USA) and this mixture was incubated for 1h at 37℃. The cells were then washed three times and adjusted to 1×10^8 cells/ml. A total of 2×10^7 lymphocytes were transfused into the tail vein of mice lightly anesthetized with ether. Recipients were killed and various organs were removed. The spleen, liver, lungs, and kidneys were weighed and assayed for radioactivity. Values were expressed by the following:

$$\%\ \text{accumulation} = \frac{\text{cpm of the each organ}}{\text{cpm of total infused lymphocytes}}$$

Isolation of parenchymal liver cells and non-parenchymal liver cells. Liver cells were isolated by the technique of Seglen (18). The liver was perfused through a cannula in the inferior vena cava with Mg^{2+}, Ca^{2+}-free Hanks balanced saline solution (HBSS) followed by the same buffer also containing 5.7 mM $CaCl_2$ and 0.1% collagenase (Wako). The liver was thoroughly cut in medium, and the dispersed cells were sedimented three times at low centrifugation force ($50 \times G$) for 3min. The remaining pellets contained pure parenchymal cells in the final preparation.

Cytotoxicity assay. Cytotoxic function was examined by ^{51}Cr-release assay. YAC-1 cells from a Moloney virus-induced lymphoma in A/St mice, JTC-11 cells (19) from Ehrlich ascites tumor and isolated murine hepatocytes were labeled with radioactive chromium and used as target cells for cytotoxicity. A number of effector cells were incubated with 10×10^4 ^{51}Cr-labeled YAC-1 cells or JTC-11 cells, or also 5×10^3 ^{51}Cr-labeled hepatocytes in a total volume of 0.2ml/well in 96-well round-bottomed microtiter plates. The plates were centrifuged at $300 \times g$ for 5min and incubated for 12h at 37℃ in a 5% CO_2. After incubation, 0.1 ml of the supernatant was counted in a gamma counter. The percentage of ^{51}Cr-specific release was determined by the following:

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

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

Results

Effects of transfusion of LAK cells on regeneration of liver cells following partial hepatectomy. All the mice survived after 70% extended hepatectomy. The mean weights and mitotic indices of hepatic remnants are shown in Fig. 1. The weights of the remaining livers have rapidly increased, and recovered preoperative values by postoperative day (POD) 7. The BrdU labeling indices of the remnant livers have also rapidly increased, reaching a maximum at 36h, and gradually decreased thereafter. Nearly same results were obtained by the ^{3}H-TdR incorporation method. The effects on liver regeneration were examined at 36h following hepatectomy too. LAK cells derived from hepatectomized mice on POD 7 were transfused into the tail vein of other mice 12h after hepatectomy. The incorporation of ^{3}H-thymidine into regenerating liver at 36h after the operation was significantly suppressed in LAK cell transfused mice as compared to control group infused with saline solution only (Table 1). The effects of LAK cells on liver regeneration were also evaluated by the BrdU labeling method following the same manner. Production of LAK cells were induced from spleen cells from non-hepatectomized mice. The infusion of LAK cells in hepatectomized mice inhibited liver regeneration in the range of 10 − 40% of the inhibition rate at doses of 5×10^7 and 1×10^8 LAK cells (Table 2).

Effects of neuraminidase treatment on LAK cytotoxicity. LAK cells incubated with different amounts of neuraminidase appeared morphologically intact and unagl-

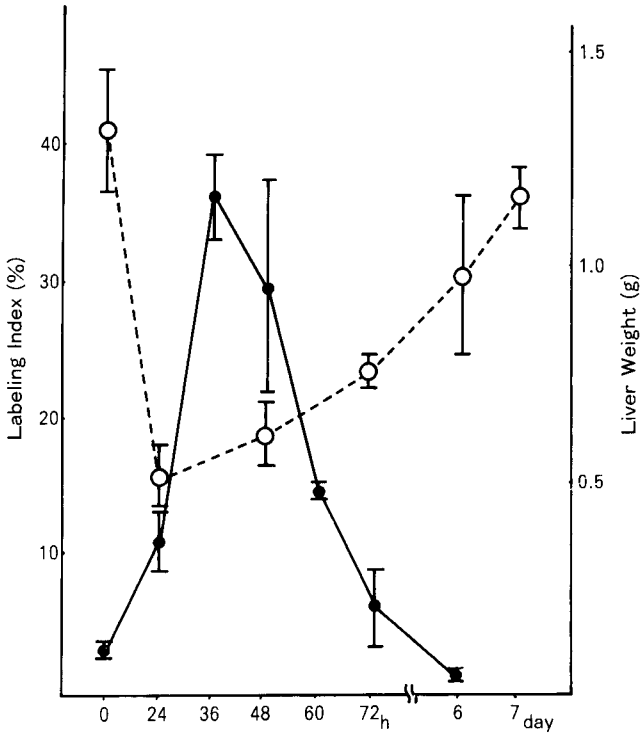

Fig. 1 Recovery of liver weights after partial hepatectomy (○) and kinetics of BrdU labeling indices of remnant liver cells (●). Each plot is a mean of 10 mice.

Table 1 Effects of transfusion of LAK cells on ^{3}H-thymidine uptake of liver cells following partial hepatectomy

Treatments[a]	^{3}H-thymidine Uptake[b]	Inhibition Rate (%)[c]
Saline	274,305 ± 45,927	
Fresh Spleen cells 5×10^7	262,766 ± 31,511	
LAK cells 5×10^7	189,743 ± 18,629[d]	30.8

a: C_3H/He mice were injected i.v. with saline only, fresh spleen cells or LAK cells at 12h after partial hepatectomy. *b*: Values are the mean ± SD of radioactivities (DPM) of 4 mice. *c*: Inhibition rate: $100 \times (1 -$ experimental DPM/saline DPM) *d*: Significantly less than the saline control, $P < 0.05$.

Table 2 Effects of transfusion of LAK cells on BrdU labeling index of liver cells following hepatectomy

Experiments	Treatments[a]	BrdU labeling index[b]	Inhibition rate (%)[c]
1.	Saline	52.8 ± 2.2	
	Fresh Spleen cells 5×10^7	50.2 ± 2.1	
	LAK cells 5×10^7	46.8 ± 4.6[d]	11.4
	LAK cells 1×10^8	31.2 ± 2.1[e]	40.9
2.	Saline	43.2 ± 3.9	
	Fresh Spleen cells 5×10^7	42.4 ± 4.9	
	LAK cells 5×10^7	41.8 ± 4.5	
	LAK cells 1×10^8	30.9 ± 5.3[f]	28.5

a: C_3H/He mice were injected i.v. with saline only, fresh spleen cells, LAK cells or LAK cells at 12h after partial hepatectomy. *b*: Values are the mean ± SD of labeling indices of mice. *c*: Inhibition rate: $100 \times (1 -$ experimental LI/saline LI). *d*: Significantly less than the saline control, $p < 0.05$. *e–f*: Significantly less than saline control, $p < 0.01$.

Table 3 Effect of neuraminidase on cytolytic activity of LAK cells

Neuraminidase treatment of LAK cells (units/ml)	Target	Cytotoxicity(%) Effector/Target 5	10	20	40	80
0	YAC-1	50.8	70.2	87.0	88.0	87.0
0.02	YAC-1	48.0	76.0	86.0	86.0	87.0
0.1	YAC-1	53.0	74.0	84.0	87.0	89.0
0.5	YAC-1	51.2	68.1	87.4	88.0	88.0
0	JTC-11	45.1	56.5	87.0	91.0	87.1
0.02	JTC-11	41.0	58.7	74.5	85.2	92.4
0.1	JTC-11	40.0	58.0	87.6	89.0	86.0
0.5	JTC-11	43.0	55.0	90.2	92.6	89.8

lutinated under a light microscope. No significant changes were observed in cytotoxic activity against JTC-11 and YAC-1 cells at any concentration up to 0.5 units/ml of neuraminidase (Table 3). LAK cells cytotoxicity against regenerating liver cells was not significantly affected by incubation with 0.5 units/ml of neuraminidase (Fig. 2).

Effects of neuraminidase treatment on the distribution of infused lymphocytes. ^{51}Cr labeled lymphocytes mainly accumulated in the liver and spleen. Neuraminidase treatment of lymphocytes greatly altered the distribution of labeled lymphocytes leading to predominant accumulation in the liver as compared with spleen, which

Table 4 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 the mean ± SD of three mice. An asterisk shows $p < 0.01$.

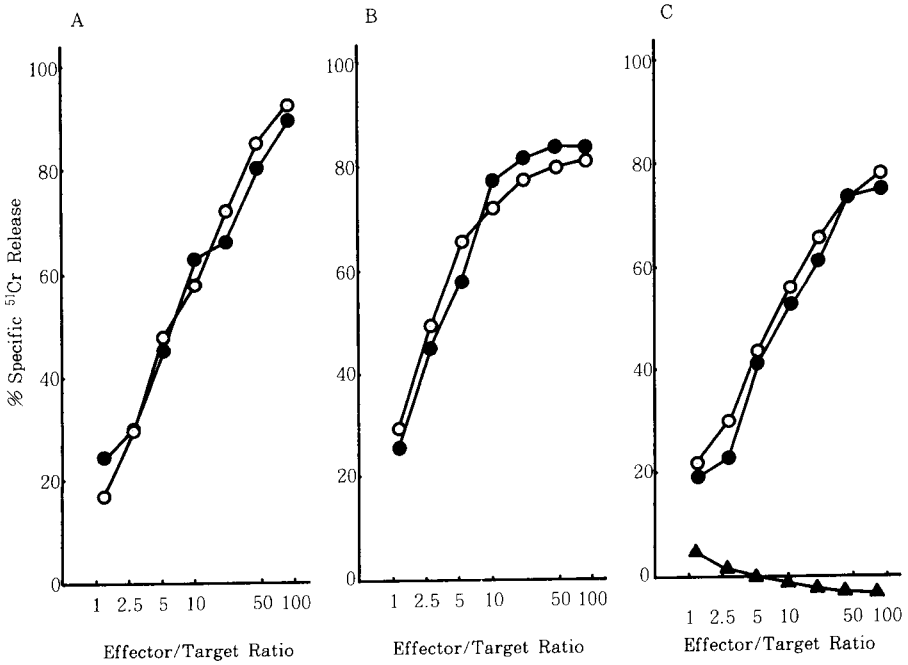

Fig. 2 Effect of neuraminidase treatment on the cytotoxicity of LAK cells against JTC-11 (A), YAC-1 (B) and regenerating liver cells (C). ○, untreated LAK cells; ●, neuraminidase-treated LAK cells; ▲, fresh spleen cells.

showed lessened radioactivity. These alterations were similar to that observed for LAK cells (Table 4).

The distribution of LAK cells was further examined in the recipients which have undergone 70 % hepatectomy 12 h before infusion of ^{51}Cr labeled LAK cells. The percentage of recovery of radioactivity in the remnant liver markedly decreased in recipients infused with untreated LAK cells. By infusion of N-LAK cells, the accumulation in the remnant liver was significantly enhanced. However, a parallel change in accumulation in the spleen was absent (Fig. 3).

Effect of N-LAK cells on the regeneration of liver cells after extended hepatectomy. The infusion of 5×10^7 N-LAK cells caused the most potent inhibition in the three separate experiments, reaching 50 % of the labeling index in the controls infused with fresh spleen cells (Table

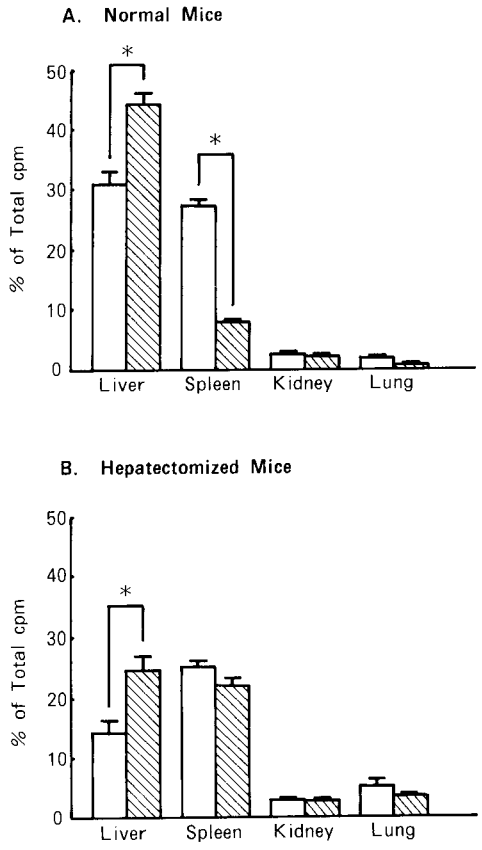

Fig. 3 Effects of neuraminidase-treatment on the *in vivo* distribution of LAK cells. LAK cells were incubated with medium alone (open columns) and in the presence of 0.5 units/ml neurarinidase (closed columns), labeled with ^{51}Cr and transfused intravenously into normal mice (A) and hepatectomized mice to which the operation was undergone before 12 h(B). Radioactivity of each organ was presented by % of the total activity of injected cells, and the value are the mean ± SD of 5 mice. An asterisk shows $p < 0.01$.

Table 5 Effects of transfusion of neuraminidase-treated LAK cells on BrdU labeling index of liver cells following partial hepatectomy

Experiments	Treatments[a]	n[b]	BrdU labeling index[c]	Inhibition rate (%)
1.	Saline	5	31.1 ± 2.8	
	Fresh Spleen cells 5×10^7	4	32.0 ± 4.7	
	LAK cells 5×10^7	5	31.2 ± 3.3	
	N-LAK cells 5×10^7	5	14.8 ± 5.0	52.4
2.	Saline	5	28.4 ± 1.1	
	Fresh Spleen cells 5×10^7	5	27.8 ± 5.3	
	LAK cells 5×10^7	5	23.7 ± 2.0[d]	16.5
	N-LAK cells 5×10^7	5	13.1 ± 5.3[e]	53.8
3.	Saline	4	43.3 ± 4.4	
	Fresh Spleen cells 5×10^7	5	41.4 ± 3.0	
	LAK cells 5×10^7	4	39.8 ± 5.1	
	N-LAK cells 5×10^7	5	19.1 ± 7.5[e]	55.9

a: C_3H/He mice were injected i. v. with saline only, fresh spleen cells, LAK cells and N-LAK cells at 12 h after partial hepatectomy. *b*: number of mice. *c*: BradU positive hepatocytes were counted at 36 h after partial hepatectomy. Values are the mean ± SD of labeling indices of 5 mice. *d*: Significantly less than the saline control, $p < 0.05$. *e*,: Significantly less than the group infused with LAK cells, $p < 0.01$.

Table 6 Effects of IL-2 administration on BrdU labeling index of liver cells following partial hepatectomy

Treatments[a]		BrdU labeling index[b]	Inhibition rate
Medium		30.95 ± 5.73	
rIL-2	1,000 units	26.80 ± 7.12	13.5 %
rIL-2	5,000 units	18.75 ± 3.12[c]	39.5 %
rIL-2	10,000 units	17.28 ± 4.94[d]	44.2 %

a: C_3H/He mice were injected i.v. with various dose of IL-2 at 12 h after partial hepatectomy. *b*: BrdU positive hepatocytes were counted at 36 h after partial hepatectomy. Values are the mean ± SD of labeling indices of 5 mice. *c*: Significantly less than the medium control, $p < 0.05$. *d*: Significantly less than the medium control, $p < 0.01$.

5).

Effect of rIL-2 administration on the regeneration of liver cells following extended hepatectomy. Mice were given intraperitonealy various doses of rIL-2 5 times every 8h after hepatectomy. Inhibition of liver regeneration was related to the amount of rIL-2. The average of inhibition of labeling index using 1×10^3, 5×10^3, 10×10^3 units of rIL-2 was 13.5%, 39.5%, and 44.2% respectively (Table 6). Similar results were confirmed by ^{3}H-thymidine assay using 10×10^3 units/body of rIL-2 (data not shown).

Discussion

NK cells mediate their cytotoxicity to malignant cells, virally infected cells, and rapidly dividing normal cells (20); and may be responsible for the regulation of hepatic growth. NK cells, predominantly existing in the peripheral blood and other lymphoid organs, were also detected in the lungs (21), liver (22) and intestine (23). Examinations of proportion and activity of NK cells among liver mononuclear cells in mice of various ages have shown that the increase in the number of liver NK cells appeared to coincide with a slowing of the rapid weight increase of murine liver. *In vitro* data have indicated liver NK cells to be cytototxic against regenerating liver cells in the partially hepatectomized liver (24). Furthermore, we have previously reported that LAK cells induced from spleen of hepatectomized mice are cytotoxic against regenerating liver cells (13,14).

In the present study, LAK cells intravenously transfused after hepatectomy were found to suppress liver regeneration, and the intensity of this effect varied depending on the dose of infused LAK cells. Since the DNA content of the liver would be rapidly changed by the trapped LAK cells, when using radioactive thymidine, regeneration of the liver was expressed in terms of radioactivity per unit weight of liver tissue instead of per unit liver DNA. These results agreed well with those of the BrdU labeling method. The latter method was more appropriate in this study because DNA replication of nonparenchymal cells could be excluded by microsopic observation.

At the time of infusion, a maximal dose of LAK cells were limited to 1×10^8 cells to avoid a risk of pulmonary embolism. Neuraminidase treatment of LAK cells is desirable to enhance selective entrapment of LAK cells in the liver. Treated lymphocytes become trapped in the liver accompanied by a reduction in the accumulation of cells in the lymph nodes and spleen (25). Most neuraminidase-treated lymphocytes do not return to lymphoid organs, but are trapped in the liver until normal membrane properties are resumed (26). Adhesion between hepatocytes and neuraminidase-treated lymphocytes is probably due to binding between mammalian hepatic membrane lectin and galactosyl residues exposed to the lymphocyte surface after removal of sialic acid residues (27). In this study, the trapping rate into the liver 24h after infusion increased about two folds by treatment with neuraminidase. N-LAK cells caused no change in cytotoxic activity toward regenerating liver cells, but infusion of these lymphocytes induced greater inhibition of hepatic regeneration. These results showed that N-LAK cells effectively affect on liver regeneration under the condition affording access to liver cells. LAK cells were supposed to secrete a growth inhibitory factor, or to mediate cytotoxic activity against hepatocytes by direct contact. Regarding the growth inhibitory factor, it was reported that tumor growth factor-beta (TGF-β) acts *in vitro* as a potent inhibitor of cell proliferation in many types of cells, including non-neoplastic and neoplastic cells. TGF-β may cause an irreversible inhibition of a normal diploid hepatocytes (28), and TGF-β mRNA increases in the non-parenchymal cells of regenerating liver, reaching its peak after a major wave of hepatocyte cell division (29). Thus, TGF-β may be involved in hepatocyte growth response *in vivo*. Since proliferating lymphocytes increase TGF-β mRNA (30), suppression of liver regeneration by infused LAK cells may also be due to TGF-β. For LAK cells, both adhesion to the sinusoidal wall and extravasation, depending on the size and deformability of cells, are pre-requisite to exhibit cytotoxicity against liver cells. However, LAK cells are significantly less deformable than other cells such as large granular lymphocytes and fresh T cells (31). Once LAK cells contact regenerating hepatocytes, they may be further activated since hepatic lectin is mitogenic for desialyated T cells (32). Thus, the killing activity of N-LAK cells may be enhanced more than would expected from the results obtained by *in vitro* assay.

The suppressive effect of LAK cells on liver regeneration demonstrated by this study is not a natural phenomenon. It has been reported that IL-2 production of spleen cells was augmented after hepatectomy (14); and IL-2 enhanced NK activity (28), generated LAK cells *in vivo*

(33,34), and also induced killer cells against hepatocytes in the liver (35). In this study, administration of IL-2 during the early phase after hepatectomy dose dependently inhibited liver regeneration, supporting the hypothesis that lymphocytes such as NK cells and Pit cells may regulate liver regeneration *in vivo*.

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