Tumor-infiltrating Lymphocytes and Prognosis of Hepatocellular Carcinoma

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Tumor-infiltrating lymphocytes (TIL) were isolated from 17 human hepatocellular carcinomas (HCC). The proliferation of TIL cultured with recombinant interleukin-2 (rIL-2) was evaluated. We also examined prognosis in relation to TIL. Successful expansion of TIL was achieved in 16 of the 17 lesions. In 10 of the 16, TIL increased more than 100-fold. Cytotoxicity to the allogeneic HCC cell line, HC-4, was demonstrated in all 13 TIL cultures tested. Maximum cytotoxicity was noted two to four weeks after culture. No statistical difference was observed either with respect to prognosis based upon growth rate or the cytotoxicity demonstrated *in vitro*. The initial number of TIL per unit weight of tumor was, however, significantly greater in the group for which the prognosis was good ($19.0 \pm 5.1 \times 10^6$ vs. $5.6 \pm 1.6 \times 10^6$, $P < 0.05$). It would appear that greater lymphocytic tumor infiltration is a prognostic marker.

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Key words: Tumor-infiltrating lymphocyte—Hepatocellular carcinoma—Interleukin-2—Prognosis

Introduction

It is suggested that tumor-infiltrating lymphocytes (TIL) represent a local immune response to cancers and are a biological defense mechanism against cancer cells.[1,2] A favorable prognosis has been noted in a number of cases in which the TIL response was high.[1–4] Others have suggested that TIL are neither cytotoxic nor influential in biological defense immediately after isolation.[5,6] The role of TIL *in vivo* remains undetermined; nevertheless, the adoptive transfer of TIL activated *ex vivo* appears to be a promising therapeutic maneuver. Adoptive transfers of activated TIL have been reported to be 50–100 times more effective than lymphokine-activated killer cells in animal models.[7] Clinical trials have been instituted for patients with malignant melanoma and other malignant tumors.[8–11] While there have been few recent studies on the character of TIL in hepatocellular carcinoma (HCC),[12,13] there have been reports on the relation between TIL isolated *in vitro* and prognosis.

We have attempted to isolate TIL from resected HCC. We have also studied growth rate and cytotoxicity of TIL after culture with recombinant interleukin-2 of TIL (rIL-2). The present study on the relation between TIL and prognosis suggests a high yield of lymphocytes collected from HCC to be a favorable prognostic marker.

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Materials and Methods

Patients

Fresh tumor tissues were obtained from 17 HCC patients who underwent surgery at Hokkaido University Hospital during the period March to September, 1988. Fourteen men (aged 37–75 yr) and three women (aged 31, 62 and 66 yr) (Table I) were the subjects of the present study. Disease was staged according to the TNM system of the International Union Against Cancer (UICC).[14] Eight of the 17 patients were HBs-Ag positive. Fifteen TIL specimens were obtained from primary sites and two from rib metastases. In most primary tumors, the lesions were classified as Edmondson's grade II. In seven of the 15 primary tumors, the liver was cirrhotic.

Preparation of TIL and Peripheral Blood Lymphocytes (PBL)

Single cell suspensions of HCC were obtained from surgical specimens weighing 0.25–20 g. Utiliz-

ing sterile techniques, tumors were sectioned into fragments of approximately 2 mm^3. These were immersed in RPMI 1640 medium containing hyaluronidase 0.0025%, deoxyribonuclease 0.002%, collagenase type IV 0.1% (Cooper Biochemicals, Malvern, PA), penicillin (50 U/ml), streptomycin (50 μg/ml), gentamycin (50 μg/ml) and amphotericin (20 μg/ml). The mixture was stirred for 2–6 h at 37°C. The cell suspensions were then filtered, washed and centrifuged over a Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient at 400 g for 20 min. All cells were counted and their viability was assessed by the trypan blue dye exclusion test.

Peripheral blood was taken *via* venipuncture from each patient at least one week prior to surgery. Blood was stored in heparinized tubes. PBL were obtained by Ficoll-Paque gradient separation.

Phenotypic Analysis of Fresh TIL and PBL

Freshly isolated TIL (1×10^6) were incubated with 5 μl fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody in 1.5 ml phosphate buffered saline (PBS) with EDTA at 4°C for 30 min. The cells were washed twice at 550 g for five min then analyzed using flow cytometry (Spectrum III, Ortho Diagnostic Systems Inc., Raritan, NJ). Cell surface phenotypes of both TIL and PBL were examined utilizing the following monoclonal antibodies: OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8), OKM1 (anti-CD11b) (Ortho Diagnostic Systems Inc.), IL-2-R (anti-CD25), anti-HLA-DR (activated T, NK cells) (Becton Dickinson, Mountain View, CA), B1 (anti-CD20) (Coulter Immunology, Hialeah, FL).

Culture of TIL with rIL-2

TIL were cultured with 1000 JU/ml rIL-2 (Shionogi & Co. Ltd., Osaka) in RPMI 1640 supplemented with 10% (v/v) heat-inactivated human serum, antibiotics and L-glutamine at 37°C in a humidified atmosphere of CO_2 in air. The initial concentration of TIL was adjusted to 1×10^6/ml and maintained at $0.5–2 \times 10^6$/ml throughout the culture period. The medium containing rIL-2 was added to the culture at intervals to adjust the cell concentrations. The expansion multiple was calculated during culture on the basis of viable cell counts.

Cytotoxicity Assay

We carried out a four-hour ^{51}Cr-release assay to measure the cytotoxic activity of TIL. In brief, target cells (cell lines: NK-sensitive K562, NK-insensitive Daudi, human hepatocellular carcinoma HC-4) were labeled with 100–200 μCi sodium ^{51}chromate (New England Nuclear, Boston, MA) for one hour at 37°C. The cells were then washed twice and used as targets at 1×10^4 cells/well. rIL-2-expanded TIL were incubated with ^{51}Cr-labeled target cells in a 96-well round-bottomed microplate (Corning, 25850) at the effector:target (E:T) ratios: 20:1, 10:1, 5:1, in 5% CO_2 in air at 37°C for four hours. Spontaneous ^{51}Cr-release was determined by the incubation of target cells without effectors, and maximal release was determined by

Table I. Tumor-infiltrating Lymphocytes Isolated from Hepatocellular Carcinoma

Patient no.	Age (yr)	Sex	TNM classification	HBs-Ag	Sources	Cirrhosis*	Edmondson's classification	Lymphocytes/g ($\times 10^6$/g)	Prognosis
1	65	M	IVB	+	Rib metastases	—	—	8.0	4 mo DOD
2	39	M	II	+	Liver	Z0	III	ND	34 mo NED
3	57	M	III	+	Liver	Z1	II	8.3	2 mo DOD
4	75	M	III	−	Liver	Z0	I	4.2	14 mo DOD
5	61	M	I	−	Liver	Z0	II	20	33 mo NED
6	66	M	III	+	Liver	Z0	II	6	33 mo NED
7	66	F	II	−	Liver	Z0	II	20	33 mo NED
8	42	M	II	+	Liver	Z3	II	8.0	33 mo NED
9	55	M	III	−	Liver	Z0	II	2.6	9 mo DOD
10	57	M	II	−	Liver	Z0	II	13	19 mo DOD
11	68	M	III	−	Liver	Z3	II	50	31 mo NED
12	67	M	II	−	Liver	Z3	II	16	30 mo NED
13	62	F	II	−	Liver	Z2	II	6	30 mo NED
14	37	M	III	+	Liver	Z1	II	26	30 mo NED
15	46	M	IVB	+	Rib metastases	−	−	0.5	13 mo DOD
16	31	F	IVB	−	Liver	Z0	II	0.3	23 mo DOD
17	46	M	II	+	Liver	Z2	II	8.2	16 mo DOD

* Macroscopic classification of cirrhosis of the liver: Z0, none; Z1, slight; Z2, moderate; Z3, severe
M, male; F, female; DOD, died of disease; NED, no evidence of disease

the incubation of target cells with 0.1 N HCl. All determinations were performed in triplicate. At the end of the incubation period, the radioactivity in 0.1 ml supernatant was counted. Percentage specific lysis was calculated as:

$$\frac{\text{Experimental release (cpm)} - \text{Spontaneous release (cpm)}}{\text{Maximal release (cpm)} - \text{spontaneous release (cpm)}} \times 100.$$

Prognosis

The long-term survival rate was obtained from the records of our out-patients' clinic. We assessed the correlation between the TIL features and the clinical prognosis of the HCC patients.

Statistical Analyses

All results are shown as mean ± SEM. Student's *t*-test and the Chi-squared test were used for statistical analyses. Survival curves after hepatic resection were obtained using the Kaplan-Meier method and analyzed by the generalized Wilcoxon's test.

Results

Recovery of TIL from Hepatocellular Carcinoma

Seventeen human hepatocellular carcinomas (15 primary sites, two metastatic sites) were processed to isolate their TIL (Table I). The mean wet weight of tumor tissue processed was 3.2±1.3 (range 0.25–20.0) g. The mean number of recovered TIL was $1.23 \pm 3.1 \times 10^6$/g (range $0.3–50 \times 10^6$/g). The viability of recovered TIL was greater than 90%, judging by the trypan blue dye exclusion test.

Comparison between Phenotypes of Fresh TIL and PBL

The surface phenotypes of fresh TIL isolated from 14 tumors were analyzed using flow cytometry. It appeared that TIL harvested from HCC contained mainly CD3 positive cells, suggesting a rare infiltration of B lymphocytes or NK cells. As for PBL (Table II), the CD25 positive population of TIL was a relatively minor cellular component (6±3%).

Expansion of TIL in rIL-2

Freshly isolated TIL were cultured with 1000 JU/ml rIL-2 for up to 60 days. Fig. 1 shows characteristic patterns of growth and expansion of TIL. Usually, the TIL starts to expand after one to two weeks of culture with rIL-2. As the lymphocytes grew rapidly, most of the tumor cells disappeared. The number of TIL increased >100-fold in 10 of the 17 cases, and in five cases the TIL increased >1000-fold. In one case, the harvested TIL increased 120,000-fold by culturing them in rIL-2. The number of lymphocytes failed to increase in case 13, but in this instance, the TIL surface phenotypes differed totally from those of the others: only 2.7% of these TIL expressed HLA-DR$^+$surface marker.

Table II. Phenotypic Analysis of Freshly Isolated TIL and PBL Obtained from Hepatocellular Carcinoma Patients

	Positive cells (%)			
	TIL (n15)		PBL (n15)	
	Mean	SEM	Mean	SEM
CD3	55	5	65	3
CD4	33	4	44	3
CD8	27	3	28	3
CD25	5	1	6	1
HLA-DR	28	4	21	3
CD16	5	2	10	2
CD11b	12	2	30	4*
CD20	6	1	13	1**

Statistically significant difference in phenotypes between TIL and PBL: *$P<0.01$, **$P<0.005$.

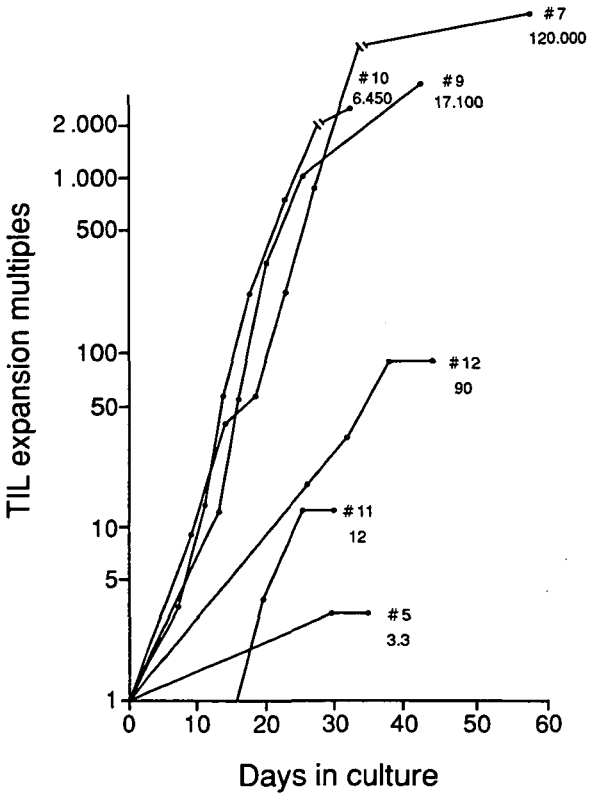

Fig. 1. Expansion pattern of tumor infiltrating lymphocytes isolated from hepatocellular carcinoma. Number after each line corresponds to patient's number in tables I and III.

Phenotypic Changes during TIL Expansion in rIL-2

In most cases the TIL surface phenotypes expanded by rIL-2 were monitored serially throughout the period of culture (Fig. 2). The majority of the rIL-2-expanded TIL in all cultures were $CD3^+$ and $HLA\text{-}DR^+$, while a substantial proportion as also $CD25^+$. The increase in the percentage of $CD3^+$, $HLA\text{-}DR^+$ or $CD25^+$ after culture was significant ($P<0.001$).

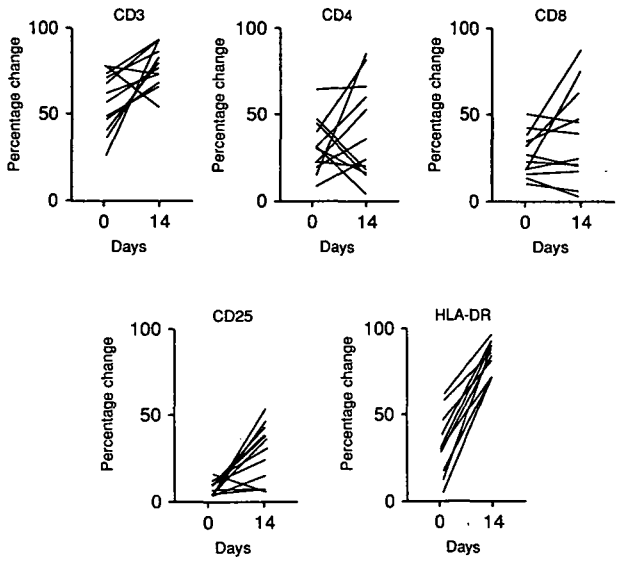

Fig. 2. Phenotypic TIL culture changes with time. The majority of TIL comprised a high percentage of $CD3^+$ cells and had a tendency for $HLA\text{-}DR^+$ to increase. The increase in the percentage of $CD3^+$, $HLA\text{-}DR^+$ or $CD25^+$ after culture was significant ($P<0.001$).

Cytotoxic Activity of rIL-2-expanded TIL

The rIL-2-expanded TIL were serially assayed for cytotoxic activity against ^{51}Cr-labeled tumor cell targets. Cytotoxicity against the allogeneic human hepatocellular carcinoma cell line, HC-4, was detected in all 13 cultures tested. The rIL-2-expanded TIL preparations reached a high level of activity after between two and four weeks in the culture (Table III). Eight of the 13 cases exhibited such a strong cytotoxic activity that they lysed over 50% of Daudi cells in a 4h- ^{51}Cr release assay (E : T = 20 : 1). At that time, six of the eight showed a reduced CD4:CD8 value of <0.9, owing to a decrease in the percentage of $CD4^+$ cells together with an increase in $CD8^+$ cells. In one case, the harvested TIL showed 46% lysis of the HC-4 cells, but only low cytotoxic activity against K562 and Daudi targets. In four cases the total cell yield was so small that we were not able to determine any cytotoxic activity.

TIL and Concomitant Liver Disease and Stage of Tumor

There were no statistical differences in the initial number of TIL per unit weight or in the cytotoxicity against allogeneic tumors between cirrhotic and non-cirrhotic livers (Table IV). While the ability of the lymphocytes from cirrhotic livers to grow tended to be lower than that of those from non-

Table III. Cytotoxic TIL Activity Assayed in 13 of the 17 Cases

Patient no.	Days in culture	CD4:CD8	Tumor targets (% lysis)								
			Daudi (E:T)			K562 (E:T)			HC-4 (E:T)		
			20:1	10:1	5:1	20:1	10:1	5:1	20:1	10:1	5:1
1	28	0.7	78	80	79	77	72	67	88	70	51
2	19	2.8	91	91	86	97	90	79	100	100	66
3	21	13.9	23	13	6	25	17	5	12	4	3
4	14	0.5	84	74	69	97	91	73	97	91	65
6	21	0.3	0	0	0	22	17	4	46	37	26
7	14	0.5	88	71	42	93	88	54	44	37	26
8	14	11.3	44	28	18	57	36	20	36	19	10
9	25	2.2	82	58	33	81	51	27	45	23	14
10	18	0.1	23	17	9	25	16	7	24	15	5
11	20	0.8	74	53	50	79	62	35	27	15	7
			(97	86	88)	(99	95	93)	(100	93	70)
12	18	0.9	75	59	38	71	54	24	44	35	12
			(72	65	62)	(85	87	79)	(99	94	70)
14	24	0.3	4	3	0	27	14	5	13	8	5
15	21	0.9	55	42	22	21	14	7	37	23	12

E:T, effector: target

Numbers in parentheses indicate lysis by LAK cell effectors in simultaneous assay.

cirrhotic livers, there was no statistical difference ($P=0.094$). No difference with respect to lymphocyte surface markers was observed (data not shown). Among stages of tumor, there was no obvious difference in the rate of lymphocyte expansion or in cytotoxic ability. We found no significant difference in the number of the harvested TIL between cases at stages I and II and those at stages III and IV.

TIL Character and Survival

By March, 1991, nine of the 17 patients remained alive, having suffered no recurrence of their disease. Eight had died from the disease within two years after surgery. An assessment of how the TIL features related to prognosis was made in 16 cases (no. 2 was not assessed because some features of the case had been omitted). The number of TIL recovered was $19.0\pm5.1\times10^6$ cell/g in the patients who survived for more than two years without recurrence, compared to $5.6\pm1.6\times10^6$ cell/g in patients who died from the disease within two years (Table V). This tendency did not relate to tumor stage. Neither the expansion rate nor the cytotoxic activity of TIL had any appreciable influence on the survival of patients according to Student's *t*-test. We therefore divided the 16 patients into two groups: one group had $>1\times10^7$ cell/g harvested TIL, the other had $<1\times10^7$ cell/g TIL. A significant advantage for survival was noted on the basis of comparing the number of TIL harvested per patient ($P<0.05$; generalized Wilcoxon test) (Fig. 3). The patients with $>1\times10^7$ cell/g harvested TIL showed a better survival pattern than those with $<10\times10^6$ cell/g. We evaluated the relation between the histological characteristics of tumors and

Table IV. TIL with Respect to Presence or Absence of Cirrhosis or to TNM Classification

		Lymphocytes gained ($\times10^6$/g)		TIL expansion >1000-fold	Lysis (E:T=20:1)*			
					Daudi		HC-4	
		Mean	SEM		Mean	SEM	Mean	SEM
Cirrhosis								
−	(n7)	9.4	3.1	4/7	61.3	13.6	59.3	10.9
+	(n7)	17.5	6.0	1/7	44.0	10.5	26.4	4.8
TNM classification								
I, II	(n7)	13.0	2.2	2/7	64.2	10.0	50.0	12.6
III, IV	(n7)	11.8	5.4	3/9	49.6	8.7	45.6	9.9

*Cytolytic activities were assayed in 13 of the 17 cases.

Table V. Prognosis with Respect to TIL

		Lymphocytes harvested ($\times10^6$/g)		TIL expansion >1000-fold	Lysis (E:T=20:1) %*			
					Daudi		HC-4	
		Mean	SEM		Mean	SEM	Mean	SEM
Stage I								
Long survival group	(n1)	20.0		0/1	ND		ND	
Stage II								
Long survival group	(n4)	12.5	3.3	1/4	69.0	13.0	41.3	2.7
Early recurrence group	(n2)	10.6	2.4	1/2	23		24	
Stage III								
Long survival group	(n3)	27.3	12.7	1/3	26.0	24.0	28.7	9.5
Early recurrence group	(n3)	5.0	1.7	2/3	63.0	20.0	51.3	24.8
Stage VI								
Early recurrence group	(n3)	2.9	2.5	1/3	66.5	11.5	62.5	25.5
Total								
Long survival group	(n8)	19.0	5.1	2/8	53.7	20.6	44.2	15.7
Early recurrence group	(n8)	5.6	1.6	4/8	57.5	16.5	50.5	19.9

* Cytotoxic activities were assayed in 13 of the 17 cases.
Statistically significant difference in the number of TIL harvested between patients who survived for more than two years without recurrence of the disease (long survival group) and patients who died from the disease within two years (early recurrence group).

the number of TIL harvested in a number of patients. The nature of the infiltrate was also examined by microscopic histology. We recovered 50×10^6 cell/g from the tumor of patient 11 whose lesion had a marked mononuclear infiltrate (Fig. 4). Conversely, we recovered only 0.3×10^6 cell/g from the lesion of patient 16, which was devoid of mononuclear infiltration (Fig. 5).

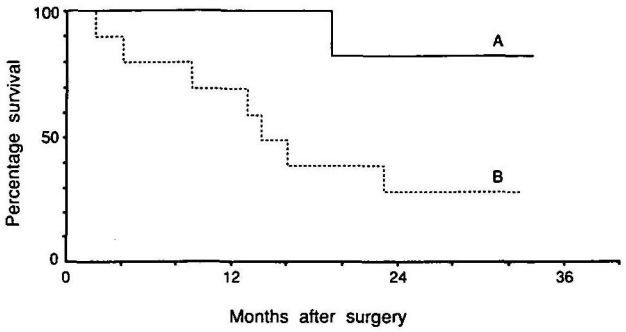

Fig. 3. Comparison of survival rates according to number of TIL harvested. A significant survival advantage was noted when we compared patients on the basis of number of TIL harvested ($P<0.05$). Patients with harvested TIL $>10\times10^6$ cell/g (—) showed a better survival pattern than those with $>10\times10^6$ cell/g (----).

Discussion

An immune state in HCC patients has been reported in several research projects. These have been, however, limited to peripheral blood studies and skin reaction reports.[15, 16] There have been few studies of TIL *vis a vis* local tumors. The present study has examined the relation between TIL and prognosis.

Our most successful expansion of TIL in rIL-2 culture reached 120,000-fold. Cytotoxicity of cultured TIL to allogeneic tumors was seen in a number of cases. Further, the prognosis was found to be better in the group from which the higher number of lymphocytes had been collected.

Phenotypic analysis of collected TIL demonstrated a preponderance of HLA-DR positive T lymphocytes, which suggests a rare infiltration of B

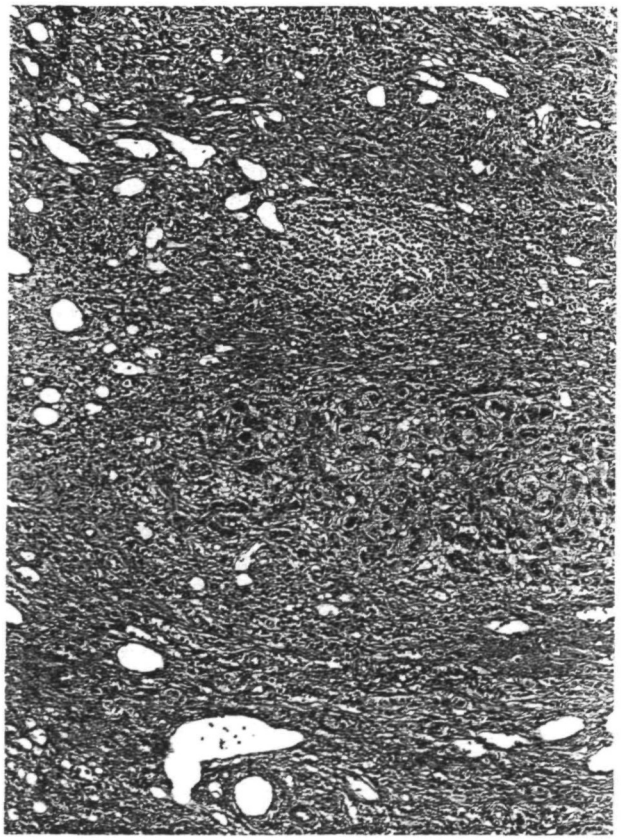

Fig. 4. Photomicrograph of hepatocellular carcinoma from patient 11. A marked mononuclear infiltrate was observed in the tumor lesion, and the harvested TIL from this tumor was 50×10^6 cell/g.

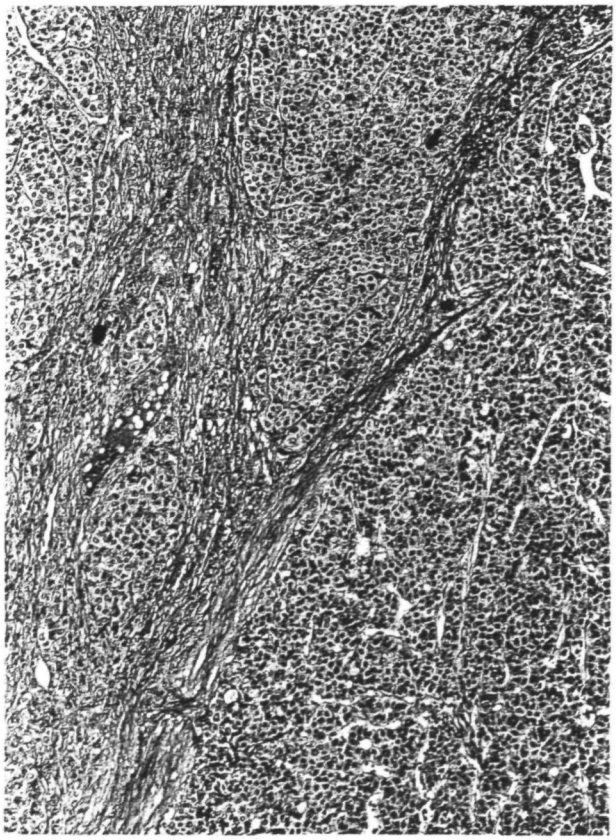

Fig. 5. Photomicrograph of hepatocellular carcinoma from patient 16. A very small number of infiltrating mononuclear cells was observed at the periphery of the tumor, and the harvested TIL from this tumor was 0.3×10^6 cell/g.

lymphocytes or NK cells. Studies of the lymphocytic subgroup of TIL in a variety of tumors have shown similar results.[17-20] We found no relation between CD4:CD8 values (*i.e.* helper:inducer and suppressor:killer cell values) and TIL collected; however, some have reported CD8 to be superior.[17, 18]

TIL growth was found in 16 of the 17 cases studied. All mixed tumor cells disappeared about 10 days into cell culture. While a high rate of TIL growth was noted, it seems that the rate of growth varies among tumors. Belldegrun *et al.*[21] reported three cases of renal cancer in which the TIL grew >500,000-fold. Topalian *et al.*[10] reported a growth of 2.8-800-fold in malignant melanoma. Itoh *et al.*[22] studied the differences in growth rates by type of tumor, and reported the growth rate in sarcoma to be significantly higher than that in metastatic melanoma, which was 5,400 times the average in five cases. While the lymphocyte growth was favorable in most cases, there were several HCC cases in which it was low. The properties of the TIL which did not expand in the rIL-2-added medium are not known to us. The HLA-DR positive rate in such cases, however, tended to be lower than that in other cases in our study. A lymphocyte subpopulation before culture may be an indicator of TIL growth.

Primary cultures of autologous tumors failed in the present study, so we are unable to report specific cytotoxicities. We found the LAK cells in two of 17 cases studied concurrently with TIL to show greater cytotoxicity, as determined by ^{51}Cr release assay on allogeneic tumor cells. This is in agreement with earlier findings[10, 20] in which the cytotoxicity of TIL was not consistently greater than that of LAK cells. Comparisons were made after culturing, suggesting TIL may prove more effective as precursor cells if growth rates are considered.

While the effect of cirrhosis in HCC should always be considered, there was no statistical difference in the initial number of TIL per unit weight or in cytotoxicity between the cirrhotic and non-cirrhotic lesions in our study. TIL growth rate tended to be low in the cirrhotic group, although we found no statistical difference between the two groups. The tumors in cirrhotic lesions were usually small and their available tissues were, therefore, limited. Hence, we need to find an improved method of culture for adoptive immunotherapy for treating HCC in cirrhotic lesions.

Favorable prognosis was associated with the higher numbers of TIL harvested per gram tumor tissue. One can postulate three reasons for the number of TIL obtained: (1) marked lymphocyte infiltration into tumor tissue, (2) soft and loose tissue, (3) favorable response to enzymatic treatment. Since more lymphocytes were collected from tumors with harder tissue, consideration of (2) can be eliminated. For (3), while enzyme treatment has not been studied quantitatively, its role in the experimental process must be negligible. This leaves us with marked lymphocytic infiltration. More precise quantifications of lymphocytic infiltration need to be made available, nevertheless, in order to achieve any degree of certainty. TIL has been identified as a host factor affecting tumor progression and prognosis. In cases of breast cancer and malignant melanoma, marked TIL infiltration has been associated with improved prognosis.[1-4] Opinions to the contrary not withstanding,[5, 6] this indicates TIL to have a role in the biological defense mechanism. In functions of antitumor activities of freshly isolated TIL remains to be elucidated.

Whether or not the number of harvested TIL depends merely on tumor stage has not been determined. It was not simply in connection with an early tumor stage that we were able to harvest a large number of TIL. It may be that the number depends upon immunological characteristics, *e.g.* frequency of expression of HLA in tumors and local production of cytokines.[23] Preoperative chemotherapy, too, may have some effect.[24]

Our study has shown that TIL in hepatocellular carcinoma can be isolated and will grow, and that the lymphocytes act with great cytotoxicity on allogeneic tumors *in vitro* after culturing with rIL-2. The present study on TIL and prognosis has shown there to be a tendency for a marked infiltration of lymphocytes among the group with a good prognosis, which suggests that TIL may actively participate in a biological defense against hepatocellular carcinoma.

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