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Liver Transplantation for Diethylnitrosamine-Induced Hepatocellular Carcinoma in Rats

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HUMAN hepatocellular carcinoma (HCC) is a highly malignant tumor with a natural history of a few months from the time of diagnosis. Curative resection offers the best chance of survival.[1] However, surgical resectability rates rarely exceed 30% to 40% because of extensiveness of the tumors or because coexisting liver disease precludes attempted partial hepatectomy.[2] Consequently, total liver removal and replacement have been used extensively as a therapeutic option.[3]

Because the rate of recurrence after liver transplantation has been high,[3–9] it has been speculated that the requisite immunosuppressive agents may enhance the recurrence of original malignancies by weakening immune surveillance.[3–10] In the present study, we have tested this hypothesis by inducing HCC in rats by multiple injections of diethylnitrosamine (DEN), followed by isogeneic liver transplantation with and without immunosuppression with FK 506 and cyclosporine (CyA). The results were inconclusive.

MATERIALS AND METHODS

Animals and Induction of HCC

Five- to 6-week-old male Fisher rats (F334) weighing 110 to 120 g were obtained from Harlan Sprague-Dawley, Inc (Indianapolis, IN) and given 75 mg/kg weekly intraperitoneal injections for 3 weeks of DEN (Sigma Chemical Co, St Louis, MO), followed by three further weekly injections of 100 mg/kg. The animals were maintained in a special room for carcinogen use with water and regular rat chow ad libitum. Isogeneic Fisher rats weighing 200 to 300 g were kept in a separate room until they were used as liver donors.

Immunologic Testing

Effector Cells. Mononuclear cells were isolated from the liver and spleen of normal Fisher rats and rats with HCC by morcellation of the specimen, and digestion for 15 to 20 minutes in RPMI medium (Gibco, Ground Island, NY), which was supplemented with 0.05% collagenase (Type 4, Sigma) and 0.002% DNase (Type 1, Sigma). After passage through nylon mesh, the mononuclear cells were separated by Ficoll-Hypaque gradient. In the rats with HCC, bits of the liver tumor were processed from which the tumor infiltrating lymphocytes (TIL) were purified.

Target Cells. The ability of the purified effector cells to lyse three kinds of cell targets was determined: (1) YAC-1 line for natural killer (NK) cell activity, (2) P815 line for lectin-dependent cell cytotoxicity (LDCC) in the presence of 5 μg/mL of phytohemagglutinin P (Sigma), and (3) HCC cells isolated from a tumor in the same rat providing the TIL. The HCC cells were separated after enzyme digestion and discontinuous (75/100%) Ficoll-Hypaque centrifugation (upper interface).

Target cells were placed in 96-well V-bottom plates (Coster, Cambridge, MA), and incubated with effector cells at effector/target ratios ranging from 50:1 to 6:1. Miniaturized 4-hour ^{51}Cr-release assays in triplicate were performed using 1×10^3 target cells, labeled with ^{51}Cr (5 mCi/mL: New England Nuclear, Boston, MA). Maximal and spontaneous release were determined by adding 5% Triton X-100 or medium, respectively. The plates were centrifuged at 65g for 3 minutes and incubated at 37°C in 5% CO_2 in air for 4 hours. Supernatants were harvested and radioactivity was counted with a beta scintillation counter. The percent specific lysis was determined according to the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Lytic units of cytotoxicity were calculated according to the formula of Pross et al[11]; one lytic unit was defined as the number of effector cells needed to lyse 20% of 5×10^3 target cells and cytotoxicity was calculated per 10^7 effector cells.

Immunosuppressive Agents

Oral FK 506 (Fujisawa Pharmaceutical Co Ltd, Osaka, Japan) dispersed with hydroxypropyl methylcellulose (a water-soluble polymer), was suspended in water with sonication and used at an oral dose of 1.0 mg/kg/d. Oral CyA (Sandoz Ltd, Basel, Switzerland) was diluted with olive oil and used at a dose of 10 mg/kg/d. Drug administration was started 1 day after transplantation and continued for 150 days by direct instillation with a gastric tube. These doses of the two drugs are effective in preventing autoimmune diabetes in rats[12,13] and in transplantation.

Orthotopic Liver Transplantation

Orthotopic liver transplantation was performed with a modification of the method of Kamada and Calne,[14] using the simplified cuff technique for the portal vein and infrahepatic vena cava anastomosis. Hepatic arterial reconstruction was not performed. Recipient animals received IM injection of cefamandole nafate (20 mg/d) for 3 days postoperatively.

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Table 1. Unaltered HCC Development by Histopathology, HCC Size, and Blood Chemistries in 24 Killed Animals

Weeks After Last DEN Injection	n	Histopathologic Diagnosis	Lung Metastasis	Mean HCC Size (cm) (Range)	Blood Chemistries GOT (U/L)	GPT (U/L)	Total Bilirubin (mg/dL)	AKP (U/L)	GGT (U/L)	Albumin (g/dL)
5–7	9	GST-P positive foci	—	—	187 ± 65	96 ± 33	0.05 ± 0.05	176 ± 126	62 ± 40	3.74 ± 0.75
9–13	7	GST-P positive foci and nodule 4 of 7 HCC	—	0.26 (0.1–0.5)	122 ± 52	65 ± 35	0.02 ± 0.04	184 ± 26	33 ± 29	3.76 ± 0.42
18–25	4	HCC with vascular invasion	1/4	1.7 (0.5–2.0)	347 ± 173	335 ± 219	0.08 ± 0.05	231 ± 56	90 ± 48	4.13 ± 0.26
32–40	4	HCC with vascular invasion	2/4	2.15 (0.6–5.0)	831 ± 1,122	690 ± 880	0.25 ± 0.13	318 ± 126	291 ± 205	3.73 ± 0.70

Note: Values are mean ± SD
Abbreviations: GST-P, glutathione S transferase placental form; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; AKP, alkaline phosphatase; GGT, gamma-glutamyl transpeptitase.

Experimental Design

Natural History. A total of 99 rats were entered, of which 6 were discarded 5 to 22 weeks after completion of the DEN course because of their morbid condition. Of the 93 remaining rats, 24 were followed and killed in cohorts to establish the natural history of the induced disease (Table 1). Liver and lung tissues were fixed in Stieve solution or Bouin's solution and paraffin embedded. Sections were stained for hematoxylin and eosin (H&E) and for glutathione S transferase placental form (GST-P), which is a marker of preneoplastic and neoplastic lesions of rat liver.[15] Staining was with the avidin-biotin complex (ABC) method using antirat GST-P antibody (Bio Prep, Dublin, Ireland).

Experimental Groups. All the other 69 rats developed HCC and were stratified by tumor size into experimental groups. Beginning 10 weeks after completion of DEN, laparotomy was performed. Rats with definite HCC nodules <1.0 cm or with nodules >1.2 cm were submitted immediately to orthotopic liver transplantation or consigned to a nontransplant group. The remaining rats with undetectable or small tumors were closed and periodically reoperated every 3 to 4 weeks until the liver tumors appeared and qualified the animal for transplantation by reaching a size >1.2 cm. A total of 42 laparotomies and 56 transplantations were performed on the 69 animals between 10 and 25 weeks after the last injection of DEN.

Eighteen of 56 transplanted animals died within 7 days after surgery, and were excluded from further analysis. Posttransplant survival rate (67.9%) was poor when compared with those of other studies at our laboratory in which normal healthy rats were used.[16]

The rats were placed into the groups summarized in Table 2. Except for the animals with small HCC (group 2) that all underwent orthotopic liver transplantation 10 weeks after completing DEN, the time of orthotopic liver transplantation was evenly distributed between 15 and 25 weeks post-DEN. The primary end point was duration of survival after transplantation.

Table 2. Experimental Groups

Group	N	OLTX (Isograft)	HCC Size at Entry* (cm)	Immunosuppression After OLTX
1	13	—	>1.2	—
2	10	+	<1.0	—
3	11	+	>1.2	—
4	11	+	>1.2	FK 506 1.0 mg/kg/d (po)
5	16	+	>1.2	CyA 10.0 mg/kg/d (po)

Abbreviations: OLTX, orthotopic liver transplantation.
*Entry: See text for time of entry.

Statistical Analysis

Differences between groups were analyzed by two-tailed Student's t test with the level of significance at $P < .05$.

RESULTS

Natural History

In the animals killed 5 to 13 weeks after completion of DEN, 4 of 16 had frank HCC and all of the others had hyperplastic nodules or foci with GST-P positive stain that identifies premalignant changes (Table 1). Between 18 and 40 weeks, eight of eight animals had frank HCC and in three there were pulmonary metastases. Although only a few animals became jaundiced or had hypoalbuminemia, the transaminases and alkaline phosphatase were progressively increased (Table 1).

Effector Cell Activity

The cytotoxicity of NK cells from the liver and spleen of HCC bearing rats was significantly reduced from that in normal animals (Table 3). The cytotoxicity of NK cells from the tumor (TIL) was even more drastically reduced. Cytotoxicity against autologous tumor cells was nil. HCC status did not affect the cytotoxicity against lectin-dependent target cells (Table 3).

Effect of Transplantation and Immunosuppression

The animals not submitted to either isotransplantation or immunosuppression lived for a median of 62 days (30 to 150); two had pulmonary metastases (Table 4). Survival was almost doubled by isogeneic transplantation, both in animals with small HCCs (group 2) and those with tumors >1.2 cm (group 3). The tumors did not recur in any of the transplanted livers in groups 2 and 3, although pulmonary metastases were present at the time of death in 3 of the 11 rats of group 3.

Table 3. Cytotoxicity of Mononuclear Cells from HCC-Bearing and Normal Rats Against Variable Target Cells

	Target Cells			
	YAC-1	P815 (−)	P815 (+)	Autologous HCC Cells
HCC-bearing rat (n = 3)				
TIL	16 ± 4*	0	0	0
Liver	55 ± 10†	1 ± 1	26 ± 5	0
Spleen	196 ± 32†	2 ± 1	26 ± 6	0
Normal rat (n = 3)				
Liver	246 ± 52	1 ± 1	10 ± 5	ND
Spleen	337 ± 43	2 ± 2	31 ± 8	ND

Note: Cytotoxicities are shown in mean ± SD ($LU_{20}/10^7$ effector cells). Abbreviations: ND, Not determined. *$P < .01$ vs liver and spleen in HCC-bearing rats. †$P < .05$ vs liver and spleen in normal rats.

The augmented survival obtained with liver replacement alone was reduced to a median of 18 days with FK 506 and to 54 days with CyA. Recurrent tumor in the transplanted liver of the immunosuppressed rats never was seen, and there were no examples of pulmonary metastases in the 15 experiments of groups 4 and 5. However, two rats developed squamous cell carcinoma of the lung (in group 4) and another (in group 2) had developed a renal cell carcinoma.

Surgical complications with the liver graft that did not have an arterial blood supply were common in all of the transplantation groups, and especially in those that included immunosuppression. Partial infarction of the livers with subsequent abscess formation was better tolerated in the nonimmunosuppressed animals than in those given FK 506 or CyA. The death of animals with infarcted liver usually was due to overwhelming intra-abdominal sepsis as well as lung infection. This was particularly evident in rats under immunosuppression.

DISCUSSION

As expected, these studies showed that isogeneic hepatic transplantation could increase survival of rats bearing carcinogen-induced HCCs. It has been argued that this experimental HCC may not be relevant to human HCC because of the large amount of carcinogen administered and because the immunogenicity of the resulting tumors is high.[17] These objections were answered in part by our demonstration that the general NK cell activity was depressed in these animals by the presence of HCC. Furthermore, the tumor cells elicited little if any cytotoxic response (autologous) from the mononuclear cells of the tumor-bearing animals. Similar depressed immunologic reactivity is typical in humans with HCC,[18,19] and has prompted a hypothesis that factors produced by human HCC cell lines are responsible.[20]

Further exploration of the host-tumor relationships with this induced tumor should be fruitful but with the important modification that the liver grafts should be arterialized. The liver transplantation technique of Kamada and Calne[14] that provides good results in our hands when used in healthy animals[16] was not so well tolerated in the rat recipients already debilitated by DEN pretreatment. In these rats, the portal hemodynamics may have been altered by the DEN-induced liver disease, including a reduction in portal venous flow and increased dependence on the hepatic artery as occurs in humans with cirrhosis. There was an extraordinary incidence of regional hepatic infarction that caused a high mortality in all animals, but

Table 4. Animal Survival After Liver Transplantation With/Without Immunosuppression

Group	N	Mean HCC Size at Entry* (cm) (Range)	Mean Time From Last DEN Injection to Entry (d) (Range)	Survival after Entry (d)	Median Survival (d)	Complications % of Animals	HCC (Primary/Recurrence) % of Animals
						Abscess in Liver	Liver
1	13	2.0 (1.2–3.2)	148.8 (114–173)	30, 37, 40, 41, 44, 58, 62, 75, 85, 112, 123, 128, 150	62.0	0	100 (13/13)
2	10	0.8 (0.5–1.0)	82.2 (66–82)	8, 15, 19, 33, 90, 127, 157, >196, >196, >205	108.5	40 (4/10)	0
3	11	1.6 (1.3–2.0)	106.3 (103–158)	8, 18, 17, 15, 62, 101, 113, 126, >164, >164, >171	101.0	36.4 (4/11)	0
4	11	1.6 (1.2–2.0)	125.6 (107–166)	7, 8, 11, 17, 18, 18, 19, 70, 122, 129, >164	18.0	63.6 (7/11)	0
5	6	1.9 (1.5–3.0)	144.6 (124–173)	8, 12, 14, 94, 118, >154	54.0	50 (3/6)	0

*Entry: See Table 2. Time of entry for group 1, HCC size >1.2 cm and no OLTX; group 2, HCC size <1.0 cm and OLTX; groups 3–5, HCC size >1.2 cm and OLTX.

especially those under immunosuppression whose death *almost always* was due to hepatic or hepatic plus pulmonary sepsis.

Thus, before it can be determined if hepatic tumor recurrence or extrahepatic metastases are accelerated either by transplantation itself or by immunosuppression, more consistent long-term survival with fully vascularized grafts will be a necessary condition. Such a study in rats is more apt to resolve these questions than attempts to study human cases, which of necessity are uncontrollably heterogeneous.[9] With the DEN method of HCC induction, the lesion being examined is producible in essentially 100% of animals, and its evolution can be determined under a wide range of controlled therapeutic variables including transplantation.

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