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Autologous Fixed Tumor Vaccine: A Formulation with Cytokine-microparticles for Protective Immunity against Recurrence of Human Hepatocellular Carcinoma

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We developed a tumor vaccine consisting of fixed hepatocellular carcinoma (HCC) cells/tissue fragments, biodegradable microparticles encapsulating granulocyte-macrophage-colony stimulating factor and interleukin-2, and an adjuvant. The vaccine protected 33% of syngeneic mice from HCC cell challenge. The vaccine containing human autologous HCC fragments showed essentially no adverse effect in a phase I/IIa clinical trial and 8/12 patients developed a delayed-type hypersensitivity (DTH) response against the fragments. Although 2 of 4 DTH-response-negative patients had recurrence after curative resection, the DTH-response-positive patients had no recurrence. The time before the first recurrence in the vaccinated patients was significantly longer than that in 24 historical control patients operated in the same department ($P<0.05$). This formulation is a promising candidate to prevent recurrence of human HCC.

Key words: Tumor vaccine — Hepatocellular carcinoma — Immunotherapy — Clinical trial

Reported tumor vaccines aiming to enhance T-cell responses consist of either irradiated tumor cells,[1–5] tumor cell lysates[2] co-injected with adjuvant,[6,7] genetically modified tumor cells,[8,9] tumor-associated antigenic peptides,[10] peptide-loaded dendritic cells,[11] or dendritic cells fused with tumor cells.[12,13] Concerning the impact in clinical practice, simplicity in preparation and bedside handling of the tumor vaccine is an important factor. In this respect, vaccines that include live cells in the formulation are disadvantageous because of the complicated preparation techniques. Unfortunately, tumor-associated antigenic peptides are only effective for patients carrying matched major histocompatibility complex (MHC) alleles,[10] and peptides and tumor cell lysates are rather weak immunogens. Antigen pre-loaded dendritic cells may be promising vaccines,[11] but they also require live cell handling. DNA vaccines occasionally provide insufficient amounts of antigens.[14]

We could successfully induce tumor-specific cytotoxic T lymphocytes (CTL) from the peripheral blood of tumor-bearing patients with autologous formalin-fixed paraffin-embedded tumor sections.[15,16] During the course of this induction, lymphocyte expansion always correlated with fragmentation of the thin tumor sections. We have also demonstrated that HLA-A-2402-restricted and carcinoembryonic-antigen (CEA)-specific CTL could be induced by culturing human peripheral blood mononuclear cells (PBMC) with autologous formalin-fixed adhesive PBMC pre-loaded with CEA-bound latex beads.[17] Antigen-presenting cells (APC), especially immature dendritic cells, phagocytose apoptotic and necrotic tumor cells,[18,19] and subsequently transfer immune signals to activate naive T cells. These observations led us to propose that solid antigens of a suitable size for APC phagocytosis would make efficient tumor vaccines. Here we report an animal experiment and a phase-I/IIa clinical trial with a proposed vaccine relevant to the recurrence of hepatocellular carcinoma (HCC).

After preliminary examinations, we selected a tumor vaccine consisting of fixed mouse Hepa 1-6 cells derived from a C57L/J mouse,[20] biodegradable microparticles encapsulating mouse granulocyte-macrophage-colony stimulating factor (GM-CSF) and human interleukin (IL)-2, and tuberculin as an adjuvant. Human serum albumin (HSA) (Baxter Healthcare Co., Deerfield, IL) was diluted to 2.5% and adjusted to pH 3.0. An equal volume of HSA solution was added to the cytokine/heparin mixture (IL-2 or GM-CSF dissolved in Heparin 1000 USP, Elkins-Sinn, Inc., Cherry Hill, NJ) while vortexing. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide was then added at 0.8 mg/ml and the mixture was vortexed for 15 min. An excess amount of 0.1 *M* glycine was added and the whole was incubated for 15 min. Microparticles were centrifuged (800*g*, 20 min), washed with water, and adjusted to contain 10^6 U/ml of IL-2 or 2.5×10^5 U/ml of GM-CSF. Hepa

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1-6 cells were fixed with 3% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) for 2 h, washed with 70% alcohol and PBS, and then incubated in Dulbecco's modified minimum essential medium at 37°C to inactivate completely residual paraformaldehyde. After 2 days, the medium was removed. Poly-L-lysine (50 μg/ml) was added and the cells were incubated for 2 h.[21] The cells were washed and adjusted to 1.3×10^8 cells/ml in PBS.

A syngeneic male C57L/J mouse was immunized by intra-dermal injection in the root of the tail with 0.05 ml of PBS containing fixed Hepa 1-6 cells (1.3×10^6 cells), GM-CSF-microparticles (1000 U as free mouse GM-CSF used for the microparticle formation), IL-2-microparticles (1000 U as free human IL-2 used for the microparticle formation), and tuberculin (10 ng) as an adjuvant on day 0 and boosted once on day 7. Seven days after the second vaccination, all mice were challenged subcutaneously in the left flank with 1×10^6 live Hepa 1-6 cells. The tumor area (mm^2) was calculated according to the largest perpendicular diameter.

The vaccination apparently reduced the growth rate of the tumor tissue, although 4 of 6 mice developed Hepa 1-6 tumors in the vaccinated group, as exhibited by the Kaplan-Meier curve for the mice bearing tumors of less than 100 mm^2 (Fig. 1A, $P<0.05$ vs. those in the control group by log rank test). Two mice in the vaccinated group remained tumor-free, although overall survival of these two groups showed no significant difference on statistical analysis (Fig. 1B).

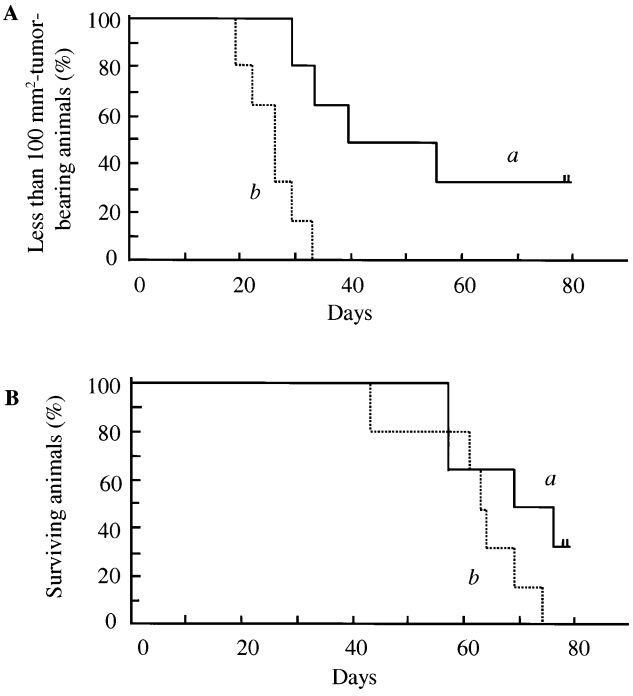

Fig. 1. Kaplan-Meier curves of small tumor-bearing mice. A, C57L/J syngeneic mice bearing less than 100-mm^2 Hepa 1-6 tumor. *a*, vaccinated mice; *b*, control mice. There is a statistically significant difference between the two curves (log-rank test, $P<0.05$). B, Survival of the mice shown in A. *a*, vaccinated mice; *b*, control mice. There is no statistically significant difference.

Then we proceeded to phase I/IIa clinical trial for patients whose HCC has been resected, since a high rate of recurrence was reported even after curative resection of HCC.[22] The following protocol was approved by the ethical authorities of Sun Yat-Sen University of Medical Science, Guangzhou. End points of this study were to confirm feasibility, to detect toxicity, and to observe the prophylactic effect of the vaccination on HCC recurrence. Patients were eligible if they had histologically confirmed HCC and adequate hepatic (Child-Pugh A and B) function. Further inclusion criteria were age 18–80 and no systemic chemo-, radio-, or immuno-therapy within 1 month prior to the vaccination. All patients gave written informed consent. Important exclusion criteria were any cardiac or significant psychiatric disease, distant metastases, second malignancies in the past 5 years, or severely impaired organ function (hematological and renal). All patients with hepatic tumors had undergone curative resection between January 13, 2000 and November 29, 2000 and were histologically confirmed as monocentric HCC stage II or lower. The patients were treated in three cohorts, each containing at least 3 patients. We gave increasing doses of the vaccine (see Table I). All contained autologous formalin-fixed HCC fragments as described below and human GM-CSF microparticles instead of fixed mouse HCC cells and mouse GM-CSF microparticles, respectively.

Resected and neutral formalin-fixed HCC tissue, 2 g or more, was homogenized. The fragments were filtered through 70 μm Nylon mesh, washed and sterilized with 70% alcohol, washed with saline, and incubated in RPMI-1640 at 37°C for 2 days to inactivate completely the residual formalin. The fragments were washed with saline and packed by micro-centrifugation at 17 000*g* for 3 min. No poly-L-lysine treatment of the fixed fragments was done, to avoid possible complications in humans. The vaccine consisted of autologous formalin-fixed HCC fragments, human GM-CSF- and IL-2-microparticles, and tuberculin.

Four weeks or more after the hepatic operation, vaccination was started by intra-dermal injection into the upper arm at a dose of 0.1 ml/site at a total of 5 sites. The patients received three vaccinations at two-week intervals. Historical control patients were those who had undergone curative resection of HCC in the same department as the vaccinated patients, from February 1998 to October 1999, and who matched exactly the eligibility and exclusion criteria for the vaccinated patients. Recurrence of HCC was detected by imaging techniques (ultrasonography and CT

Table I. Doses and DTH Responses in the Phase-I/IIa Clinical Trial

	Dose 1	Dose 2	Dose 4
Vaccine components per injection site[a)]			
Autologous fixed HCC fragments (μl)[b)]	10	20	40
hIL-2 microparticles (Units)	1000	2000	4000
hGM-CSF microparticles (Units)	1000	2000	4000
Tuberculin (ng)	25	50	50
PBS (μl)	90	80	60
Volume per injection site (ml)	0.1	0.1	0.1
DTH responses after 3 vaccinations (erythema, mm in diameter)[c)]			
Patient 1	25		
Patient 2	7		
Patient 3	7		
Patient 4		6	
Patient 5		40	
Patient 6		4	
Patient 7			31
Patient 8			25
Patient 9			10
Patient 10			12
Patient 11			48
Patient 12			12

a) Vaccination (5 injection sites in the upper arm) was repeated 3 times with a 2-week interval.
b) Packed fragment volume after micro-centrifugation at 17 000*g* for 3 min.
c) DTH skin tests were performed two weeks after termination of the vaccine injection by intra-dermal injection in a forearm with a suspension of 10 μl of autologous fixed HCC fragments and 90 μl of saline. Observation was carried out 48 h later. For patients 10, 11, and 12, DTH skin tests were also performed 48 h before the first vaccination, but none of the patients developed any measurable erythema.

scan) and confirmed by pathological inspection after needle biopsy or secondary resection.

A delayed-type hypersensitivity (DTH) skin test was performed 48 h before the first vaccination and/or two weeks after the third vaccine injection. A suspension containing 10 μl packed volume of the autologous fixed tumor fragments and 90 μl of saline was injected intra-dermally into the forearm. If erythema and induration occupied a skin area of 10 mm or more 48 h later, this was defined as DTH-response positive.

A total of 12 HCC patients received different doses of tumor vaccine preparations after curative resection of histologically confirmed monocentric HCC (see Table I for doses). In the dose-1, -2 and -4 tests (there is no dose 3, because we approximately doubled and quadrupled the contents of the vaccine components), all patients (3 per group) tolerated the vaccination well. We observed no adverse effect in the patients except patient 3 (dose 1) and patient 8 (dose 4), both of whom showed dry desquamation and pruritus (grade II) at the injection site 2 weeks after the second and first vaccine injection, respectively. These weak adverse effects disappeared after 2 weeks.

We then performed a DTH skin test with autologous formalin-fixed HCC fragments suspended in saline two weeks after the third vaccination. Dose 1 patients showed one positive and two negative responses (Table I). One patient in the dose 2 group displayed a strong response with erythema of 40 mm in diameter (patient 5, Table I). In the dose 4 test, we added 3 patients (patients 10, 11, 12) with whom we tried the DTH skin test with autologous fixed HCC fragments before the first vaccination. None of these patients developed any measurable erythema 48 h after the injection. All of these dose 4 patients exhibited a positive DTH response 2 weeks after the third vaccination (Table I).

In the follow-up observation, two DTH-response-negative patients in the dose 2 group revealed the first recurrence 9 and 10 months after the curative resection. However, we observed no HCC recurrence in any of the other patients when the majority of these patients had survived one year after the resection. We compared these HCC recurrences with those of historical control patients. To avoid potential bias owing to technological advances in the curative resection of HCC, we restricted historical control patients to those with histologically-confirmed monocentric HCC operated in the same department as the vaccinated patients closest to the period preceding the present clinical trial, and who had received no further treatment before the time of HCC recurrence.

After applying strictly the same eligibility and exclusion criteria as in the case of the phase I/IIa patients, we enrolled 24 patients in the historical control group. No essential difference was observed between the 12 vaccinated patients and the 24 historical control patients in the base-line data, except that numbers of HBV-positive patients are mismatched, i.e., the former and the latter groups included 10 (83%) and 14 (58%), respectively (Table II). Although the vaccinated patients were all males, while the historical controls include 4 females, age, cause of liver injury, Child-Pugh classes, serum alanine-aminotransferase level, percentage of patients with cirrhosis, AJCC stages, and blood loss and transfusion in the operation are all closely similar between the groups. Possibly the most influential factor, the resected tumor size, was 46 ± 22 and 43 ± 12 mm in the major axis in the vaccinated and historical control patients, respectively.

The recurrence in these historical control patients was consistent with the observation of Lai *et al.* (Table IX in ref. 22, after 1987) 1 year after operation; percentages of tumor-free patients were 37.5% and 35.9% in the present historical controls operated in Guangzhou and those

able II. Base-line Data of the Vaccinated and the Historical ontrol Patients

	Vaccinated (n=12)	Historical control (n=24)
Age, years	52±7	52±13
Male	12 (100%)	20 (83%)
Female	0 (0%)	4 (17%)
Cause of liver injury		
Hepatitis B	10 (83%)	14 (58%)
Unknown	2 (17%)	10 (42%)
Child-Pugh class A	12 (100%)	22 (92%)
class B	0 (0%)	2 (8%)
Alanine aminotransferase, U/ml	47±30	77±135
Cirrhosis	8 (67%)	14 (58%)
α-fetoprotein		
<400 ng/ml	9 (75%)	16 (67%)
≥400 ng/ml	3 (25%)	8 (33%)
Tumor size, mm	46±22	43±12
AJCC stage I	2 (17%)	2 (8%)
AJCC stage II	10 (83%)	22 (92%)
Blood loss in operation, ml	617±666	621±520
Blood transfusion in operation, ml	342±601	548±501

reported for the 194 patients operated in Hong Kong (near Guangzhou) between 1987 and 1994,[22] respectively (Fig. 2, the dotted line and the asterisk at 12 months). Takayama *et al.* reported a percentage of 59.5% at the National Cancer Center, Tokyo (calculated from Fig. 2 in ref. 23). By 36 months, no further patients in the present historical controls had developed HCC recurrence, although the percentage of disease-free patients decreased to 22.8% in ref. 22 and 32.4% in ref. 23.

The Kaplan-Meier curves of the recurrence-free patients among the 12 vaccinated patients (cumulatively shown in Fig. 2A for dose 1, 2, and 4) and among the 24 historical controls from the same department showed a statistically significant difference (log-rank test, $P<0.05$). To avoid the mismatch of HBV-positive patients shown in Table II, we compared the recurrence-free patients among the HBV-positive patients extracted from the two groups (Fig. 2B), and that also resulted in a statistically significant difference (log-rank test, $P<0.05$). All of the 8 patients who developed a positive DTH response (Table I) have remained recurrence-free for 11–21 months (Fig. 2C, log-rank test, $P<0.01$ vs. the historical control).

The present results suggest that 1) a vaccine composed of fixed tumor cells/tissue fragments, cytokine-encapsulated microparticles, and an adjuvant can elicit anti-tumor immune responses in a murine HCC model and in human phase I/IIa clinical trials (Figs. 1 and 2); 2) the vaccine containing autologous fixed HCC fragments has essentially no adverse effect; 3) the time to first recurrence in the vaccinated patients, especially in the DTH-response-

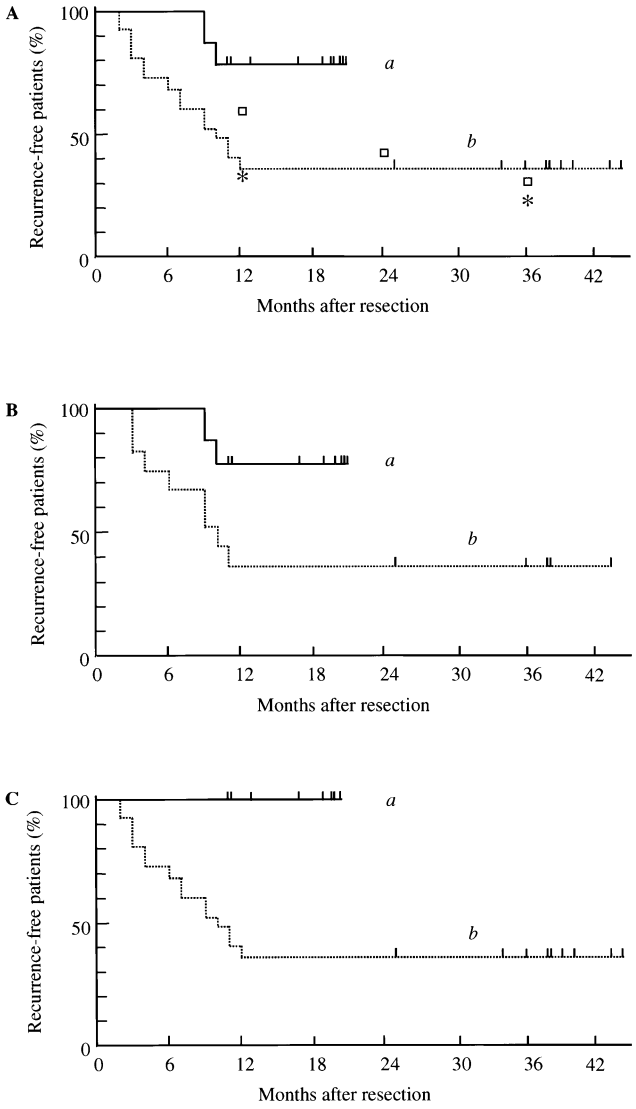

Fig. 2. Recurrence-free patients in the present phase I/IIa study. A, Kaplan-Meier curves of, *a*, 12 patients vaccinated with dose 1, 2, or 4 (see Table I) operated during January and November, 2000, and, *b*, 24 historical control patients, enrolled under the same eligibility and exclusion criteria, operated during February, 1998, and October, 1999, in the same department in Guangzhou. The difference of the two curves is statistically significant (log-rank test, $P<0.05$). Asterisks (*) at 12 and 36 months indicate data cited from Lai *et al.* after HCC resection between 1987 and 1994 in Hong Kong (see Table VI of ref. 22). Open rectangles (□) show the data calculated from 74 patients operated in the National Cancer Center, Tokyo, after 1992 (see Fig. 2 in ref. 23). B, To avoid the mismatch of HBV-positive patients shown in Table II, we compared the recurrence-free patients among HBV-positive patients extracted from the vaccinated (*a*, n=10) and the historical control (*b*, n=14) groups. The difference between the two curves was statistically significant (log-rank test, $P<0.05$). C, From curve *a* in A, DTH-positive patients (n=8) were extracted. The difference of the two curves, *a* and *b*, was statistically significant (log-rank test, $P<0.01$).

positive patients, was significantly longer than that in the historical control group (Fig. 2).

The major advantage of the present human HCC vaccine formulation is that it contains autologous (therefore strictly "personalized"), fixed (therefore very stable) tumor antigens and no fragile live cells. The present vaccine consisting of unidentified tumor antigens stabilized by simple fixation with formalin would be highly preferred in many medical institutions in which in-house tumor vaccine preparation using MHC-class matched antigenic peptides and/or live cells would be difficult. Compared to the tumor vaccine containing live dendritic cells, which are considered the essential component,[24, 25] non-living cell-containing stable vaccines provide ease of handling at the bedside and, therefore, increase the practicality of application.

At present, the mechanisms of the anti-tumor immune response are obscure. However, we prepared the fixed HCC as a fragment suspension in the vaccine. Phagocytosis of the fragmented antigen is likely to be an important pathway for presentation of the antigenic peptide on MHC-class I molecules, since antigens in particulate form can elicit $CD8^+$ MHC class I-restricted CTL response,[18, 19, 24–26] though soluble antigens elicit mainly responses of $CD4^+$ MHC class II-restricted lymphocytes.[27] The $CD4^+$ T cells then transfer their immune information to stimulate antibody production. Therefore, direct induction of CTL against soluble antigens is difficult, except when the antigenic peptide is loaded on the antigen-presenting dendritic cells.[28] However, macrophages can efficiently induce CTL responses *in vitro* when particulate carriers are used to deliver short antigenic peptides.[29, 30] Still, of course, there remains a possibility that NK cells may also have contributed to the present immune response.

HCC is essentially different from most other cancers, since hepatitis virus contributes the tumorigenesis. Therefore, there are two possible mechanisms by which the vaccination may prevent HCC recurrence; 1) virus-specific immunity, and 2) HCC-specific immunity. At present we have no evidence to indicate which mechanism is more likely. If the former operates, and if there are a large number of HBV-latently-infected hepatocytes which express HBV-derived antigens but still function, these hepatocytes might be attacked by the autologous CTL specific to the viral antigen and the vaccinated patient might then develop impairment of liver function. However, so far as we have observed for more than a year, no adverse effect has been found in the hepatic function of the vaccinated patients, perhaps implying that the former mechanism is unlikely to play a major role.

In the vaccination of HCC patients, dose 4 is a feasible dose for further clinical trials since 1) only one patient at dose 4 showed short-term dry desquamation and pruritus and no other adverse effect appeared; 2) we measured the DTH response as a monitoring method for detecting potential HCC antigen-specific immunity. Although two of three patients who received the dose 1 or dose 2 vaccines gave negative DTH responses, all of the dose 4 patients displayed a positive DTH response (Table I); 3) as regards serum α-fetoprotein levels, which could be another marker for potential HCC recurrence, none of the dose 4 patients exhibited a level of 20 ng/ml or higher after the vaccination (data not shown); 4) preparation of a higher concentration of the tumor fragments was not practical, since the tumor fragments in the dose 4 preparation accounted for 40% (v/v) of the vaccine suspension; 5) all six patients injected with the dose-4 vaccine were DTH-positive (Table I) and recurrence-free (Fig. 2C).

At present, we do not know whether or not the vaccine is able to suppress development of multi-centric and/or second-primary HCC. More advanced clinical trials seem warranted, not only for HCC, but also for many other tumors, since the present vaccine formulation is applicable to any type of tumor which retains its antigenicity after fixation.

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