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Growth potential of human hepatocarcinoma cells in the liver of neonatal immunocompetent mice and its relation to immunological tolerance

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Abstract

To determine the pathological behavior of human hepatocarcinoma cells in the liver microenvironment of neonatal non-immunodeficient mice, three human hepatocarcinoma cell lines (Bel7402, HepG2, and SK-Hep-1), traced by DiI, were transplanted into the intrahepatic or subcutaneous tissue of neonatal and adult Kunming mice. Histopathological observations showed that cells in the adult liver induced a severe immune response as early as the second day after the implantation, while the subcutaneous neoplasm underwent extensive necrosis by the end of the study. Only the cells injected into the neonatal liver underwent a delayed immunologic rejection in the organ microenvironment. These cells retained recognizable tumor features over the first seven days, and displayed an intrahepatic invasive pattern. The expression of tumor markers including alpha-fetoprotein and survivin was maintained. The quantitative ELISA for the expression patterns of IL-2 and IL-10 also confirmed that the intrahepatic immunity was non-susceptive during this period. The high serum alpha-fetoprotein level was inversely correlated with the change in immune response. Our study provided a bio-system for the research of immune responses to xenografts in the liver.

Keywords: Human hepatocarcinoma cell; Immunologic rejection; Liver microenvironment; Neonatal mice; Xenograft

1. Introduction

Hepatocellular carcinoma (HCC) is a global healthcare concern. Human–animal models have improved the understanding of the mechanisms that underlie the development and pathogenesis of human HCC [1,2]. To reduce the immunogenicity of human xenografts or prolong their survival, methods to establish a more suitable microenvironment for xenografts have been evaluated. Some success has been achieved as a direct result of the improved understanding of the biological mechanism of the immune system. Mouse–human tumor models have been developed using methods such as immunosuppression [3], transplantation into immunologically privileged sites [4], and induction of immunological tolerance [5]. But the most valuable model is the transplantation of human tumors into immunodeficient mice such as athymic or severe combined immune-deficient mice [6,7]. This is a pivotal step for *in vivo* research on the interactions between tumor cells and microenvironments where the tumor cells grow. Many biological aspects of human cancer are amenable to investigation. Using these classical xenograft models, it has been shown that the local microenvironment, particularly local tissue factors [8,9], profoundly influences the biological behavior of human tumors.

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However, the models mentioned above represent, to some extent, an immune-deficient system. Limited attention has been focused on the behaviors of the tumors transplanted into an immunocompetent organic microenvironment. One of the particular interests of our laboratory is the development of transplantation strategies that overcome the specific immune susceptibility of the liver microenvironment to human tumor cells in neonatal non-immunodeficient mice.

The liver is known for its unique ability to accept allogenic livers without the need for pharmacological immune suppression [10]. The transplanted liver not only withstands its own rejection, but also induces donor-specific immune tolerance for the sequential transplantation of other organs such as the heart, kidney and skin [11]. Therefore, the liver is considered as an immune-privileged organ for transplantation. Moreover, the liver is the main source of the high level of alpha-fetoprotein (AFP) that occurs during embryonic and neonatal development [12], and many studies have revealed that AFP can function as a suppressant on the immune system and can help hepatocarcinoma cells escape from immune surveillance [13,14]. Therefore, we presumed that the intrahepatic microenvironment at the neonatal stage may be appropriate for the survival of human hepatocarcinoma cells.

In this study, the immune susceptibility of neonatal (3-day-old) and adult (6-week-old) Kunming (KM) mice to human hepatocarcinoma cells was compared. We focused on characterizing the specific biological behaviors of human hepatocarcinoma cells after transplantation into the liver of neonatal non-immunodeficient mice.

2. Materials and methods

2.1. Human tumor cells

Human hepatocarcinoma cell lines (HepG2, AFP^+, Bel7402, AFP^+, and SK-Hep-1, AFP^-) were obtained from the Cell Bank of the Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, USA) containing 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate. All cultures were incubated in a humidified 5% CO_2 incubator at 37 °C. Cells were routinely examined and were shown to be free of mycoplasma and viruses prior to use.

2.2. Experimental animals

KM mice were used as recipients for cell transplantation. All mice were purchased from the Experimental Animal Center of Shandong University. They were maintained under pathogen-free conditions, and were fed standard food and sterilized water.

Mice were studied at two different developmental stages: three days and six weeks old. Equal numbers (90) were used in three experimental groups: the adult intrahepatic group, the neonatal intrahepatic group and the neonatal subcutaneous group.

All the animal procedures were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee.

2.3. DiI labeling

The fluorescent carbocyanine dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes, USA) was used to trace the transplanted cells *in vivo*. Cells were incubated in 20 μg/ml DiI for 30 min at 37 °C, and were agitated gently at 10-min intervals. Then, they were washed three times with sterilized phosphate buffered saline (PBS) to remove the residual dye. Cells were suspended in basic culture media and were adjusted to a concentration of 0.5×10^6 cells/μl. Cell viability was assessed by trypan blue dye exclusion, and only single cell suspensions with viability over 90% were used for implantation.

2.4. Intrahepatic implantation

Three-day-old and 6-week-old KM mice were used for intrahepatic implantation. The 3-day-old mice were anesthetized locally with lidocaine, while the adult mice were anesthetized with pentobarbital. The DiI-labeled tumor cell suspension (20 μl) was transplanted with a 27-gauge glass syringe into each mouse. The needle was injected at a 30° angle into the capsule of the left-lateral hepatic lobe. The injection was performed at a depth of 3–5 mm under the skin surface over a 2–3 min period.

2.5. Subcutaneous implantation

Three-day-old mice were used for subcutaneous injections. The cell suspensions as described above were implanted subcutaneously into the anterior left-lateral wall of the mice. The injection was performed at a depth of 5–7 mm under the skin surface.

After the implantation, all the animals were returned to their respective cages and were watched.

2.6. Macroscopic assessment at autopsy

Mice were examined daily for signs of morbidity. The presence of tumor was checked macroscopically on days 2, 4, 7, 11, 15 and 21 after the injection. Five mice from each group were sacrificed by deep pentobarbital anesthesia on each of the days listed. Regional and distant lymph nodes, the lungs, as well as other organs were routinely inspected for metastases.

2.7. Histopathology assessment

Tissue samples including tumor tissues and adjacent normal tissues were collected at each time point. All the tis-

sue samples were fixed in 4% paraformaldehyde solution for 6 h. Then, the samples were dehydrated, embedded, sectioned, and stained with hematoxylin and eosin for microscopic examination and fluorescent observation at an excitation wavelength of 560–590 nm.

2.8. Immunohistochemical examination

Tumor samples from the neonatal intrahepatic group were collected. The expression of AFP and survivin was assayed by immunoperoxidase staining with the ABC method (ABC kit, Zhongshan Biocompany, China). Monoclonal antibodies were diluted 1:200 for AFP (Serotec, UK) or 1:100 for survivin detection (Santa Cruz, USA). The samples were counterstained with hematoxylin.

2.9. ELISA

In the neonatal intrahepatic group, blood samples (10 μl) were collected from the tail veins and were suspended in distilled water (40 μl). All the samples were stored at −80 °C. A quantitative ELISA kit (Xitang, China) was used to measure the expression level of AFP, IL-2 and IL-10, in accordance with the manufacturer's protocols. AFP concentration was expressed as ng/ml. IL-2 and IL-10 concentrations were expressed as pg/ml.

2.10. Statistical analysis

The results were expressed as the mean ± SEM and accompanied with the number of independently performed experiments. Statistical analysis was performed using the Student's *t*-test and *p*-values of less than 0.05 were considered statistically significant in all experiments.

3. Results

3.1. Survival state

No animals died from the xenograft burden during the experiment. In the subcutaneous group, necrotic fluid could be seen flowing from ulcerated skin from day 5 onwards. Overall, the average body weight of the mice that were implanted was less than that of the control, and the hair was drier and duller.

3.2. Autopsy analysis of tumor location

At autopsy, the injected tumor cells caused disparate morphological changes in each group (Fig. 1). There was no significant difference among the xenografts of the three cell lines.

In the neonatal intrahepatic group, the injected tumor cells generated externally visible single orthotopic nodules in all mice. They had well-defined limits within the liver tissues (Fig. 1(a)). The tumor mass was often less than 0.5 cm in diameter with irregular growth patterns. Cells also showed different degrees of distant dissemination to other lobes in 21 mice at different time points. Based on visual observation, none of the mice developed invasive tumors except those in the neonatal intrahepatic group (Fig. 1(b)).

The subcutaneous xenograft had a pattern of expanded growth during the first seven days, which then decreased by the end of the experiment (Fig. 1(c) and (d)). In the adult group, visible nodules were detectable in six cases. But, in the majority of mice, the normal liver tissue was always partly replaced by yellow fatty masses (Fig. 1(e) and (f)). Histological analysis revealed that the mass was inflammatory tissues.

3.3. Histological characteristics

The location of the injected cells was traced using the *in vivo* fluorescent dye DiI throughout the experiment. The highly lipophilic nature of DiI allowed its immediate integration into membranes and even into the cells, resulting in complete "orange–red-like" delineation of cells (Figs. 2–4).

Microscopic characteristics of the tumor generated in the neonatal intrahepatic group are presented in Fig. 2. A tumor nodule was formed in the injected lobe with a clear boundary. Consistently, the xenograft cells hardly formed any architectural disposition in the first four days. The cells exhibited an immature and malignant appearance, with characteristics of high mitotic activity, high nuclear-to-cytoplasm ratio, and marked nuclear polymorphism (Fig. 2(a)). These cellular characteristics coincided with the growth of cells in the monolayer culture. From the seventh day onwards, infiltration of lymphocyte cells was apparent. The tumors clearly comprised sheets of densely packed, epithelioid cells. Visible necrotic areas appeared in the center of the tumor area (Fig. 2(b)); however, there was no evidence that these necrotic areas were enlarged to the whole tumor. Furthermore, local and distant dissemination of the injected cells occurred as early as the ninth day after implantation. These intrahepatic-invasive cells had an amorphous form similar to the cells at the initial transplanted stage (Fig. 2(c)). Consistently, they did not show any necrotic tendency, even by the end of the experiment. Extensive tumor burden of the liver could be observed throughout the experiment.

In the adult group, the xenograft cells evoked significant inflammatory cell infiltration on day 7 after implantation (Fig. 3(b)). The transplanted cells showed an abundant, foamy, eosinophilic cytoplasm form, which was surrounded by an extensive inflammatory cell capsule (Fig. 3(d)), and were necrotized over subsequent days. Histological examination of these tumors did not display any evidence of growth.

The subcutaneous lump was always surrounded by a fibrous capsule. The infiltration of lymphocyte cells necrotized the injected cells rapidly from day 3 onwards (Fig. 4). By the end of this experiment, the injected cells were thoroughly eliminated.

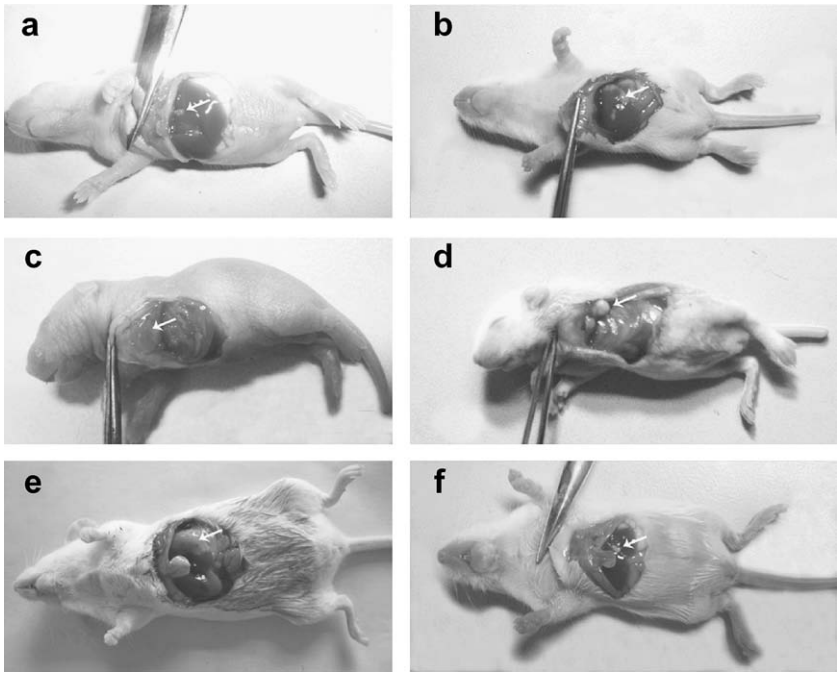

Fig. 1. Autopsy confirmation of successful transplantation. The growth and invasion behaviors of the intrahepatic xenograft on days 5 (a) and 9 (b) in the neonatal liver after subcutaneous injection. Solid xenograft lumps were observed on days 2 (c) and 5 (d) after subcutaneous injection. (e and f) Morphological changes caused by the xenograft in the adult liver.

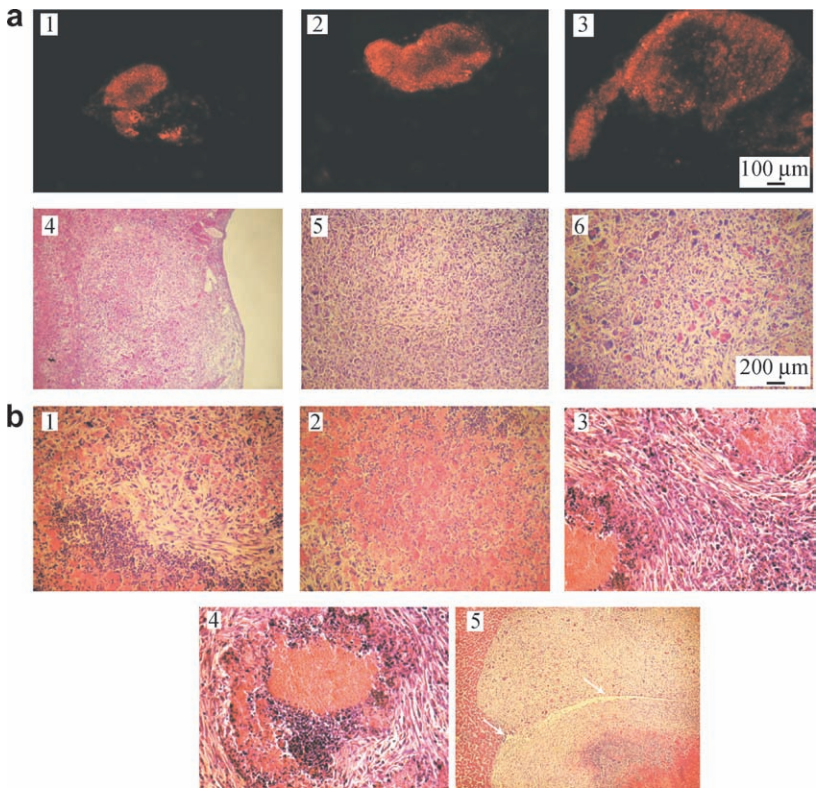

Fig. 2. Pathological process of human hepatoma cell in the liver of neonatal mice. (a) Fluorescent (1–3) and microscopic features (4–6) of the formed tumors at the initial stage. 1 and 4, Bel7402; 2 and 5, SK-Hep-1; 3 and 6, HepG2. (b) Histopathological characteristics of lymphocyte infiltration (1 and 2), the packed epithelioid cells (3), necrotic areas (4) and extensive intrahepatic invasion (5).

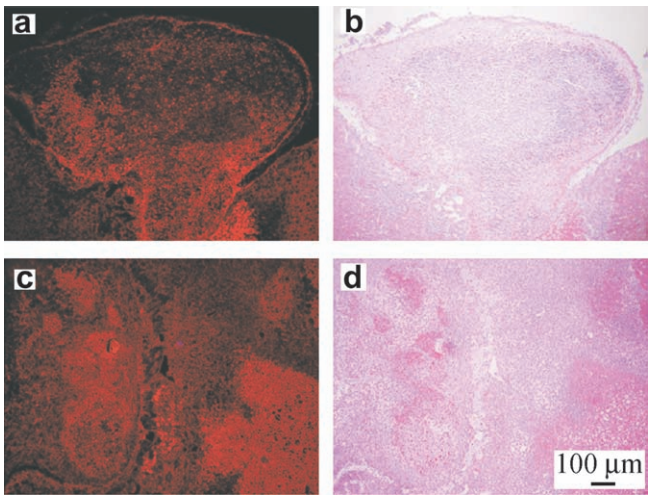

Fig. 3. Fluorescent and microscopic features of the intrahepatic xenograft in the adult group. (a and b) Lymphocyte infiltration appeared on day 2 after implantation. (c and d) Numerous inflammatory cells surrounded the xenograft cells on day 5.

There was no evidence of tumor metastasis in the lung, brain, heart and pericardial cavity in any group. The three human hepatocarcinoma cell lines in each group underwent a similar growth rate. Therefore, the results of the Bel7402 and SK-Hep-1 transplanted groups are not mentioned specifically.

3.4. Immunohistochemical results

Histological results demonstrated that only the cells injected intrahepatically were tumorigenic in the neonatal mice. The tumor sections from this group were selected for further experiments. The immunohistochemical results suggested that the injected cells maintained their *in vitro* AFP and survivin protein expression patterns.

The expression of AFP antigen was moderate in the HepG2 (Fig. 5(b)) and Bel7402 cells, and was absent in the SK-Hep-1 cells (data not shown). In normal tissues, AFP was only expressed in the cytoplasm of hepatic oval cells, which have characteristics of small size, oval shape and ovoid nuclei and are located around the bile duct (Fig. 5(c)). The expression of survivin antigen was higher than that of AFP in the human tumor cells. Survivin was detectable as a strong staining signal in a majority of the tumor cells (Fig. 5(e)), but there was no expression signal in the observed normal tissues (Fig. 5(f)).

3.5. Serum AFP expression patterns

The changes in serum AFP levels are shown in Fig. 6(a). In the normal KM mice, the AFP level was very high during the first three days of life, and began to decrease in 5-day-old mice. Then, the concentration declined to the basal level two weeks later. The AFP level in blood samples of the normal adult mice tended to be undetectable.

AFP expression was stimulated and changed dramatically in response to the intrahepatic injection. Initially, the decreased rate of AFP concentration appeared to be similar to the control. However, from day 4 onwards, its concentration increased greatly and reached a maximal level on day 6. By approximately day 11, the AFP level had decreased to baseline levels.

3.6. Serum IL-2 and IL-10 expression patterns

The serum IL-10 level increased steadily starting shortly after implantation and reached a peak on day 3, while IL-2 decreased to a nadir over the same time (Fig. 6(b)). The IL-10 level subsequently and gradually returned to its normal level. In contrast, the expression of IL-2 was stimulated and reached a peak on day 7, and thereafter returned to its normal level.

4. Discussion

To our knowledge, little is known about the subtle interplay between cancer cells and their partial tissue microenvironment during the process of cancer growth, metastasis, or anti-cancer immunity. Our results showed that the liver microenvironment at different developmental stages of non-immunodeficient mice had a profound effect on the growth fate of the transplanted hepatocarcinoma cells. In particular, the neonatal liver showed a delayed rejection to the hepatocarcinoma cells and supported them for a short period of time. These results may represent an advantageous system for the research of immune responses to xenografts in the liver.

Several studies have been done to develop xenograft models of liver cancer; however, only orthotopic transplan-

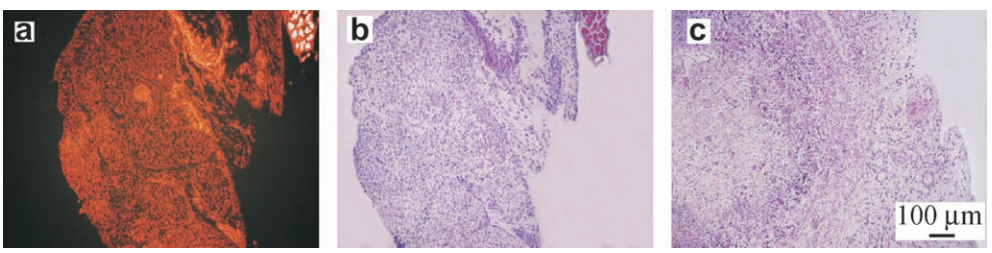

Fig. 4. Fluorescent and microscopic features of the subcutaneous lump on days 2 (a and b) and 5 (c) after implantation.

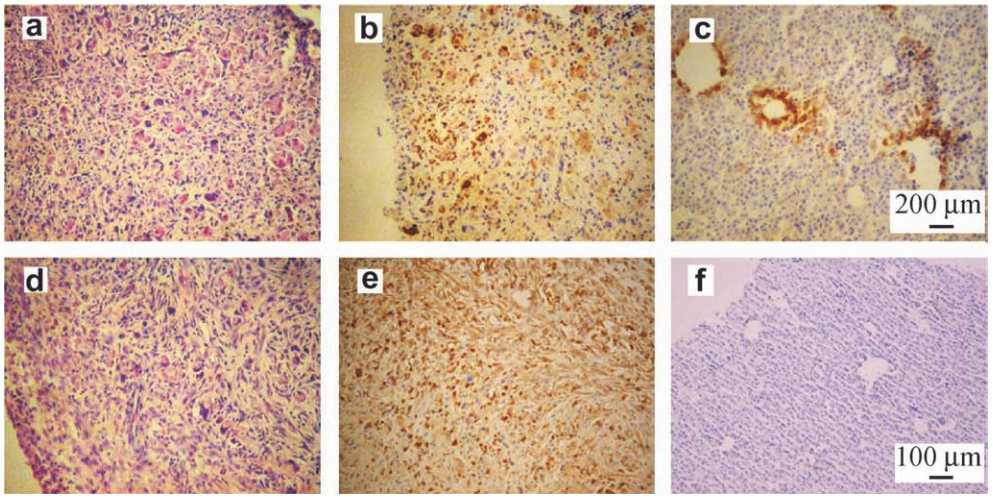

Fig. 5. Microscopic features of the intrahepatic xenograft (a and b) and immunohistochemical staining against AFP (b and c) and survivin (e and f) for samples from the neonatal intrahepatic group (b and e) or from normal mice (c and f).

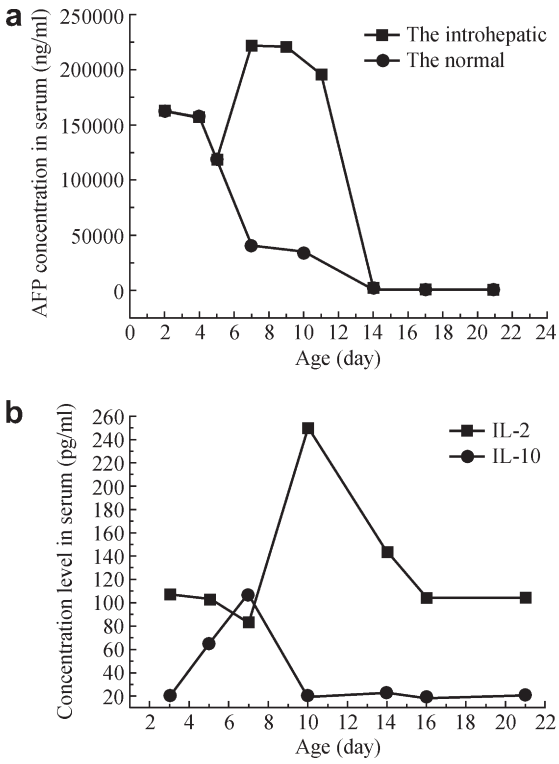

Fig. 6. (a) The AFP level changed with time in the intrahepatic group (-■-) and the normal KM mice (-●-) during the first neonatal 21 days. (b) The levels of IL-2 (-■-) and IL-10 (-●-) changed with time after the neonatal intrahepatic implantation ($p < 0.05$).

tation provides a suitable microenvironment for the study of the biological and clinical properties of tumors [15]. It is well established that the relevant organ environment governed the phenotypic properties of transplanted cells in the nude mouse model [8,16]. The results of our study are consistent with those of the previous studies using the nude mouse xenograft system.

The growth rate of the cells was prominently different between the three experimental groups. We used three kinds of HCC cell lines in this study. As the histological results showed, only the microenvironment of the neonatal liver allowed the cells to maintain their original *in vitro* tumor characteristics. In particular, the cell line HepG2 was non-tumorigenic in immunosuppressed mice as described in ATCC. But in this study, these cells not only survived for approximately seven days in the neonatal liver, but also maintained their invasive behaviors (Fig. 2(c)). Moreover, immunohistochemical analysis confirmed that the expression profiles of AFP and survivin were maintained in the growing tumor cells (Fig. 5). These two antigens are not only tumor markers [17,18], but are also believed to play key roles in tumorigenesis, tumor invasion and metastasis [19,20]. Therefore, we can confirm that the cells have and retained their *in vitro* phenotype when growing in the neonatal liver. In contrast, necrotized cells appeared in the other two groups as early as day 3. Although the subcutaneous lumps showed rapid growth during the first seven days, histology suggested that this tendency did not always correlate with their long-term growth potential, which might be due to the inflammatory reaction. In other words, the neonatal liver should provide a more suitable target tissue for these human hepatocarcinoma cells than others.

In the second step, we tried to identify the specific immune state in the neonatal liver. Many studies have confirmed that immunological factors play important roles in tumor growth in the human–nude mouse system [9]. In this study, the expression patterns of AFP, IL-2 and IL-10 were determined after the intrahepatic injection, using immunochemistry assays. In particular, IL-2 and IL-10 are two powerful immune regulatory cytokines. It has been shown that these two cytokines play different roles in the progress of graft inflammation and tissue remodeling. IL-2 can enhance T-cell activation and promote graft rejection after

organ transplantation [21,22]. In contrast, IL-10 is a cytokine with broad anti-inflammatory properties. A deregulated level of IL-10 may limit the host immune and inflammatory responses to graft rejection [23,24]. After the intrahepatic injection, we found that the IL-2 level was reduced whereas the IL-10 level was stimulated (Fig. 6(b)). Consistent with this tendency, the pathological results also revealed that no infiltration of lymphocyte cells or inflammation occurred in this period (Fig. 2(a)). From this point of view, we believe that the immunity during the first seven days may be abnormally suppressed or non-susceptive in the neonatal intrahepatic microenvironment.

During the first seven days of the neonatal KM mouse model, we documented a high AFP concentration in the serum (Fig. 6(a)). This is similar to the expression pattern of other strains of mice including BALB/c/J, BALB/c/BOM and C3H/He mice [25,26]. Furthermore its expression level increased abnormally just after intrahepatic injection (Fig. 6(b)). It is well known that AFP was one of the first tumor serological markers identified for HCC in clinical practice [17]. Recent studies also indicated that this antigen had multiple pleiotropic activities. AFP can promote the proliferation of liver cancer cells by influencing cAMP- and Ca^{2+}-mediated signal transduction pathways [27], and shield HCC cells from apoptosis [28]. Furthermore, many studies have confirmed the inhibitory effect of AFP on immune responses *in vivo* [13]. Thus, as AFP seems to play an intriguing role in tumorigenesis and development of HCC, we assumed that the high AFP level may play an important role in the subsequent host immune response described above. Results of the ELISAs revealed a dramatic change in AFP level, which was inversely correlated with the change in immune response. The decrease in AFP levels occurred before IL-2 reached its peak level at around day 7 after implantation. Interestingly, from this time onwards, obvious necrosis appeared in the neonatal intrahepatic xenograft (Fig. 6). Breaching of the original immune "silence" should therefore be closely associated with the increased expression of IL-2 and the decreased expression of AFP. In contrast, AFP was hardly detectable in adult mice. After being injected into the liver, the cells stimulated a rapid and severe immune response and rapidly underwent necrosis (Fig. 3). Hence, we believed that the high level of AFP expressed in the neonatal liver and the dramatic changes in IL-2 and IL-10 levels could partly explain the survival of the xenograft HCC cells and the delayed immune rejection from the host. However, other regional tissue factors might participate in creating this immune status.

In conclusion, tumorigenesis is a mysterious story with too many network-distributed aspects. Here, we described the relevant pathological process of human hepatoma cells in the liver of neonatal normal mice. The neonatal liver microenvironment shows a delayed immune susceptibility to xenograft tumor cells. Further studies are needed to better understand the mechanism of this process.

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