Int J Clin Exp Med 2016;9(2):3693-3698
www.ijcem.com /ISSN:1940-5901/IJCEM0015049

Original Article

Establishment of human hepatocellular carcinoma CAM xenograft model and observation of angiogenesis characteristics

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Received August 25, 2015; Accepted December 11, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: To investigate the establishing method of human hepatoma cell line Bel-7402 CAM transplantation model and to observe and analyze the characteristics of angiogenesis. Different concentrations of human hepatoma Bel-7402 cells were inoculated on chick embryo chorioallantoic membrane (CAM) to observe CAM tumor survival factors, tumor growth characteristics and angiogenesis. Human hepatocellular carcinoma CAM xenograft model was established. In the model, tumor was easy to grow, with a strong angiogenic effect. The model is easy to copy, and can dynamically observe HCC angiogenesis process, which can be used to study the biological behavior of HCC and screen drugs.

Keywords: Hepatoma, chick chorioallantoic membrane, models, animal, angiogenesis

Introduction

Angiogenesis is the essential biological process in tumor growth [1]. Liver cancer is one of the most common malignant tumors in the digestive system; its growth, metastasis and prognosis is closely related to tumor angiogenesis [2, 3]. Therefore, anti-angiogenesis has become one of the promising way to treat liver cancer; the research premise of tumor angiogenesis and anti-angiogenesis therapy is to establish a suitable model of angiogenesis. In this study, human hepatoma cell line Bel-7402 was used to establish CAM transplantation model and observe angiogenesis features.

Material and methods

Material

Human hepatoma cell line Bel-7402, a gift from the Traditional Chinese Medicine laboratory, Changhai Hospital of Second Military Medical University.

Reagents RPMI 1640 medium: Solarbio Company; sterilizing newborn calf serum without mycoplasma: Hangzhou Sijiqing Biological Engineering Material Company; trypsin: Solarbio Company.

Eggs were purchased from Shanghai flagpole farm.

Methods

Tumor cell culture BEL-7402 cells were incubated with RPMI 1640 medium containing 10% inactivated newborn calf serum, conventionally cultured in incubator.

Chick embryo incubation After routine disinfection, with the chamber end up, eggs were incubated in a 37.6°C incubator with 66% humidity; Turn the eggs once a day. Homemade eggbeater was used for egg inspection; delineate the window position at 0.5-1.0 cm from the bottom of fetal head at and set aside.

CAM fenestration The well-developed 8-day-old Chick embryo was placed in clean benches and disinfected; open a hole in chamber end and penetrate the shell membrane with sterile ophthalmic tweezers; with the above delineated window position as the center, open a 1 cm × 1 cm window; add 1 drop of sterile with saline syringe to wet the egg shell membrane, and then pierce the shell membrane, so that CAM sagged and separated with egg shell membrane; CAM was exposed after removing the egg shell membrane to make a fake gas cham-

Table 1. Effects of the concentrations of the tumor cells BEL-7402 on the embryo survival rate

Groups	The experiment chicken embryos (only)	Live chicken embryo number (only)	Survival rate (%)
PBS	6	6	100
$1*10^6$	6	6	100
$2*10^6$	6	5	83.33
$4*10^6$	6	5	66.67
$8*10^6$	6	5	83.33
$1.6*10^7$	6	2	33.33

Table 2. Tumor formation rate of inoculated in CAM different numbers of Bel-7402 cells

Groups	Live chicken embryo number (only)	Chicken tumor number (only)	Tumor rate (%)
PBS	6	0	0
$1*10^6$	6	0	0
$2*10^6$	5	1	20
$4*10^6$	5	2	40
$8*10^6$	5	4	80
$1.6*10^7$	2	2	$100^{▲,☆}$

Note: ▲Compared with 2×10^6 group, $P < 0.05$; ☆Compared with 8×10^6 group, $P > 0.05$.

ber, with sterile transparent tape to seal window for spare.

Tumor cell inoculation and experimental groups BEL-7402 cells in exponential growth phase were digested with 0.25% trypsin and centrifuged at 1000 r/min for 5 min; the number of cells was adjusted with 0.1 mol/L PBS. 20 mL cell suspension was inoculated into the relatively avascular regions of CAM; a total of six groups: PBS, 1×10^6, 2×10^6, 4×10^6, 8×10^6, 1.6×10^7; PBS group was taken as negative control, 9 Chick embryo per group. After addition of the cells, it was sealed with a sterile transparent tape and placed in an incubator with constant temperature and humidity to continue the incubation, without rotating the eggs.

Fixation After removing the membrane and inoculation, Chick embryo survival and tumor growth were observed every day. Chick embryonic survival was judged based on the embryo activities; solid tumors with a diameter of 2 mm was regarded as positive tumor-formation; embryo was collected from each group every day since the completion of vaccination; CAM pictures were taken by orthotropic digital camera; then it was fixed with a 1:1 mixture of methanol and acetone for 10 min; with CAM ophthalmic scissors to cut the experimental area, which was tiled in dish and photographed by a digital camera; computer image analysis system was used to process the pictures; some specimens were fixed with neutral formalin for 10-15 min in situ, embedded in paraffin and sectioned to perform histological examination (HE staining).

Statistical analysis

SPSS 13.0 statistical package was used to test and process data. Count data were presented in n (%), and X^2 test was used to compare the difference between each group. $P < 0.05$ was considered statistically significant.

Results

Effects of the number of the BEL-7402 cells on the embryo survival rate and tumor formation rate

In this study, five different numbers of Bel-7402 cells were inoculated into the relatively avascular zone of CAM; after inoculation, incubation was continued for 7 d; the results showed that different inoculated numbers of Bel-7402 cells had no significant effect on the survival of Chick embryo; Chick embryo survived well in each experimental group; the survival rate of $1.6*10^7$ group was slightly lower; there was no significant difference in the effect of different inoculated numbers of cells on Chick embryo survival ($P > 0.05$) (**Table 1**).

When the number of cells was lower than the $4*10^6$, it was hard to form tumor; tumor formation rates in $1*10^6$ and $2*10^6$ were 0 and 20%; compared with the PBS control group, the difference was not statistically significant ($P > 0.05$); with the number of Bel-7402 cells increased, tumor formation rate also increased; when the number of Bel-7402 cells increased to $1.6*10^7$, tumor formation rate increased to 100%; and compared with $2*10^6$ group, the difference was statistically significant ($P < 0.05$); but compared with $8*10^6$ group, there was no significant difference in tumor formation rate ($P > 0.05$) (**Table 2**).

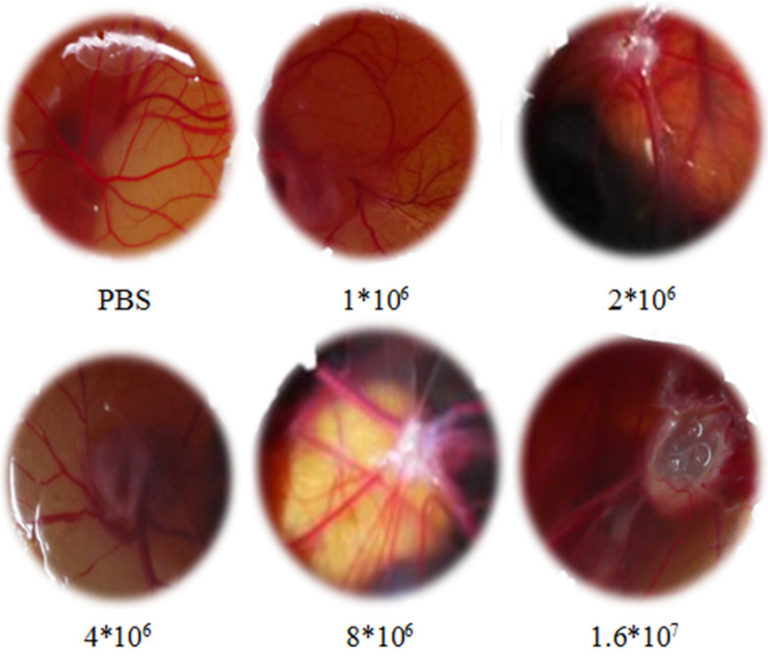

Figure 1. Tumor formation of the CAM inoculated by different numbers of BEL-7402 cells (SP*100).

Situ growth characteristics of xenografts

At 24 h after BEL-7402 cells were inoculated, BEL-7402 cells attached to the vaccination area of chick chorioallantoic membrane, showing pale white; after 48 h, BEL-7402 cell aggregated in inoculation area, and CAM transparency decreased slightly; after 72 h, the tumor appeared; its growth rate significantly accelerated, and volume increased significantly; there was obvious angiogenesis and vessels grew into the vaccination area; after 96 h, blood vessels showed radial growth around the tumor; tumor growth came into the peak period, with a significant increase in volume; 7 days later, the increase rate of the tumor slowed; on the 8th day, with the recession of chorioallantoic membrane vascular network, tumor growth was beginning to recess; there was no significant changes in tumor volume.

Angiogenesis features of liver transplantation model

In 24 h after BEL-7402 cells were inoculated, in addition to the normal development of CAM vascular network, no significant vascular changes had been observed; 48 h later, weak blood vessel reaction was observed, and a small amount of fine blood vessel grew to vaccination zone of Chick embryo; after 72 h, on the CAM blood vessels markedly dilated and increased, but disorganized; blood vessels centralized to the tumor on the CAM, and some grew into the tumor; on the 4th day, the blood vessels showed a radial arrangement taking the newborn tumor as the center; some were thick and coiled. On the fifth day, the tumor entered into the growth peak; there was significant difference in the number of vessels compare with the first day; later the number of vessels was increasing every day; on the 8th day, with the recession of chorioallantoic membrane vascular network, the number of vessels within the selected range around the tumor had decreased significantly (**Figure 1**).

Histological features of liver cancer CAM transplantation model

Under light microscope, paraffin sections and HE staining showed that cell structures of tumor tissue in the model were similar to those of human hepatoma tissue. Tumor cells were round or oval, with a large volume; tumor giant cell can be observed; little cell cytoplasm, large and deeply-stained nuclei; significant atypia; mitotic was common. Some tumor cells formed duct-like tissue. Angiogenesis and lymphangiogenesis could be observed around the tumor (**Figure 2**).

Discussion

A variety of vivo and vitro models are widely used in tumor angiogenesis and anti-angiogenesis studies. Vitro models often require a combination of appropriate vivo models due to lack of environmental simulation of tumor growth and blood vessels. CAM xenograft model is a widely used model in vivo studies [4, 5]; this study inoculated human hepatoma cell line Bel-7402 into the CAM model, explored the modeling conditions of BEL-7402, and successfully established hepatocellular CAM xenograft model; the growth of transplanted tumor and angiogenesis feature could be observed from the window of the eggshell; the effect was visu-

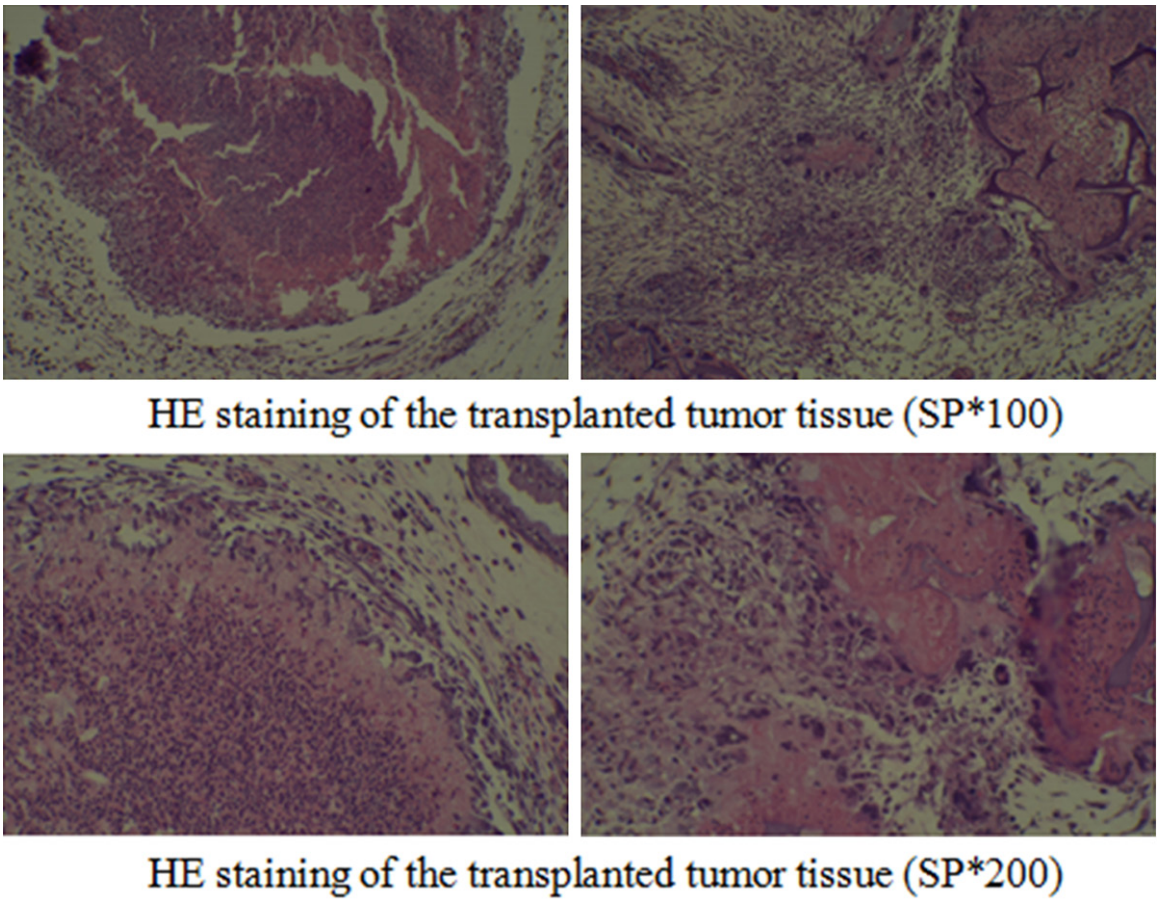

Figure 2. HE staining of CAM transplanted hepatocarcinoma.

ally shown. The experiment had low requirements on the equipment conditions, and the experimental cycle was short. Therefore, CAM angiogenesis is a good animal model for the study of angiogenesis and anti-angiogenesis inhibitor, which can be applied to the angiogenesis and anti- angiogenesis studies in hepatic carcinoma.

But the conditions and success rates of different tumor types and different tumor cell lines in CAM xenograft model process vary. Its success rate is affected by the window method, carrier selection, window time, the number/density of cell seeding and many other factors [6]; before establishing the model, various factors should be considered to.

In this study, BEL-7402 cells showed island-like growth and proliferated actively; the number of seeded cells had a significant effect on the tumor rate of xenograft models. But the survival of chick embryo and operability of the experiment should also be considered; in the 1.6×10^7 group with a tumor rate of 100%, the workload and difficulty of cell culture would increase in batch modeling. When applying the model and selecting appropriate magnitude of tumor cells, the survival, tumor formation rate and operability should be considered, ensuring a high tumor rat without increasing the burden on experiment because of the too large number of cancer cells. Our experimental results showed that 8×10^6 was the ideal inoculation number for BEL-7402 cell CAM xenograft model.

Studies have found that the days of age of selected chicken embryos could affect the success rate effects of modeling [7]; the experiments showed that too little chick embryo was still at “single bead” stage, large and the embryonic activity was large, so vaccination site was difficult to fix and often shifted, which had great influence on the experimental results and observations; and little embryo had high

requirements on experimental ambient and temperature; the mortality was also high after fenestration. With embryonic age increasing, the survival rate of embryo will be significantly increased after windowing, but when the chick embryo age was too old, it occupied a larger space within the shell, and CAM also began to degenerate, and chicken incubation period was only 21 d, which would limit the application greatly after modeling; so it should not be vaccinated too late. Comprehensively considering the state of chick embryos, angiogenesis feature and experimental purposes, the ideal embryo age for BEL-7402 cell CAM inoculation was 7-9 days. In addition, aseptic operation is also critical for successful modeling; some scholars have suggested dropping the liquid containing antibiotics to prevent infection. But if we can ensure strict aseptic operation, not only the success rate of the experiment could be ensured, but also the impact of preventive antibiotics on cell growth and late pharmaceutical intervention could be avoided.

In the experiment we observed that vascular response was prior to the growth of transplanted tumor, in line with the theory of tumor angiogenesis of Folkman [8, 9]; tumor growth is dependent on new blood vessels to transport nutrients to a certain extent; when significant vascular reaction occurs, new blood vessels ensure the peak of tumor growth; with the recession of CAM angiogenesis, tumor angiogenesis would decline and tumor growth stops. The main drawback of CAM transplantation model is modeling the relatively fixed observable experimental cycle after modeling, without sustainability. But as vivo experimental model, conventional fixing, paraffin section and HE staining showed that human hepatocellular cell line BEL-7402 CAM xenograft model reserved the histological features of liver cancer excellently, increasing the credibility of the intervention study on biological behavior.

Xenograft model established by inoculating BEL-7402 cells directly into the chick chorioallantoic membrane can be used to study the growth characteristics of hepatoma, angiogenesis, invasion, metastasis, and a variety of chemotherapeutic drug screening and radiation sensitivity [10, 11]. Especially it is suitable for screening and studying tumor angiogenesis factors and drugs; it provides a good model for anti-angiogenic therapy in liver cancer.

Acknowledgements

This work was supported by the Project of National Natural Science Foundation of China (81202958, 81473624, 81473628), Key specialized subject construction project of national Chinese Medicine (ZJ0901ZL020), Scientific research project of Shanghai Municipal Bureau of health (20124Y018) and innovation project of Shanghai Municipal Board of education (12YZ057).

Disclosure of conflict of interest

None.

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