

Angiogenesis characteristics of infantile hemangioma and feasibility observation of transplantation model of human hemangioma on mice

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Abstract. – **OBJECTIVE:** To study pathogenic features of pediatric hemangiomas, we successfully established a model in mice, by transplanting human hemangioma tissues.

MATERIALS AND METHODS: The hemangioma from the leg of a two-month-old infant was dissected and sliced into several pieces. During a careful surgical procedure, the hemangioma tissues were individually transplanted into skin incisions in anesthetized mice. The volume of the transplanted tumors was measured and the changes in shape recorded at 1 day, and at 1, 2, 3, 4, 5 and 6 months after the transplantation. HE dyeing, CD31 and Glut1 IHC were applied to tumors in the proliferation and involuting phases. Also, 10 survival tumors and 10 normal tissues from infants undergoing circumcisions (control tissues) were used to determine their Angiotensin 1 (Ang1), Angiotensin 2 (Ang2), Tie2, and endothelium growth factor (VEGF) expression levels by IHC method.

RESULTS: We observed all the tumors going through the same stages, where after two months their volumes increased sharply and then after 4 months they all began to recede. During the proliferative phase, newly born capillaries could be seen and the tumor elasticity increased (bright red color). During the involuting phase, the color faded away and the tumors became harder and were almost gone by 6 months. During the first two months after transplantation, HE dyeing showed hypertrophied and proliferating endothelial cells accumulating inside the tumors with irregular cavities inside blood vessels being filled by them. During the involuting phase (at 4 months), the lumen in blood vessels was distinctly enlarged while fiber and adipose tissue had significantly deposited. The transplanted and original tumors tested positive for CD31 and Glut1 dyeing, without significant differences. Compared with control samples, the Ang1 expression of the transplanted tumor in both the hyperplasia and proliferative phases was stably low ($p<0.05$), while expressions of Ang2 and Tie2

were both stably high ($p<0.05$). The VEGF expression in the tumors, however, was high during the proliferative phase ($p<0.05$), while the VEGF of the involuting phase showed no significant differences from that of the normal samples ($p>0.05$).

CONCLUSIONS: We showed the reliability of the mouse model in reflecting the pathologic evolution of the proliferation and involuting phases of infantile hemangiomas. Angiogenic mediators Ang1, Ang2 and Tie2 may be abnormally expressed and play important roles in the development of this angiogenic disease.

Key Words

Hemangioma; Infant; Mice; Angiotensin; Endothelial cells.

Introduction

Infantile hemangioma is a common benign tumor occurring in 1-10% of children. The prognosis for a hemangioma is very good since 90% of them disappear naturally¹. Most hemangiomas first develop within several weeks after birth and get enlarged continuously within a short period of time (about half a year) before starting to recede naturally. For the overwhelming majority special intervention is not required. Nevertheless, tumors in a few infants lead to complications such as malformation, or organ dysfunction during the process of enlargement. In these cases, clinical intervention should be actively taken. Even though there are various approaches for treating hemangiomas, including systemic or topical glucocorticoids, a beta-blocker or surgical removal, none of the alternatives are widely recommended for children². In the 1990s, researchers looking at the pathophysiology of hemangiomas performed important correlation studies, and came up with

Ideas linking hemangiomas to developmental defects, gene mutations or involvement of placental chorionic villi endothelial cells³. However, the basic understanding of hemangioma development, proliferation and involution is still lacking, slowing down research for targeted therapeutic strategies. Researchers have sought out the establishment of a good animal model with no satisfactory results⁴. In this study, a transplantation model of human hemangioma on mice was established to observe the changes in shape, size and nature of the transplanted tumor and to test the expression of angiopoietin and its Tie2 receptor family proteins. We gathered information that successfully explains how the interaction of mediator proteins marks the transition from the proliferating to the involuting phase in hemangiomas.

Materials and Methods

The list of essential materials for this study included: mouse anti-human CD31 monoclonal antibody (Lab Vision/NeoMarkers, Fremont, CA, USA), rabbit anti-human Glut1 polyclonal antibody (Lab Vision/NeoMarkers, Fremont, CA, USA), goat anti-human Ang1 antibody (RnD Group, Indianapolis, IN, USA), goat anti-human Tie2 antibody (RnD Group, Indianapolis, IN, USA), goat anti-human Tie1 antibody (RnD Group, Indianapolis, IN, USA), rabbit anti-human VEGF antibody (Shanghai Anyan Biology Co., Ltd., Shanghai, China).

The list of equipment included: a Vernier caliper (Shanghai Precision Instrument Factory, Shanghai, China), full automatic immunohistochemical staining equipment (Sigma-Aldrich, St Louis, MO, USA), Nikon E600 research microscope (Nikon, Tokyo, Japan), section bleach and heat temperature controller QP-B1 type (Anhui Electronic Science Institute, Anhui, China), an image analyzer (American Diagnostic Company, Hauppauge, NY, USA).

A two-month-old male infant in our hospital was selected as the tissue donor. The hemangioma lesion on the infant's leg was carefully excised from the subcutaneous tissues under aseptic conditions. A portion of the sample was used for immunohistochemistry and another one was saved for transplantation, kept at 4°C in a humidified box for less than an hour before the procedure.

The immunohistochemistry sample was fixed and embedded into paraffin for sectioning and HE dyeing. Immunohistochemistry (IHC) was performed with antibodies for CD31 (located in the

vascular endothelial cell membrane) and for Glut1 (located in the hemangioma endothelial cell membrane). For each section, 10 fields of high power lens were chosen; the images were analyzed to calculate the integral optical density, the average optical density and the proportion of IHC positive reactions. In the case of Glut1, positivity was judged based on the presence of dye in relation to the amount of red blood cells included (a negative sample showed no dye, a weak positive sample showed less dye area than red blood cells' area, and a strongly positive sample had as much dye as red blood cells area).

The hemangioma sample ready to be transplanted was cut into 5×4×3 mm patches. Each mouse was anesthetized by injecting it with 45 mg/kg of 2.5% pentobarbital sodium into the abdominal cavity. Then, incisions were made at the neck, waist and back, and both armpits of each mouse. There were 10 mice, with 4 transplant places each, for a total of 40 transplantations. The operations all happened within 1 hour after the hemangioma was originally dissected from the infant's leg. After the transplantation, no other special measures were taken. Each patch was left to grow naturally and its volume was regularly recorded. The Vernier caliper was used to measure the transverse diameter (a) and longitudinal diameter (b) of the tumors. The computational formula for the volume was $\pi/6 \times a \times b^2$. And the volume of each tumor was observed after 1 day, and 1, 2, 3, 4, 5 and 6 months after the transplantation. Additionally, two healthy tumors were chosen and dissected to conduct HE, CD31 and Glut1 dyeing 2 and 4 months after the transplantation.

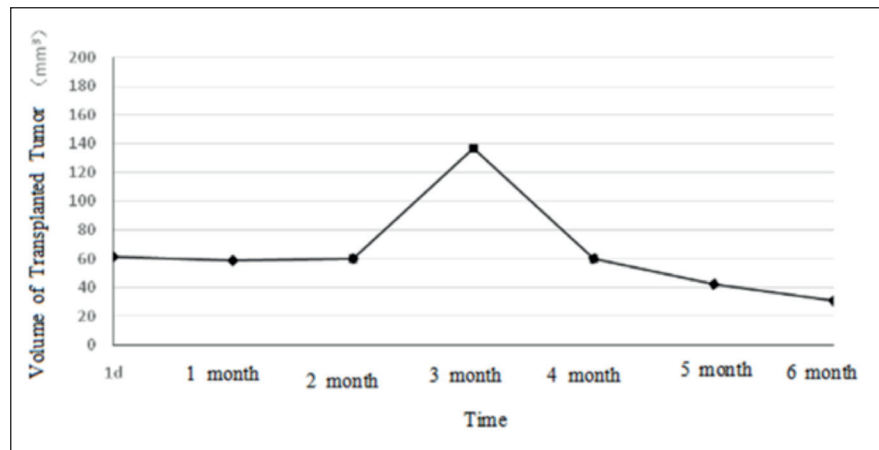
10 survival tumors from mice were chosen 2 months and 4 months after the transplantation model was established. They were fixed with 4% paraformaldehyde. The IHC method was applied to test for Ang1, Ang2, Tie2 and VEGF. Meanwhile, 10 samples of removed prepuce tissue were chosen from normal infants after circumcision for comparison to normal control tissue.

The Nikon E600 microscope, SPOT Cool CCD camera was chosen to capture and observe the images. In terms of image analysis, the Image pro plus 4.5 software was used. 10 fields of high power lens were chosen for each section after observation. The integral optical density (IOD) was recorded; the computational formula multiplied the tested area times the average optical density.

Statistical Analysis

The statistical software SPSS 19.0 was used to analyze the data. The IOD values of Ang1, Ang2, Tie2 and VEGF were calculated and shown as

Figure 1. Average volume changes of the transplanted tumor within 6 months.



media $\pm$ standard deviation. The *t*-test was applied for pairwise comparisons and $p < 0.05$ was considered as statistically significant.

Results

Dynamic observation of changes in the volume and form of the transplanted tumor

Volume: during the first 2 months, there were no distinct changes in the volume of the transplanted tumor. Then after 2 months, the volume got larger quickly and reached a peak value after about 3 months. Later, during the 4th, 5th and 6th month the tumors followed a shrinking trend. At 6 months, the volumes were about 1/3 of the largest volume they had attained. Please refer to Figure 1.

Form: there were no distinct changes during the first 2 months. Then, newly born capillaries started to appear and the tumors got larger quickly, the elasticity increased and a light blue color could be observed. 3 months after the transplantation, the tumor elasticities reached the highest value and the colors were bright red. Later, as the tumors began to shrink again, the color began to fade away, the elasticity decreased and the tumors became harder. At 6 months, most of the tumors had disappeared, the small number of tumors left over were light yellow or white, and most of them were in the form of fiber or fat tissue and relatively hard (Figure 1).

HE, CD31 and Glut1 Dyeing Result Analysis

HE dyeing: From the first day after transplantation to two months later, HE dyeing revealed hypertrophied endothelial cells proliferating and

accumulating inside the tumor, and the irregular lumen inside the blood began to narrow. During the proliferative phase (2 months-3 months), the endothelial cells accumulated in large amounts in a nest-like form, and the irregular lumen inside the blood vessels further narrowed. During the regression phase (4 months), the endothelial cells became fewer. The lumen inside blood vessels expanded and fibrous and fatty tissue deposition occurred.

CD31 dyeing: The expression of CD31 in the vascular endothelial cell membrane of transplanted tumors and donor's hemangiomas was positive. The average IOD values during the proliferative phase of the two kinds of tumors were respectively 5288.1 ± 450.3 and 5166.6 ± 724.9 , showing no statistically significant difference ($t = 0.637$, $p > 0.05$).

Glut1 dyeing: The transplanted tumors and human hemangiomas of the donor both showed strong positive expression of Glut1 as expected.

Comparison of IOD value of Ang1, Ang2 and Tie2 during the proliferative and involuting phases and in normal tissues.

Compared with normal samples, the expression of Ang1 in transplanted tumors was low while the expressions of Ang2 and Tie2 were high. The average expressions of Ang1, Ang2 and Tie2 showed no significant differences between the proliferative and the involuting phases in tumors ($p > 0.05$). The VEGF average expression in tumors was high during the proliferative phase and low in the involuting phase, showing significant differences ($p < 0.05$). However, the expression of VEGF during the involuting phase showed no significant difference from that in the normal control samples ($p > 0.05$). Please refer to Table I.

Table I. Comparison of average IOD values for Ang1, Ang2 and Tie2 during the proliferative and receding phases and in normal tissues.

Sample Type	Ang1	Ang2	Tie2	VEGF
Proliferative Phase (at 2 months)	802.3±211.4 ¹	5943.6±4194.0 ¹	4301.5±1164.8 ¹	3346.8±711.1 ^{1,2}
Receding phase (at 4 months)	744.5±142.3 ¹	4971.1±1762.1 ¹	4148.5±514.7 ¹	121.6±72.3
Normal samples	1458.7±476.3	541.0±411.9	811.6±124.8	97.1±64.3

¹The IOD values in transplanted hemangiomas were compared to the same values in normal samples, $p < 0.05$.

²The IOD values in the proliferative phase were compared to those in the receding phase, $p < 0.05$.

Discussion

Infantile hemangiomas are a common type of benign tumors, occurring in 2-10% of infants. The incidence in one-year-old infants is a little higher than that in newborns, while premature infants have the highest incidence of all. The main manifestation of this disease is the abnormal hyperplasia of vascular endothelial cells. In a classical clinical picture a hemangioma appears 1 month after birth and continues to enlarge within the next half or 1 year. Then, most of the tumors recede naturally, but a small amount of residual tissue may lead to a partial skin scar, atrophy or angiotelectasis. Despite the fact that most hemangiomas do not require special intervention, due to individual differences, a small amount of hemangiomas proliferate too fast or grow in inconvenient places, and require active intervention⁵. Currently, there are no specific treatment methods. Furthermore, little is known about the pathological development characteristics of hemangiomas and establishing an accurate animal model has become a necessary approach to elucidating the pathogenesis of these tumors.

In the hemangioma transplantation model on mouse, it must be guaranteed that the transplanted tumor can survive continuously and the pathological development characteristics such as the shape are similar to those in the human body. In this study, we have established a transplantation method of human hemangiomas on mice. The recorded changes in shape at different time points, confirmed that the trends are similar to those in humans, which matches earlier reports by other researchers^{6,7}. Moreover, HE dyeing showed distinct hyperplasia of endothelial cells with a small and irregular cavity gap between vessels during the early (proliferating) phase; and flattened endothelial cells with an enlarged cavity gap between vessels during the involuting phase. Such characteristics are similar to the histological features of human hemangiomas⁸. In addition, CD31 dyeing results

also proved the components of the transplanted hemangioma derived mainly from human hemangioma endothelial cells, and exclude the possibility that the tumors proliferated from murine tissues. Finally, other studies have found Glut1 is expressed specifically in human hemangiomas and does not get expressed in normal tissues^{9,10}. Our results, showed Glut1 expression in both survival tumors and in the original human hemangioma of the donor. Based on all these considerations, we believe the mouse transplantation model for hemangioma is highly reliable and very useful.

Studies have shown that Ang1, Ang2 and Tie are involved in the growth and maturation process of new blood vessels, and that VEGF is directly involved in angiogenesis^{11,12}. Based on that knowledge, this paper examines the expression of Ang1, Ang2, Tie and VEGF (encompassing the whole process of the formation of blood vessels) in hemangiomas. By comparing the average IOD values of Ang1, Ang2 and Tie2 at different time points after transplantation and in control tissues, we found the expression of Ang1 is lowest in the hemangiomas¹³, which suggests that the low expression of Ang1 is an indicator for abnormal angiogenesis. Ang1 is mainly expressed in peripheral and parietal cells of the blood vessels. It is possible that Ang1 interacts with Tie2 leading to activation of phosphatidylinositol 3 kinase (PI3K)/Akt¹⁴. Activated Tie2 stabilizes endothelial cells and promotes their survival¹⁵. Ang2 is expressed in endothelial cells; it prevents Tie2 activation and promotes blood vessel destabilization (a first step before VEGF angiogenesis). However, mainly influenced by pro-angiogenic factors such as VEGF, Ang2 can vary its functions on the blood vessel¹⁶. Some studies suggest that without VEGF, high concentration of Ang2 can have the same function as Ang1, that is to say, helping with the survival of the endothelial cells¹⁷. This study shows that Ang2 expression is significantly higher in the transplanted tumors than in the normal control samples. However, during the transition from the proliferating phase to the involuting phase,

the expression level shows a declining trend. Therefore, we think dynamic changes of Ang2 might be playing an important role in the normal evolution process of the hemangioma.

Our IHC results showed low expression of Ang1 during the proliferative phase, while the expression of Ang2 and Tie2 was high. The combination of the latter two can inhibit the activation of Tie2 by Ang1, leading to a destabilization of the blood vessels (a step necessary for angiogenesis to begin). Meanwhile, during the proliferative phase, the VEGF expression is high. The high expression of VEGF can accelerate the hyperplasia of hemangiomas. The average expressions of Tie2 and Ang2 during the proliferation and involuting phases were high and showed no significant difference amongst the two phases, while the expression of VEGF was significantly lowered during the involuting phase. As a result, the lower VEGF levels during the involuting phase could be determinant for the resolution of the hemangioma.

Conclusions

We observed that the hemangiomas are basically a type of angiogenic disease, and that interactions between the mediators Ang1, Ang2, Tie2 and VEGF are the key players in the process of angiogenesis. By showing how VEGF changes distinctly during the proliferative and involuting phases, we begin to elucidate a key pathway of the pathological process in hemangiomas, linking the mediator proteins Ang2/Tie2 to the angiogenesis factor VEGF in the vessels of hemangiomas.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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