Angiogenesis and cell proliferation in human craniopharyngioma xenografts in nude mice

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Object. Craniopharyngioma is one of the most common congenital tumors of the sellar and suprasellar regions and accounts for between 4 and 6% of all intracranial tumors. Its oncogenesis and biological behavior have not been well studied, and neither a cell line nor an animal model have been established. To better understand the tumor and improve its clinical management, the authors investigated the angiogenesis and cellular proliferation in subcutaneous craniopharyngioma xenografts obtained by implanting human tumor cells into athymic nude mice.

Methods. Human craniopharyngioma cells obtained from surgical specimens were subcutaneously implanted into BALB/c-nu/nu nude mice to establish a preliminary animal model of a transplanted tumor. Immunohistochemical staining with streptavidin–peroxidase complex was used to identify the cell phenotype and to evaluate the angiogenesis and proliferation in the xenografts. Expression of cytokeratin, minichromosome maintenance deficient 6 (MCM6) protein, and endothelial cell marker CD34 on the xenograft sections were assayed quantitatively by computer-assisted microscopy.

Twenty-seven surviving subcutaneous xenografts were obtained in 15 nude mice. The total implantation success rate was 28.12% (adamantine epithelioma [AE], 37.50%; squamous papillary tumor [SPT], 18.75%). Formation of capillaries and cell proliferation were observed in all of these xenografts. Microvessel density and degree of MCM6 immunostaining were positively correlated in the surviving grafts (r = 0.410, p < 0.05), but there was no significant difference in these variables between the AE and SPT groups (p > 0.05).

Conclusions. A preliminary animal model of human craniopharyngioma was established in the nude mouse by heterotopic implantation. Surviving xenografts maintained their vascularization and proliferation activities until harvesting at 12 weeks.

Key Words • craniopharyngioma • angiogenesis • cell proliferation • animal model • xenograft • pediatric neurosurgery • mouse

Craniopharyngioma, one of the most common congenital intracranial tumors, accounts for between 4 and 6% of all intracranial tumors and for approximately 54% of sellar tumors in children.\(^2,16\) Although it was first reported more than 150 years ago, the pathogenesis and histological classification of craniopharyngioma remain controversial, and until now neither a cell line nor an animal model has been established. To better understand the tumor and improve its clinical management, the authors investigated the angiogenesis and cellular proliferation in subcutaneous craniopharyngioma xenografts obtained by implanting human tumor cells into athymic nude mice.

Methods. Human craniopharyngioma cells obtained from surgical specimens were subcutaneously implanted into BALB/c-nu/nu nude mice to establish a preliminary animal model of a transplanted tumor. Immunohistochemical staining with streptavidin–peroxidase complex was used to identify the cell phenotype and to evaluate the angiogenesis and proliferation in the xenografts. Expression of cytokeratin, minichromosome maintenance deficient 6 (MCM6) protein, and endothelial cell marker CD34 on the xenograft sections were assayed quantitatively by computer-assisted microscopy.

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Abbreviations used in this paper: AE = adamantine epithelioma; CK = cytokeratin; MCM6 = minichromosome maintenance deficient 6; MVD = microvessel density; SPT = squamous papillary tumor.
Human craniopharyngioma xenografts in nude mice

cells such as the athymic nude mouse is more practicable. Although there is a need for further study of the biological characteristics of the xenografts, they have been shown to maintain the inherent characteristics of the human tumor.33

In the present study, we implanted human craniopharyngioma cells subcutaneously into the bilateral axillae of nude mice in order to establish an animal model of the tumor. We assessed immunoreaction to anti-CK antibody in xenograft sections to confirm that the graft was derived from craniopharyngioma and then evaluated cell proliferation and angiogenesis immunohistochemically by using streptavidin–peroxidase complex.

Materials and Methods

Tumor Tissue and Antibodies

Twelve AE and 12 SPT tissue specimens from resected tumors were obtained from the Department of Neurosurgery at West China Hospital, Sichuan University. Written informed consent was obtained from all patients or their parents. Patients in the AE and SPT groups were matched for sex, age, and tumor size. The specimens were examined histologically and the diagnosis of craniopharyngioma was confirmed. Portions of the fresh tumor tissue were immediately cut into pieces of approximately 1 mm³ and put into culture capsules containing penicillin, streptomycin, amphotericin B, and 10% fetal calf serum. The samples were then stored at 4˚C.

A concentrated solution of goat anti–MCM6 antibody (clone C-20/sc-9843) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and concentrated solutions of broad-spectrum mouse anti–human monoclonal antibody (clone AE1/AE3[Q]) and mouse anti–human monoclonal antibody CD34 (clone QBEnd/10) were purchased from Zymed Laboratories, Inc. (San Francisco, CA).

Animal Preparation

The study was approved by the Bio-Medical Ethics Committee of Sichuan University. Twenty-four female and 24 male BALB/c-nu/nu nude mice (4–6 weeks old, weighing 18~22 g) were kept under pathogen-free conditions at 22˚C with a 12-hour light–dark cycle. They were provided with a standard laboratory diet and access to water.

Implantation and Xenograft Observation

To generate a tumor cell suspension for implantation, the pieces of tumor tissue were washed in phosphate-buffered saline, and minced with microscissors, then washed again and mixed by repeated pipetting in Hanks solution. The mice were anesthetized by intraperitoneal injection of 4% chloral hydrate, and the tumor cell suspension was injected into their axillae. Susensions of cells from each tumor were injected bilaterally into the axillae of one female and one male mouse, so four subcutaneous xenografts were expected to be obtained from each case.

Xenograft size was measured weekly by micrometer or vernier caliper. The volume was estimated according to the following formula: vol = π × abc/6 mm³ (a = length, b = width, c = height).

Twelve weeks after implantation, the surviving xenografts (27 in all) were removed for immunostaining and histological examination.

Immunohistochemical Studies

Series of 4-μm-thick histological sections were prepared from each of the 27 xenografts. Two sections from each graft were stained with H & E; the remaining sections were stained with anti-CD34 antibody, anti-CK antibody, anti-MCM6 antibody or used for controls.

For immunohistochemical staining, deparaffinized sections were treated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase, blocked with 5% bovine serum albumin, and then incubated with primary goat polyclonal or mouse monoclonal antibodies for 12 hours at 4˚C. The sections were then washed and incubated with biotinylated goat or mouse anti–human immunoglobulin G antibodies. The cells were visualized using 3,3’-diaminobenzidine and slight counterstaining with hematoxylin. Expression of CD34, CK, and MCM6 was detected by using the streptavidin–peroxidase method. Sections incubated without the primary antibodies served as negative controls.

Evaluation of Cell Proliferation and Angiogenesis

Images of MCM6-and CD34-stained vascular endothelial cells were obtained using an inverted biological microscope and camera (Nikon Corp., Tokyo, Japan). Mean density values were calculated as continuous indices with the aid of Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). Three representative sections were selected for each case. In each of these sections, five high-power fields were chosen in the most strongly stained areas, and then mean values were calculated for the xenograft.

Statistical Analysis

Statistical analyses were performed with SPSS version 12.0 (SPSS, Inc., Chicago, IL) and STATA version 8.0. (Stata Corp., College Station, Texas) software. Continuous data were expressed as means ± standard deviations. Rates of successful implantation were analyzed by chi-square test for AE and SPT. The correlation coefficient for the mean densities of MCM6 protein and MVD in the xenografts was calculated by the least squares method. The survival rates of xenografts from primary and recurrent SPT and AE were evaluated using the Fisher exact and chi-square tests. The Student t-test for independent samples was used to compare the continuous variables among groups. Differences were considered statistically significant at a probability value of less than 0.05 (two-tailed).

Results

Cranioopharyngioma Xenografts

Cells from 24 tumors were subcutaneously implanted into 48 BALB/c-nu/nu nude mice by bilateral axillary inoculation. These 96 inocula resulted in 27 xenografts that survived at least 12 weeks. The surviving grafts were between 4 and 12 mm higher than the epidermis, without livedo, hard nodes, cysts, or abscesses. Microscopic examination showed that all the grafts were infiltrated by small blood vessels and that most of the original structural features of craniopharyngioma had been maintained. There were distinct boundaries between tumor and neighboring tissue, but no peels, cysts, or calcification foci were observed. The xenografts were generally homogeneous, with some microvessels among enamel epithelial cell groups or squamous epithelial cell layers. Other cell types were distributed randomly and loosely. In the mesenchyma of the grafts, the stellate reticulum and inflammatory cells appeared degenerated. In immunostained graft sections, the epithelial cells reacted to anti-CK antibody, indicating that they kept their original epithelial characteristics of craniopharyngioma.

Xenograft Survival and Cranioopharyngioma Subtypes

Cells from 12 resected SPTs were implanted into the axillae of 12 male and 12 female nude mice, producing 48 inocula. In 12 mice, all the SPT inocula appeared to have been fully absorbed within 3 weeks. However, in one of these mice (a male), a graft was observed to develop from the 4th week on. In eight additional mice, the inocula were absorbed more slowly and were no longer evident at 7 weeks. A total of nine SPT xenografts in three male and two female mice remained viable 12 weeks after implantation. Among the 48 AE inocula, there were 20 subcutaneous hemorrhages in 10 mice within the first 2 weeks, and by 11
weeks there was no evidence of the implant in these animals. An additional eight implants in four mice were no longer detectable after 8 weeks. Growth was obvious in 11 grafts in six mice from the 2nd week after implantation of the cells. Seven xenografts in four mice grew slowly from the 6th week. Eighteen AE grafts in 10 mice survived to the end of the 12-week study period; only one graft survived in each of two of these 10 mice.

The 12-week graft survival rates were significantly different for SPT and AE (p = 0.041). The AE xenografts survived more readily than the SPT xenografts in this nude mouse model.

**Xenografts From Recurrent Craniopharyngiomas**

Tumors recurred postoperatively in eight of the patients (three with SPT and five with AE) who contributed tumor tissue for the present study. Tissue from the recurrent tumors was also implanted subcutaneously into nude mice. The xenograft survival rate for inocula from recurrent SPT (four [33.33%] of 12 implants) was significantly higher than that for inocula from recurrence-free primary SPT (five [13.88%] of 36 implants; p = 0.019). Similarly, the survival rate for the inocula from recurrent AE (11 [55%] of 20 implants) was significantly higher than that for recurrence-free AE (seven [25%] of 28 implants; p = 0.034).

**Expression of MCM6 and MVD in Xenografts**

Cell proliferation was evaluated in the surviving SPT and AE grafts by immunohistochemical methods with anti-MCM6 antibody (Fig. 1), but MCM6 protein was also found in the cytoplasm of some cells, indicating that cell apoptosis occurred in parallel with the proliferation. In contrast, MCM6 protein was not expressed in the scarce inflammatory cells in the xenografts. When the MVD was evaluated by CD34 labeling of vascular endothelial cells, many unevenly distributed capillaries were detected in the central portion of the AE graft and around the epithelial lamina of SPT graft. This microcirculation could provide tumor cells with oxygen and nutrition (Fig. 2).

**Correlation Between MCM6 and MVD**

As we mentioned earlier in Materials and Methods, mean densities were calculated as continuous indices using the Image-Pro Plus software in conjunction with the Nikon microscope. In nine SPT grafts, the mean MCM6 density was 0.270 ± 0.124 (range 0.098–0.312), and the mean MVD was 0.149 ± 0.04 (range 0.065–0.234). In the 18 AE grafts, the mean MCM6 density was 0.291 ± 0.146 (range 0.137–0.384), and the mean MVD was 0.165 ± 0.07 (range 0.085–0.262). The mean MCM6 densities and MVDs correlated positively in 27 xenografts (r = 0.41, p < 0.05); there was no significant difference in mean MVD and MCM6 density between the AE and SPT xenografts (p > 0.1).

**Discussion**

**Residual Immune Potential and Xenografts**

In this study, the cells from 24 craniopharyngiomas were inoculated subcutaneously into the axillae of 48 nude mice, and 27 xenografts (in 15 tumor-bearing animals) were obtained in 12 weeks. Some inocula seemed to grow slowly or not at all initially but then enter a more active growth phase. Subcutaneous hemorrhage occurred in 10 of the mice. Other adverse reactions such as abscess or swelling were not observed, except in four mice that had bilateral swollen axillary and inguinal lymph nodes. The inflammatory reaction in the lymph nodes, identified by histological and immunohistochemical methods, indicated that the inoculation might have activated residual immune potential in the athymic mice. The rate of successful implantation might be improved through removal of residual immunity in the mice or the use of other more compatible animals.

**Xenograft Survival According to Tumor Subgroups**

The mean final volume of the 18 AE xenografts was 712.31 ± 4.26 mm³, with a mean increase of 621 ± 5.17 mm³. The mean final volume of the nine SPT grafts was 592 ± 3.20 mm³, with a mean increase of 482 ± 6.27 mm³. There was a significant difference in the mean increase in volume between the AE and SPT xenografts (p = 0.000).

The overall xenograft survival rate was 28.13%; the survival rates for AE and SPT grafts were 37.50 and 18.75%.
In the present study, fresh human craniopharyngioma cells were implanted subcutaneously into BALB/c-nu/nu nude mice and some xenografts were obtained. These tumors showed most biological characteristics of craniopharyngioma, and cell proliferation as well as the development of microcirculation were documented. Although a preliminary animal model of human craniopharyngioma was established, the rate of successful transplantation was still relatively low for preclinical studies of craniopharyngioma. The animal model should be further improved to provide a better understanding of the oncogenesis and recurrence of craniopharyngioma.

**Conclusions**

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**Acknowledgments**

We thank the neurosurgeons who helped us to obtain surgical specimens, and the personnel in the pathology laboratory who helped us identify the subtypes of craniopharyngioma.

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Manuscript received November 17, 2005. Accepted in final form June 26, 2006. This work was supported in part by Grant 05JY029-005-7 from the Science and Technology Bureau, Sichuan Province, People’s Republic of China.

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