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A Three-Dimensional Micro-Organ Culture System for Microtumor Spheroids from Human Malignant Glioma Specimens†

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= Abstract =**Tumor tissue obtained from seven human malignant gliomas was minced and explanted into agarose-coated culture plates. After five to seven days, these microtumor fragments emerged as spheroids in four tumors and were maintained as multicellular organotypic spheroids for more than eight weeks. The morphological features and growth characteristics of different spheroids were studied and compared with the histology of the original tumor specimens. Light microscopic and ultrastructural studies of the spheroids demonstrated that morphological structures were similar to those of the original tumor tissue in vivo. The microtumor spheroids contained connective tissue, blood vessels, and macrophages, maintaining a three dimensional-architectural resemblance to the original tumors. Volumetric measurement of the spheroids showed that the size decreased initially and did not change thereafter over a period of time. This growth pattern of the spheroids was consistent with that of tumors in vivo, suggesting the linkage of cell proliferation and loss. This in vitro culture system for surgically removed brain tumor specimens may serve as an alternative to the in vivo xenograft model for the research of brain tumor biology, invasion and immunology and provide a valuable technique for the evaluation of new therapies, such as biologic response modifiers.**

Key Words; *Micro-organ culture, Tumor spheroids, Malignant glioma*

INTRODUCTION

Malignant gliomas are composed of heterogenous populations of tumor cells (Darling *et al.* 1983) and normal host cells, including normal glial, mesenchymal, endothelial, and microglial cells, as well as lymphocytes and macrophages. Interactions among tumor cells or between tumor cells and host cells by virtue of direct cell-cell contact or through the extracellular matrix may therefore modify the biological properties of the tumor cells, such as chemosensitivity (Tofilon *et al.* 1987) and invasiveness (Bjerkvig *et al.* 1986).

In this respect, conventional culture methods such as monolayer (Rosenblum *et al.* 1978) and agarose (Hamburger and Salmon 1977) cultures of established cell lines or multicellular tumor spheroids from permanent cell lines (Sutherland *et al.* 1981; Sutherland

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1988) have many limitations in chemotherapeutic trial and studying tumor biology. In addition recent developments in the search for biologic response modifiers demands a culture system more similar to in vivo conditions. Recently, one of the authors reported the optimization of a three-dimensional micro-organ culture system for in vitro growth of microtumors from human glioma xenografts (Jung *et al.* 1991). In this study, cells cultured from human brain tumors were found to have the ability to form multicellular spheroids with the three-dimensional architectural complexity and diversity of cell types imitating the complexity of actual tumors. However, in this in vitro model, the residing host cells (macrophages) maintained in tumor spheroids were not human body cells but those of athymic mice. Thus, we initiated the development of a long-term micro-organ culture system for in vitro growth of multicellular tumor spheroids from human brain tumor specimens. A recent investigation from Norway with a background very similar to the present study showed that tumor tissue from human gliomas was maintained in long-term agar overlay culture as multicellular organotypic spheroids (Bjerkvig *et al.* 1990).

Here we report a long-term micro-organ culture system that supports the proliferation of human glioma cells from surgical specimens while sustaining normal host cells. We observed that tissue removed surgically from human malignant gliomas easily forms tumor spheroids in agarose overlay culture. Their light microscopic and ultrastructural morphological features and the individual growth pattern of different spheroids were studied.

MATERIALS AND METHODS

1. Tumor tissue

Tumor fragments 1.0 cm or smailer in size were obtained directly from the surgical specimens of four patients with glioblastoma multiforme and three patients with anaplastic oligodendroglioma. Under a surgical microscope, the most grossly viable portions of the tumor excluding the necrotic region or the area adjacent to the normal brain were resected. Specimens were taken in tumor areas corresponding to regions with contrast enhancement on preoperative computerized tomography or magnetic resonance images. The specimens obtained at surgery were immediately placed in a test tube containing the minimum essential medium (MEM) at 4°C and transported to the cell culture room. The MEM consists of Dulbecco's modified Eagle's medium (GIBCO, New York, U.S.A.) supplemented with 20% fetal calf serum (FCS) (GIBCO, New York, U.S.A.).

2. Microtumor culture

Specimens selected for three-dimensional culture were thoroughly cleaned of blood clots and connective or grossly necrotic tissue and then minced with sterile scalpels into pieces smaller than 1 mm in diameter. Minimizing the time between removal of tumors and incubation in the culture plate proved to be a critical determinant of successful microtumor growth. The tumor fragments were selected by size exclusion sieving (Nytex mesh filters, Tetko, Inc., Elmsford, New York), and the pieces between 200μm and 500μm were washed with cold PBS (4°C) and transferred to 6-well plates (Nune, Denmark). The wells were base-coated with 2.5ml of 0.5% agarose (BRL, U.S.A.) in MEM (MEM-agarose). The volume of the overlay suspension was 3 ml of MEM. The MEM was changed every week replacing 90% of the medium. The spheroids were cultured for up to 14 weeks, and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and checked daily with a phase-contrast microscope. Light microscopic, electron microscopic, and immunohistochemical investigations were performed after long-term culture.

3. Spheroid growth

The growth of the microtumors was examined periodically with an inverted microscope. Single spheroids (250 to 450μm in diameter)

were transferred with a sterile custompulled micropipette (200-500μm internal diameter) from the culture plates into 24-well plates. The wells were base-coated with 0.5 ml of MEM-agar. The volume of overlay suspension was 1 ml. To determine the spheroid size, individual spheroids were then selected after two to three days and seeded into the wells. The size of the spheroids was measured during the culture period with a calibrated reticle. The average diameter of the spheroids was recorded as measured by two diameters, the minimum and maximum radii which were at right angles to each other. Fragments that grew in irregular, asymmetric shapes were excluded from the data analysis.

4. Light microscopy

After long-term culture, five spheroids from each tumor were collected for light microscopic examination. They were gently rinsed three times in PBS and fixed for 4 hours in 3% paraformaldehyde and 0.1% glutaraldehyde in a 0.1 mol/L cacodylate buffer, pH 7.4. In order to discriminate the spheroids, they were stained with eosin and then embedded in 1.0% agar in phosphate buffered saline after fixation. Fixed spheroids in agarose block were embedded in paraffin. Sections from the paraffin blocks were stained with hematoxylin and eosin and were used for immunohistochemical study. The light microscopic observations of spheroids from each tumor were compared to the histologic findings of corresponding operative specimens obtained from the same patient.

5. Electron microscopy

At least three spheroids from each tumor were collected for transmission and scanning electron microscopic studies. They were fixed for two days in 2% glutaraldehyde in 0.1 M sucrose-adjusted cacodylate buffer (pH 7.4). Postfixation was performed in 1% O_5O_4 and spheroids were prepared for electron microscopic investigations.

6. Immunohistochemical study

Immunohistochemical staining was performed to detect leukocytes such as lymphocytes and macrophages after long-term culture. Sections from the paraffin blocks were treated with monoclonal antibodies (DAKO, Denmark) against CD45, L26, kappa, and lambda chains. The ABC Elite Kit (Vector) was used to process the slides for chromogenic reaction. The slides were counterstained in Mayer hematoxylin and examined under a conventional light microscope.

RESULTS

1. Spheroid formation

Of seven human malignant gliomas studied, four formed spheroids, after five to seven days in culture (Fig. 1). The fraction of the fragments that formed spheroids varied from 58% to 91% for the tumor studied (Table 1). Fragments that did not form spheroids became dark and died in the culture plates. Only tissue fragments that became spherical within seven days in culture were used for further study.

2. Spheroid growth

The spheroids from the four human gliomas revealed a relatively uniform growth potential. Differences in growth potential were not observed between glioblastoma multiforme

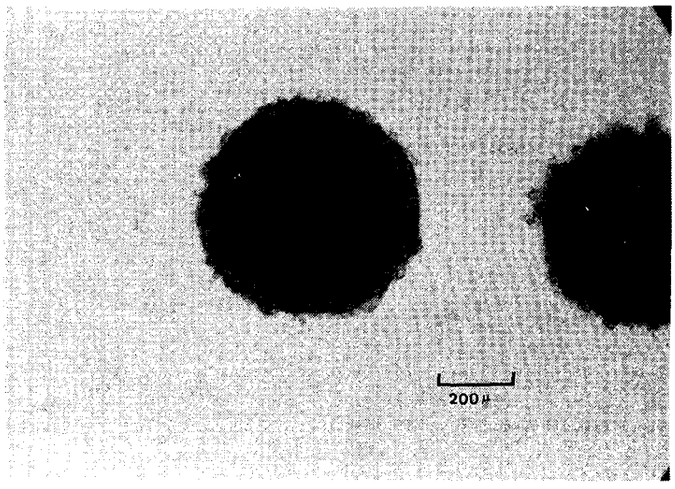

Fig. 1. Photomicrograph of the spheroids (GM2) in medium after eight weeks in culture (phase contrast microscope, x 100).

Table 1. Human gliomas grown as microtumor spheroids in vitro

Tumor no.	Patient's sex/age	Pathologic diagnosis	Fraction of spheroid formation	Duration of growth (wks)
GM 1	F/42	Glioblastoma multiforme	88%	8
GM 2	M/55	Glioblastoma multiforme	91%	9
OG 1	M/44	Oligodendroglioma, anaplastic	58%	14
OG 2	M/30	Oligodendroglioma, anaplastic	62%	14

and anaplastic oligodendroglioma. The size of the spheroids decreased during the first one to two weeks and was relatively stable thereafter over a period of culture time (Table 2).

Table 2. Spheroid growth

Tumor no.	No. of spheroids	Initial diameter* (μm)	Duration of growth (weeks)	Last diameter* (μm)
GM 1	13	390 ± 53	8	328 ± 58
GM 2	20	378 ± 46	9	319 ± 42
OG 1	15	346 ± 41	14	312 ± 49
OG 2	11	338 ± 52	14	286 ± 56

*: diameter as mean ± 1 standard deviation

3. Light microscopy

Spheroids obtained from the same biopsy had similar morphological features. However, some degree of morphological variation was observed between spheroids from different tumors (Figs. 2 & 3). The morphology of the spheroids remained similar to the morphology of the original tumor tissue (Figs. 2 & 3). After long-term culture, a varying degree of cell de-

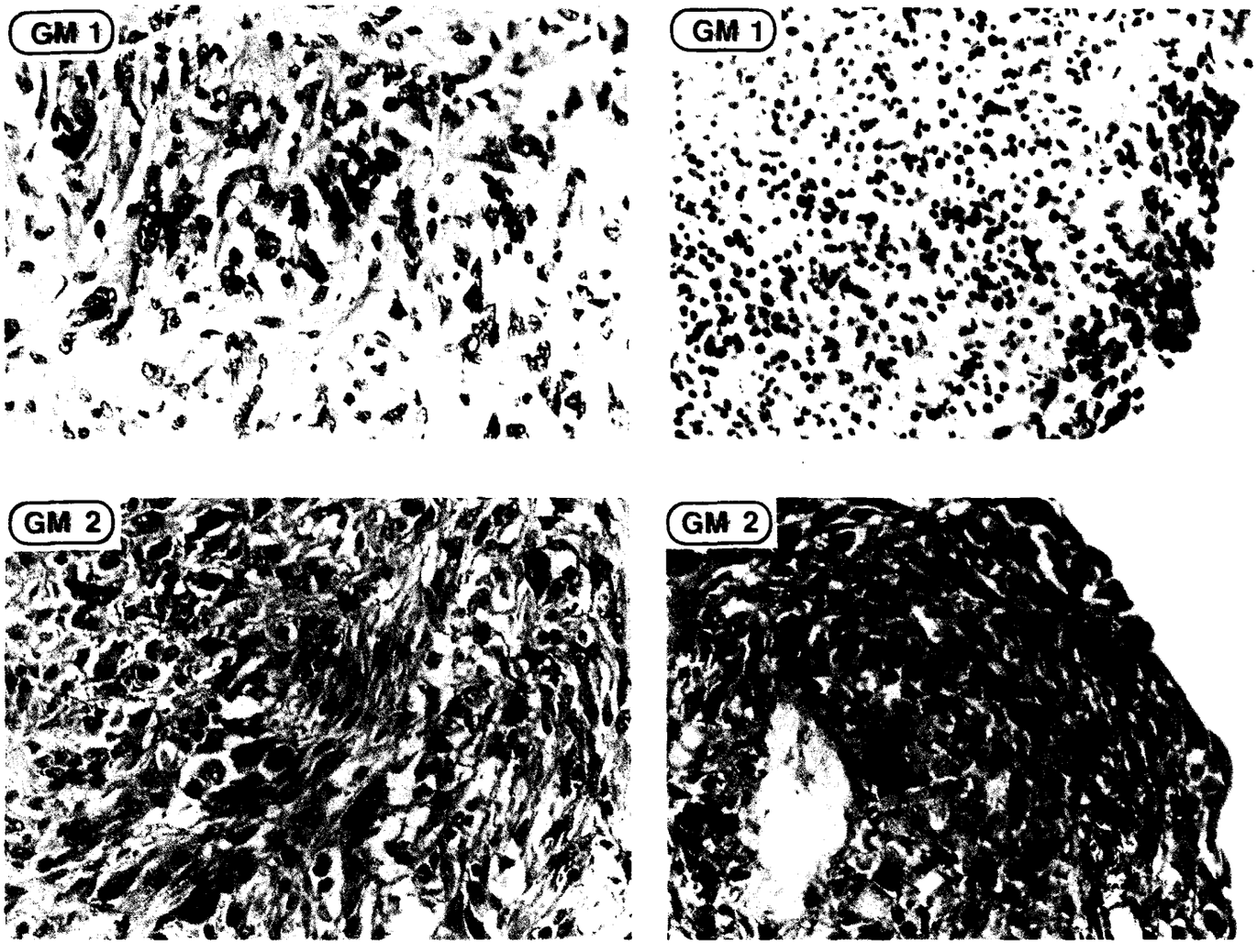

Fig. 2. Photomicrographs of the tumor biopsy material from two glioblastomas (left column) and of semi-thin sections of the tumor spheroids (right column) (H & E, x 400).

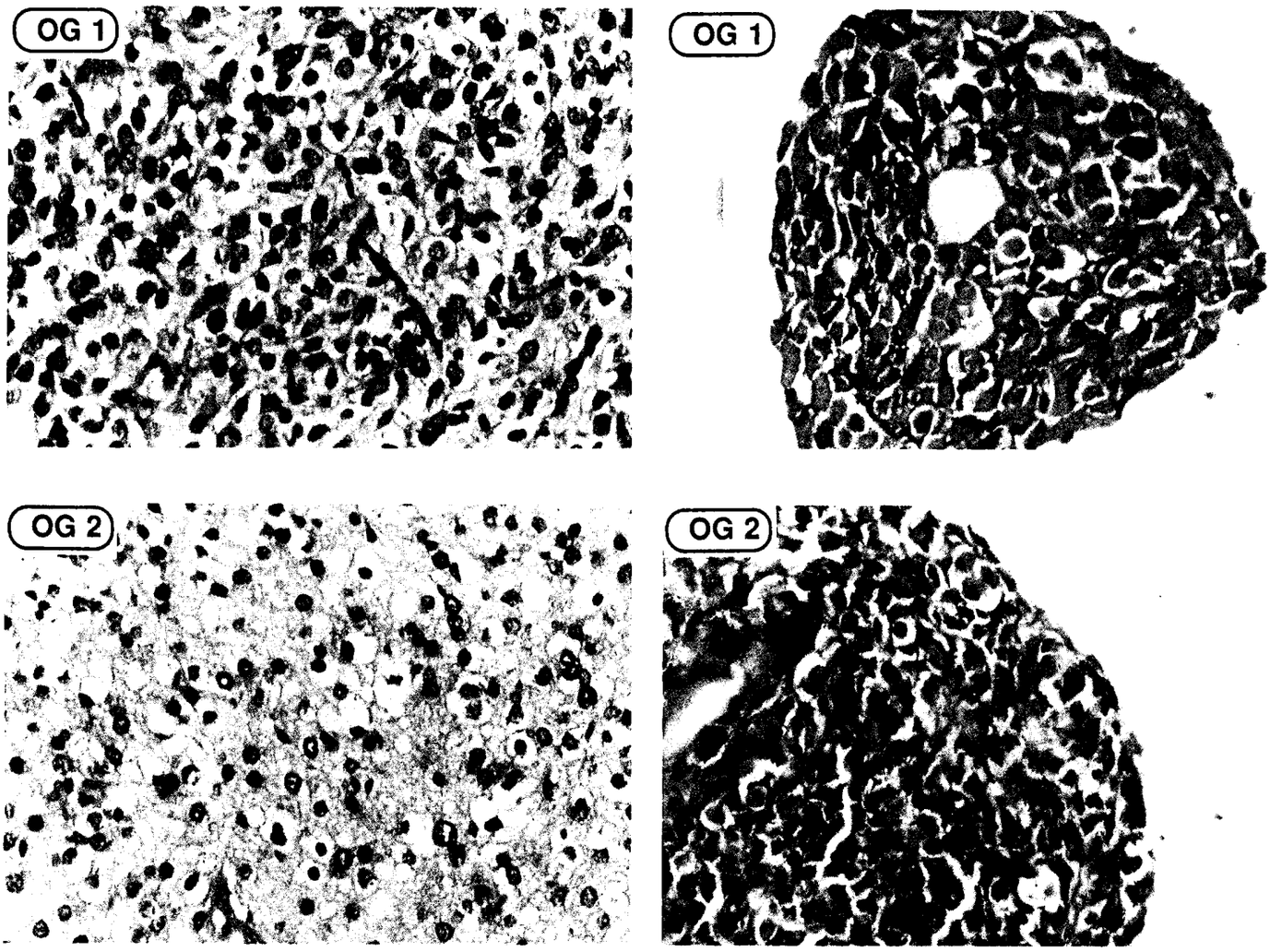

Fig. 3. Photomicrographs of the tumor biopsy material from two oligodendrogliomas (left column) and of semi-thin sections of the tumor spheroids (right column) (H & E, x 400).

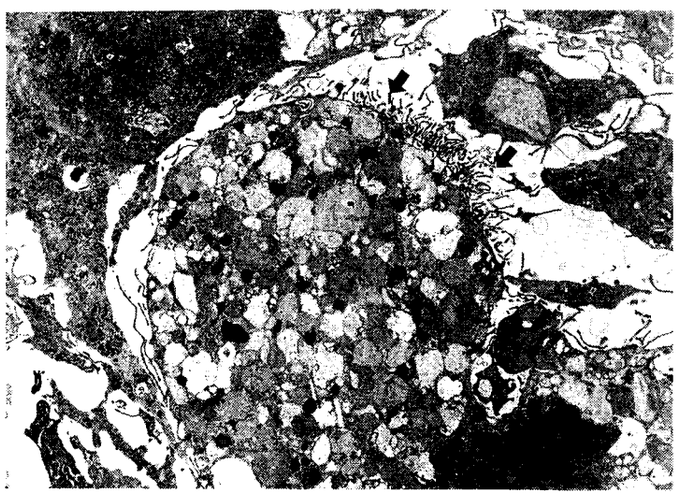

Fig. 4. Transmission electron micrograph from a GM1 spheroid closed up for a tumor cell with numerous microvilli (arrows) (x 2500).

generation with pyknotic cells was seen, including areas of necrosis and hyalinization in the center of the spheroids. The spheroids consisted of viable cells which were located mainly in the periphery of the spheroids. Some mitotic figures were also observed.

4. Transmission electron microscopy

Atypical cells were observed in all spheroids with varying ultrastructural characteristics. A considerable pleomorphism in cell shape as well as in the nuclear configuration was observed even after long-term culture. In the glioblastoma spheroids, cells exhibited ultrastructural features indicating an astrocytic origin (Fig. 4). In all of the spheroids, vascular elements and collagen fibers in association with

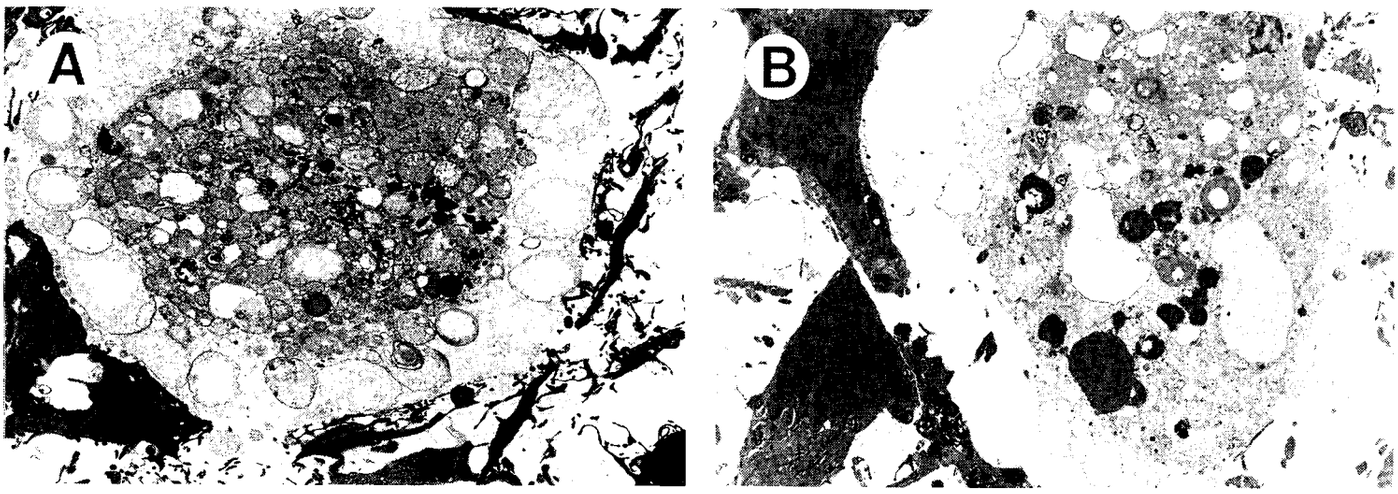

Fig. 5. Transmission electron micrograph from a OG2 spheroid closed up for a macrophage. A (x 3500) B (x 4000).

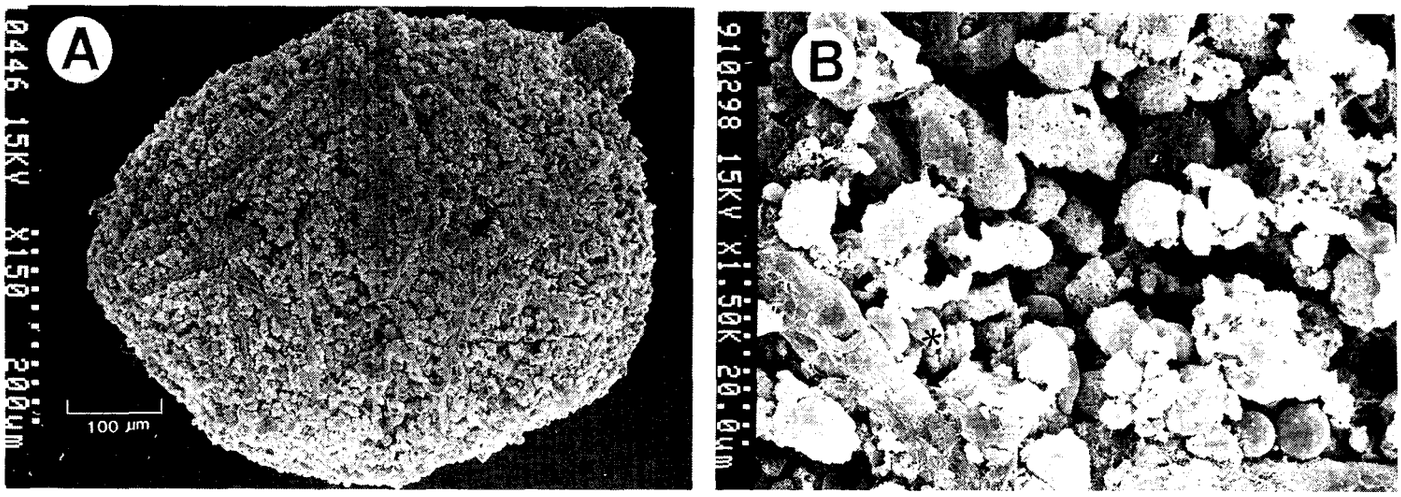

Fig. 6. Scanning electron micrographs of a spheroid from GM1. A (x 150), B (x 1500) Note numerous vascular elements (arrows) and red blood cells (asterisks).

the fibroblasts and macrophages were observed (Fig. 6).

5. Scanning electron microscopy

The surface architecture of the spheroids from the same tumor was fairly uniform. However, variations in surface structures were obvious when spheroids from different tumors were observed. Viable tumor cells as well as numerous vascular elements were seen on the spheroid surface (Fig. 6A). Scattered red blood cells and vascular elements were also found (Fig. 6B).

6. Immunohistochemical study

Some cells suggestive of positive reaction were observed in the spheroids after long-term culture, but in general these were interpreted as negative (Fig. 7).

DISCUSSION

Current methods used for in vitro drug sensitivity testing of malignant brain tumors are unable to account for the influences of cell-cell and cell-matrix interactions on tumor response (Miller *et al.* 1981). Human tumors grown as xenografts in immunodeficient mice have also been used to investigate the efficacy of chemotherapeutic agents. In vivo model system, however, is extremely costly and requires specialized animal care facilities. In vitro tumor

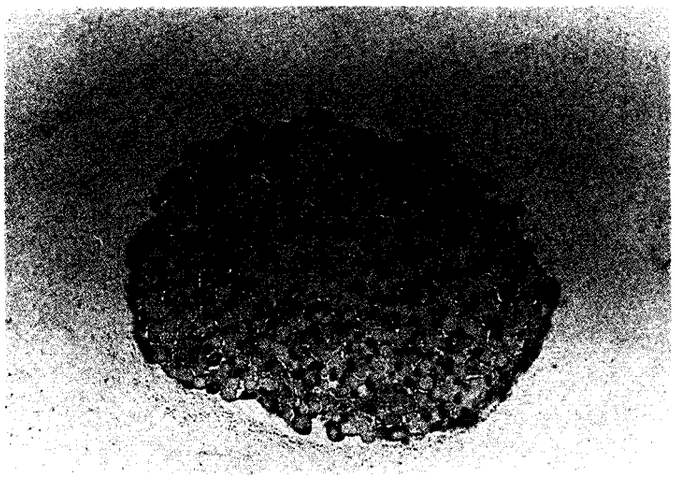

Fig. 7. Photomicrograph of semi-thin section of a spheroid from OG1 immunohistochemically stained for CD45. Some cells suggestive of positive reaction are observed but in general these are interpreted as negative (ABC, x 200).

models that imitate the complexity of solid tumors more closely may allow more realistic evaluation of drug therapy and may serve as a less costly alternative to in vivo models. Tumor tissue fragments with intact cell-cell relations may be grown in organ culture, where the specimens are kept in a moist gas or air phase on the surface of a relatively large volume of stationary nutrient medium (Archer *et al.* 1968; Kalus *et al.* 1968; Roller *et al.* 1966). Early attempts to propagate brain tumors in vitro as micro-organ cultures were reported by Rubinstein *et al.* (1973) and Saez *et al.* (1977). However, these culture methods had the disadvantages of ponderous procedures and limited use for chemosensitivity testing. Several authors started to grow tumor cells as multicellular spheroids, using cells obtained from monolayer culture (Yuhas *et al.* 1977; Carlsson *et al.* 1983). It has been shown that such spheroids, maintaining three-dimensional architecture, have several biological characteristics which are similar to in vivo tumors. Multicellular spheroids mainly obtained from permanent cell lines have several limitations. The biological properties of tumor cells from permanent cell lines might be quite different from those of the original tumor in that the cells could consist of stem cells during three-dimensional growth and normal host cells are not present within spheroids.

An advantage of explanting solid tumor tissue in fragments is that cell and tissue components, which have the risk of disappearing in monolayer culture, have a chance to survive for a prolonged period in organotypic culture. The simple liquid overlay culture method can also be used for organotypic glioma culture. Mackillop *et al.* (1985) reported spheroid formation in short-term culture of biopsies from pediatric brain tumor. Freeman *et al.* (1986) used this method on other tumor types and reported findings comparable to those from brain tumors. Recently, Jung *et al.* (1991) reported a micro-organ culture system optimized for in vitro growth of microtumors cultured from human glioma xenografts in athymic mice. They observed histological findings of microtumor spheroids similar to the tumor cell nests in the xenograft. Furthermore, the presence of macrophages after 15 days of culture was of considerable significance in light of reports of macrophages and microglial cells in the developing nervous system and of extensive macrophage infiltration into astrocytomas. Thus, using this model, immunohistochemical studies on human macrophage and lymphocyte in both long-term and short-term culture should be performed in the future.

Recently, Bjerkvig *et al.* (1990) presented detailed information on the morphology and proliferation of human gliomas maintained as multicellular tumor spheroids in vitro. In their study, they successfully maintained host cells within spheroids for more than 80 days. These host cells included both immune and vascular elements. Although cellular proliferation was seen, the spheroids did not consistently increase in size, which suggests that cell proliferation was balanced by cell loss through death or shedding.

In this study, tumor tissue obtained from human malignant gliomas was maintained in long-term agar overlay culture as multicellular

organotypic spheroids. Light microscopic and ultrastructural observation of spheroids displayed morphological features similar to those of the original tumor tissue in vivo. Spheroids contained vascular elements, connective tissue in association with fibroblast, and macrophages. The presence of macrophage could not be confirmed by immunohistochemical study. It is thought that these immunohistochemical findings are probably related to cellular changes or loss during long-term culture as well as technical problems during slide preparation. More cases will be studied with a special emphasis on immunohistochemical verification of host immune cells in this microtumor spheroid model. To prove the presence of tumor-infiltrating lymphocytes in the spheroids, it will be necessary to include interleukin-2 into the culture medium, especially after forty days in culture. This microtumor spheroid could then be established as a relevant model revealing a close resemblance to the condition of the original tumors. The present preliminary observations suggest that this in vitro model may serve as an alternative to the in vivo xenograft model for research of brain tumor biology, invasion and immunology and provide a valuable technique for the evaluation of new therapies, such as biologic response modifiers.

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