Three-dimensional Cell Culture of Glioma and Morphological Comparison of Four Different Human Cell Lines

YOSHINOBU MANOME[1], SHUICHI MIZUNO[2], NOBUTAKE AKIYAMA[3], KOUKI FUJIOKA[1], HIDEKI SAITO[1], YOSHIAKI HATABA[4], TOSHIAKI KOBAYASHI[5] and MICHIKO WATANABE[1]

Departments of [1]Molecular Cell Biology, [3]Molecular Immunology, Institute of DNA Medicine, Research Center for Medical Sciences, Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan; [4]Integrated Imaging Research Support, Chiyoda-ku, Tokyo 102-0093, Japan; [5]Cancer Screening Technology Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Chuo-ku, Tokyo 105-0045, Japan; [2]Department of Orthopedic Surgery, Brigham and Women's Hospital, Boston, MA 02115, U.S.A.

Abstract. *Background: To explore the intracranial behaviors of glioma, a three-dimensional culture was devised and the morphology of four cell lines was examined. Materials and Methods: Bioabsorbable and degradable gelatin was used as the scaffold and T98G, A172, KNS42, and U118MG representative standard malignant glioma cell lines were cultured three-dimensionally. Results: When grown, the cells demonstrated characteristic conformations. The U118MG cells dispersed with numerous fiber formations. In contrast, the KNS42 and A172 cells aggregated, adhering to each other, resulting in the formation of balloon-like structures. The T98G cells demonstrated an intermediate character. Conclusion: The cell lines showed distinct characteristics in three-dimensional culture. This culture method may have a role in elucidating the fundamental character of cells in the human body.*

Tissues in the human body are derived from certain types of cells and accordingly, the characters of the individual tissues are determined by the constituent cells. This especially may be true in organs such as the central nervous system, because the tissue consists of relatively homologous types of cells, albeit with many functions. Based on this understanding, methods for cell culture have been utilized for biomedical studies and cell biology experiments. However, most of these studies have been conducted using a monolayer or two-dimensionally cultivated cells. Many researchers nowadays notice that two-dimensional cells on flat and hard plastic dishes or flasks do not represent natural cells in living tissues or organs (1). First of all, monolayer cells do not have steric cell connections, unlike in the human body. Such three-dimensional cell-to-cell connections are important for proliferation, adhesion, migration, invasion and phenotype presentations. In addition, cellular function is regulated by the microenvironment. The environments in monolayer cells are far from those *in vivo*. The extracellular matrix is also known to be important for morphogenesis as it interacts in the cell-to-cell connection and anchoring of cells. However, it differs between cells cultured *in vitro* and *in vivo* (2). From this point of view, three-dimensional cell culture has gained popularity and is now being used in a wide range of cells (3). Representatives of the three-dimensional cell culture method include reconstituted basement membrane (rBM, commercially know as Matrigel) (4-6) and spheroids (7, 8). Many other devices with a collagen scaffold have also been used. However, in spite of the number of studies and the acceptance of three-dimensional cultures as mainstream methods, better systems that simulate the inside of the human body are still required. In this context, we established a three-dimensional cell culture and demonstrated the chondroinduction of human fibroblast cells with demineralized bone powder (9, 10). In that series of studies, when cells were cultured in three dimensions, demineralized bone powder induced an extracellular matrix similar to that deposited around *bona fide* chondrocytes. Kinetic analyses of gene expression during chondroinduction also confirmed the effect (11, 12). We have emphasized that this culture system has the advantage of using bioreactors that can easily control medium perfusion, as well as enable the application of hydrostatic pressure, and/or exposure to low oxygen tension (13-20). Using this device, we also identified messenger RNAs that were regulated by hydrostatic pressure in three-dimensional culture. In the human

Correspondence to: Yoshinobu Manome, MD, Ph.D., Department of Molecular Cell Biology, Institute of DNA Medicine, Research Center for Medical Sciences, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo, 105-8461, Japan. Tel: +81 334331111 ext. 2360, e-mail: manome@jikei.ac.jp

Key Words: Malignant glioma, cell culture, three-dimension, morphology.

body, hydrostatic fluid pressure constantly oscillates in certain types of tissues or organs, including the central nervous system, that is exposed to intracranial pressure and the vascular system or blood cells exposed to systolic or diastolic blood pressures. Genes of the regulator of G-protein signaling 5 (RGS5) in neuroblastoma cells and chromosome condensation 1-like (CHC1-L) in lymphocytes increased after exposure to hydrostatic pressure (21).

However, during the development of the three-dimensional culture, little was known about the morphology of cultured cells other than chondrocytes. Malignancy is one of the prospective uses for the culture. In this study, human malignant glioma cells were cultivated and their morphologies were observed. The differences between four different cell lines were also compared.

Materials and Methods

Cell lines. The human glioblastoma cell lines, T98G (22), A172 (23) and U118MG (24) were purchased from the American Type Culture Collection (Rockville, MD, USA). The human glioblastoma KNS42 cell line was provided by Dr. Takeshita, Kyushu-University (25). These cell lines were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose supplemented with 10% fetal bovine serum. The cells were grown in a normal culture flask until the start of the experiments.

Three-dimensional cell culture. The scaffold material for three-dimensional culture was bioabsorbable and degradable gelatin. The method for the experiments was a modified version of a previously described procedure (9, 10). Dispersed human glioma cells (1×10^4 cells/100 μl of DMEM) were injected into the scaffold and left to stand for 4-6 hours at 37˚C in a 5% CO_2 incubator without additional cell culture medium. After attachment of the cells to the scaffolds, the meshes were transferred into a 10-cm dish, immersed in 10 ml of culture medium and further cultivated for 3 to 20 days.

Morphological evaluations. The cultures with scaffolds were fixed with 10% phosphate buffered-formalin for light microscopy, 1.2% glutaraldehyde for scanning electron microscopy (SEM) in 0.1 M phosphate buffer (pH 7.3, 400 mOsm) or 2% glutaraldehyde for transmission electron microscopy (TEM), and then used as specimens. Paraffin-embedded specimens were sliced at 6-μl thickness, stained with hematoxylin and eosin, and examined under a light microscope. Freeze fractured- or non-fractured specimens in ethanol were dehydrated by a critical point dryer (Hitach-Hightec, Tokyo, Japan), coated by Au-Pd magnetron sputter and examined at 15 kV under a JSM-5800LV scanning electron microscope (JEOL Ltd., Tokyo, Japan). Other specimens were examined at 80 kV under a H-7500 transmission electron microscope (Hitachi-Hightec) after ultramicrotomy.

Results

Three-dimensional culture of T98G glioma cells. A cross-section of a three-dimensional culture on day 10 is shown in Figure 1a. The inoculated cells proliferated in the three-dimensional scaffold and were crammed into the compartment and their fibers also filled in the extracellular spaces. When the cells were crowded in the seeded tracks, they tended to migrate to unoccupied adjoining space and re-proliferate (Figure 1b). At the beginning of culture, injected or seeded cells adhered to the material and started to grow in the attached spots. After several days, these multiplied cells were detected as groups of cells (Figure 1c) and at this early stage of the culture, they actively grew, rather than producing extracellular matrix. Unattached and dead cells were sloughed away and not observed. When the cellular density increased, the cells produced more extracellular matrix. Simultaneously, the cells started to migrate to neighboring space by anchoring to the scaffold. The T98G cells in three-dimensional culture were vibrant and plicae, microvilli and fibers were clearly observed (Figure 1d). The three-dimensional spatial environment conferred dynamic viability on the cultured cells. The adaptability of the cells to the scaffold was evaluated by TEM. The glioma cells attached to the scaffold with many processes (Figure 1e). Although the cells retained their ability to migrate as well as their mobility on the scaffold surface, cell attachments to the scaffold were abundant. A study at higher magnification revealed that fine matrix fibers intervened between the cell process and the scaffold (Figure 1f). The matrix was produced by the glioma cells and deposited in the extracellular spaces. The cells bonded to the scaffold by means of the extracellular matrix. Similar intervention was also observed on process-to-process connections between the cells (Figure 1g). These connections arose in a complicated and sophisticated manner. Extracellular matrix was used for the connections. Steric connections were observed throughout the culture. The features demonstrated here were rarely detectable in conventional cell culture (data not shown).

Morphology of four representative glioma cell lines. Similar to the T98G cells, human KNS42, A172 and U118MG cell lines have commonly been used in previous studies. When the cells were grown, they presented distinctive appearances. T98G cells grew initially as a group, then migrated into and accumulated in neighboring spaces. A cluster of T98G cells is shown in Figure 2a. The cells aggregated and rolled up, which was the typical appearance of T98G in three-dimensional culture. In contrast, the KNS42 and A172 cells aggregated, adhering to each other, leading to the formation of balloon-like structures (Figure 2b, c). These structures were not formed by the T98G cells. While both the KNS42 and A172 cells conglomerated, the KNS42 cells bound more tightly than the A172 cells. Unlike KNS42, individual cell shapes were maintained in A172 which grew more independently and thus each cell was distinguishable. The U118MG cells presented drastically different characteristics. They dispersed, grew

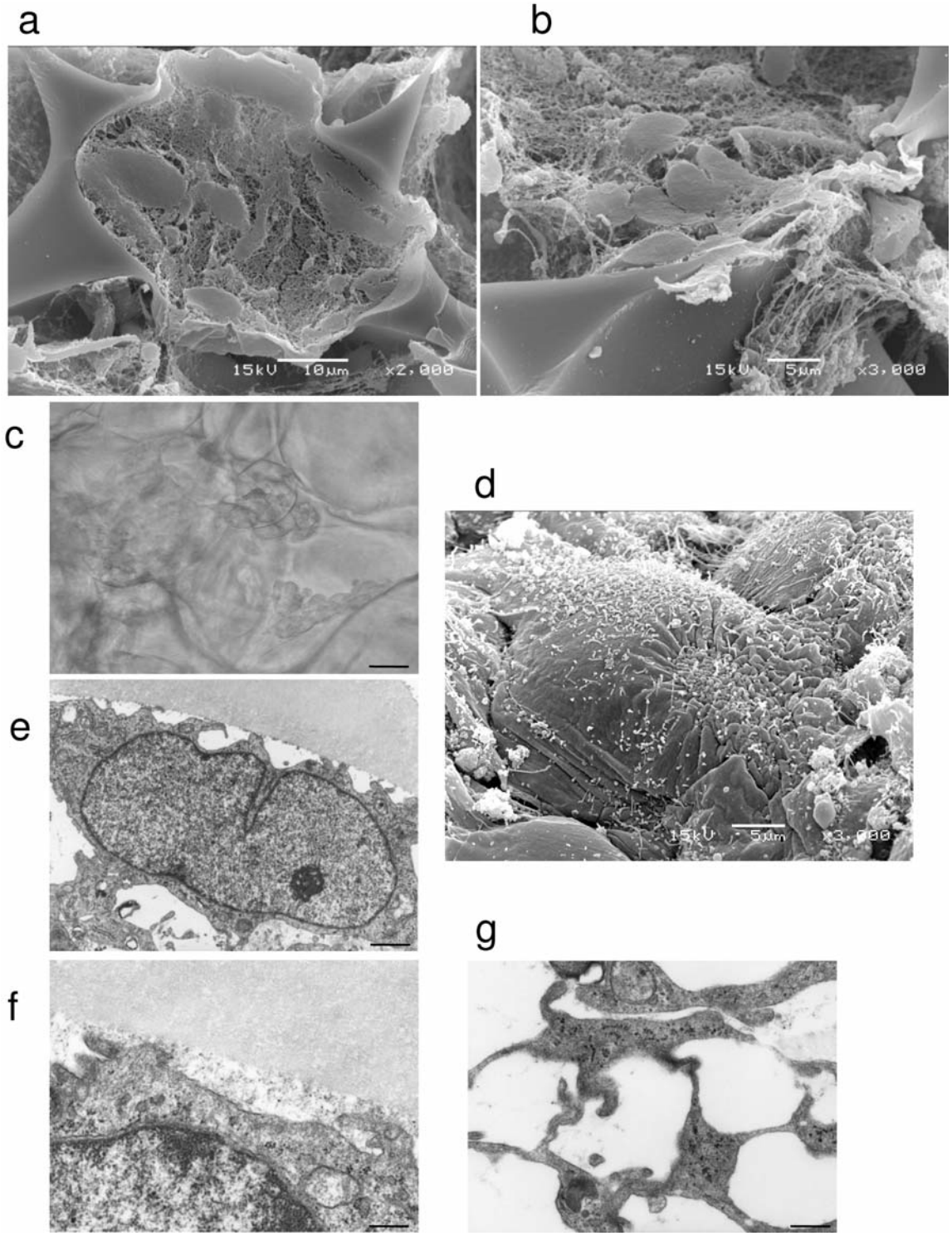

Figure 1. *Morphology of three-dimensionally cultured human T98G glioma cells. a: SEM of freeze-fractured cells on day10 of culture, the fractured scaffold presenting a smooth and flat structure. Processes and fibers fill the intracellular space. b: SEM on day 5 of the culture. Most of the glioma cells also fractured. c: Appearance of the glioma by phase-contrast microscopy on day 4 of culture. The cells were tightly attached to the scaffold and starting to proliferate. Magnification, ×40; bar, 40 µm. d: SEM of the glioma on day 5 of culture. Fine structures of the fibers, plicae, and microvilli on the surface of T98G cells. e: TEM of attachment of a glioma cell to the scaffold on day 8 of culture. More than eight processes projected from the cell body cradling tight adherence. Bar, 1.2 µm. f: Higher magnification TEM of the attachment revealing extracellular matrix fibers between the cell projections and the scaffold. Bar, 317 nm. g: TEM of connection of cell to cell processes on day 8 of culture. Bar, 380 nm.*

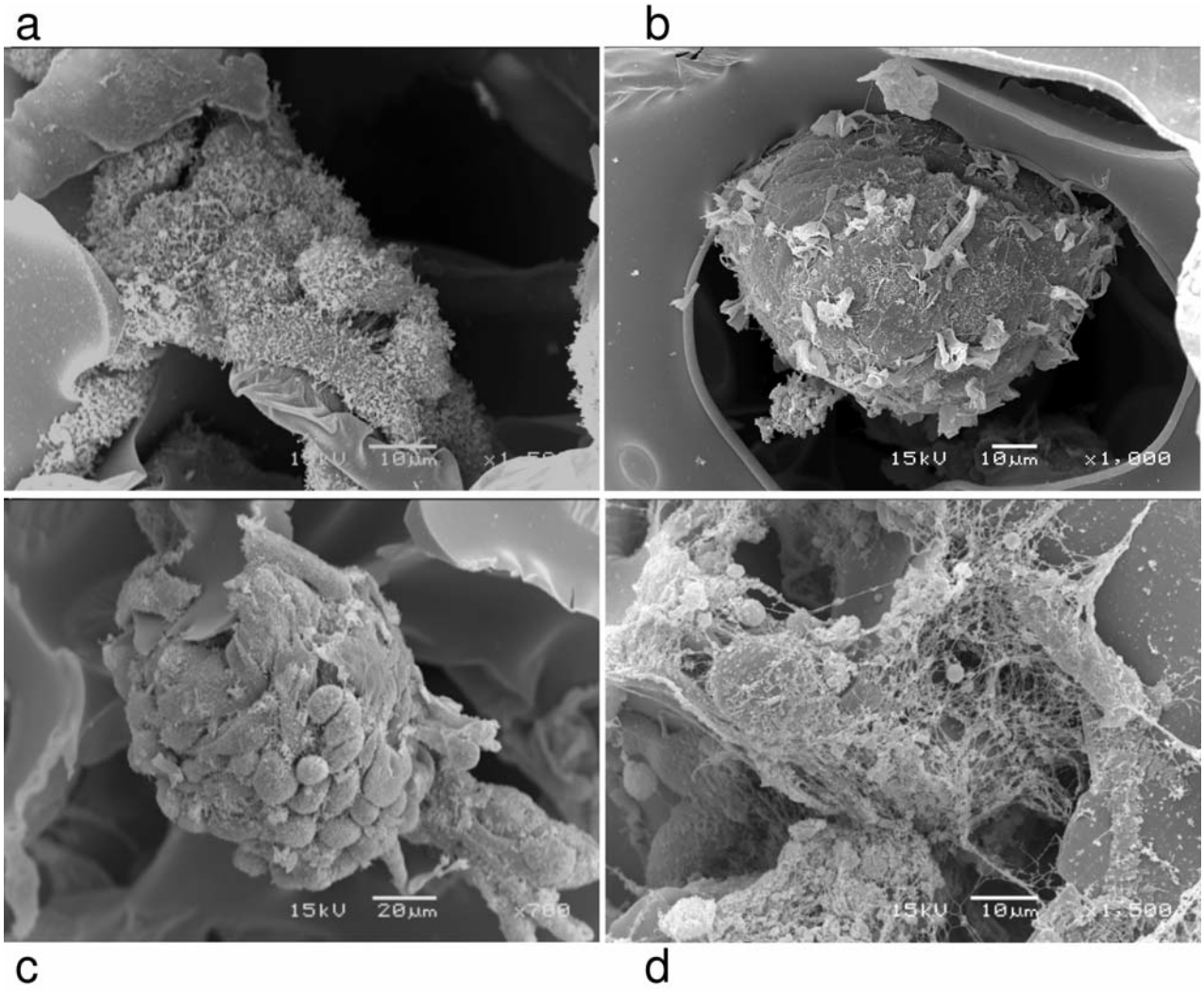

Figure 2. *Appearance of three-dimensional cultures of separate glioma cell lines. a: T98G, appearance intermediate between that of KNS42 and U118MG cells. b: KNS42, cell boundary obscure compared to T98G or A172. c: A172, shape of each cell clearly apparent. d: U118MG, strongly attached to the scaffold with numerous fibers.*

separately and did not pile up or conglomerate (Figure 2d). The cells attached to the scaffold with numerous fiber formations. These features were observed from the beginning of the cell culture.

Characteristics of three-dimensional culture of KNS42, A172 and U118MG cells. TEM was used to observe the inside of the aggregated KNS cell structure (Figure 3a). Numerous microvilli were observed on the surface of the cells and they also protruded into the extracellular spaces in the inner side of the aggregate. The individual cells processed their own extracellular spaces, which might be an advantage for maintaining the micro-environment of individual cells. Even after 15 days of culture, the cells still proliferated. Despite the cell numbers and size of the aggregates, very few cells underwent apoptosis or necrosis (Figure 3b). Although the cells tended to adhere to each other, many attachments to the scaffold were also observed (Figure 3b). TEM also demonstrated tight bonding to the scaffold of the cell (Figure 3c) which was closer than that of the T98G cells. The features of A172 were basically similar to those of KNS42. The cells formed a globoid structure (Figure 3d) and microvilli were found on the surface or intra-structural spaces. However, the overall structure of the A172 complex was different from that of KNS42 since unlike KNS42, the cells protruded beyond the outline of the globoid structure in A172 cells giving a more ragged appearance. In contrast, U118MG cells did not form a complex, grew independently, and even immediately after dividing, the cells dispersed and produced massive fibers (Figure 3e). Long processes

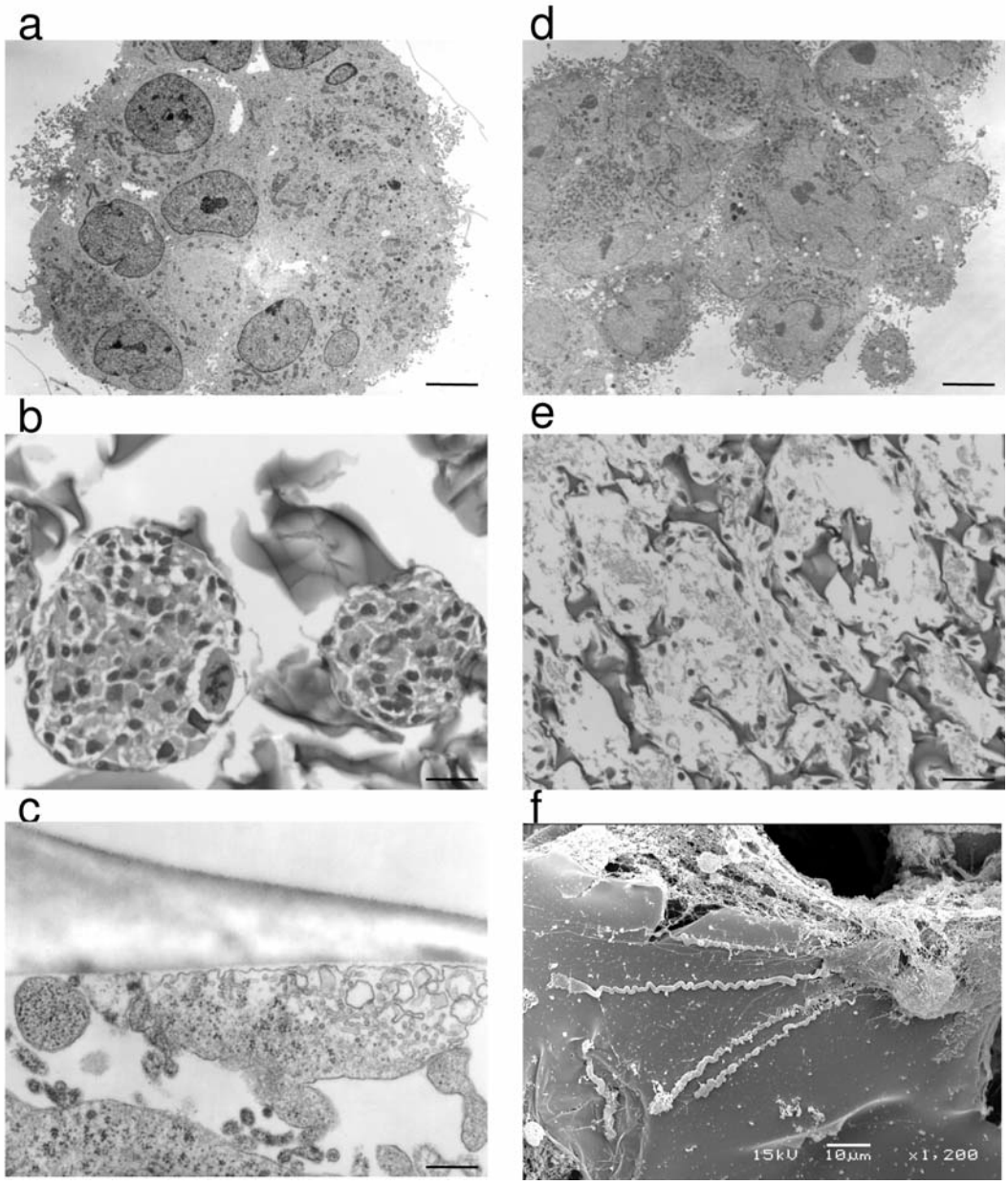

Figure 3. *Morphology of three-dimensionally cultured KNS42, A172 and U118MG cells. a: TEM image of KNS42 cells. Cells aggregated with numerous microvilli in the extracellular spaces as well as on the outside surface of the structure. Bar, 8 µm. b: Cross-section of KNS42 cells forming a spheroid structure (hematoxylin-eosin staining). Note the presence of attachments to the scaffold. Magnification, ×40; bar, 40 µm. c: TEM image of a KNS42 cell attached to the scaffold with distinct extracellular matrix. Bar, 500 nm. d: TEM image of A172 cells with a ragged and lumpy surface and prominent microvilli. Bar, 8 µm. e: Cross-section of U118MG cells (H-E staining) demonstrating an infiltrative pattern and abundant extracellular fibers. Magnification, ×10; bar, 160 µm. f: SEM image of U118MG cells firmly attached to the scaffold by fibers and long processes projecting from the cell body. Microvilli occurred on the tips of the processes.*

extended from the cells and attached to the scaffold with further extension of small fibers that also attached to the scaffold (Figure 3f). Many microvilli were seen on the tips of the processes. These features were unique to U118MG. Cellular connection was usually achieved *via* the cell processes.

Discussion

Malignant glioma is one of the intractable diseases of the human body. In spite of recent advances in medical technologies, malignant glioma is refractory to most current therapies and alternative therapies are required. One

advantage for the development of an effective treatment is that malignant glioma cells seldom metastasize to organs other than the central nervous system and long-term survival or even a cure of the disease could be expected by inhibiting local tumor recurrence. Therefore numerous studies have been carried out using glioma cells. Under such circumstances, the development of an experimental model that simulates an intracranial glioma is important (26, 27), because most studies have been performed on cultures with ordinary monolayer cells.

In a recent study, we optimized therapeutic ultrasound against malignant brain tumor, especially glioma, using a three-dimensional cell culture (28). The data obtained were directly applicable to *in vivo* animal studies and we noted the importance of the nature of the culture. In the present study, the tendency of T98G cells to enter a viable G_1-arrested state when crowded (22) was confirmed in an ordinary two-dimensional cell culture (data not shown). In contrast, glioma cells in three-dimensional culture kept on proliferating at least until day 20 under the current experimental conditions. The difference might be attributable to the abundant scaffold spaces of the three-dimension cell culture. The morphology observed in three-dimensional culture was quite different from that of cells in a conventional monolayer culture. Each glioma has different properties for growth and infiltration into brain parenchyma *in vivo* and these abilities may influence the prognosis of host patients. When the different glioma cells were grown in the scaffold, they displayed notably different characteristics. The U118MG cells dispersed, while the KNS42 and A172 cells conglomerated. After the cells were inoculated on to the scaffold, only those that were attached to the scaffold started to divide, and these cells proliferated *ad locum*. Whether the cells dispersed or conglomerated could have been attributable to different migratory or locomotive abilities. While the U118MG cells tended to separate, the KNS42 and A172 cells did not migrate even after division.

The cells used in this study were representative of standard glioma lines widely used experimentally, both *in vitro* and *in vivo*. These cells were much alike in two-dimensional cell culture. However, different characteristics became evident only after three-dimensional cell culture. It is concluded that culture experiments should be planned with consideration to the individuality of each tumor cell type. There may be many different types of three-dimensional culture methods (29-33) other than the one presented here, suitable for the evaluation of expansion or invasion of glioma. While the morphology of the gliomas was demonstrated using the present scaffold method, a better system that replicates the intra-cerebral environment for malignant cells is required. This may also apply to cells other than gliomas. Accordingly, further study is warranted.

References

1 Pampaloni F, Reynaud EG and Stelzer EH: The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol *8*: 839-845, 2007.

2 Kleinman HK, Philp D and Hoffman MP: Role of the extracellular matrix in morphogenesis. Curr Opin Biotechnol *14*: 526-532, 2003.

3 Pilkington GJ, Bjerkvig R, De Ridder L and Kaaijk P: *In vitro* and *in vivo* models for the study of brain tumour invasion. Anticancer Res *17*: 4107-4109, 1997.

4 Kleinman HK and Jacob K: Invasion assays. Curr Protoc Cell Biol Chapter *12*: Unit 12-2, 2001.

5 Kleinman HK and Martin GR: Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol *15*: 378-386, 2005.

6 Marques MM, Martins MD and Franca CM: Effect of Matrigel on adenoid cystic carcinoma cell line differentiation. Int J Exp Pathol *87*: 405-410, 2006.

7 de Ridder L, Cornelissen M and de Ridder D: Autologous spheroid culture: a screening tool for human brain tumour invasion. Crit Rev Oncol Hematol *36*: 107-122, 2000.

8 Kunz-Schughart LA, Kreutz M and Knuechel R: Multicellular spheroids: a three-dimensional *in vitro* culture system to study tumour biology. Int J Exp Pathol *79*: 1-23, 1998.

9 Mizuno S and Glowacki J: Three-dimensional composite of demineralized bone powder and collagen for *in vitro* analysis of chondroinduction of human dermal fibroblasts. Biomaterials *17*: 1819-1825, 1996.

10 Mizuno S and Glowacki J: Chondroinduction of human dermal fibroblasts by demineralized bone in three-dimensional culture. Exp Cell Res *227*: 89-97, 1996.

11 Allemann F, Mizuno S, Eid K, Yates KE, Zaleske D and Glowacki J: Effects of hyaluronan on engineered articular cartilage extracellular matrix gene expression in 3-dimensional collagen scaffolds. J Biomed Mater Res *55*: 13-19, 2001.

12 Yates KE, Mizuno S and Glowacki J: Early shifts in gene expression during chondroinduction of human dermal fibroblasts. Exp Cell Res *265*: 203-211, 2001.

13 Glowacki J and Mizuno S: Collagen scaffolds for tissue engineering. Biopolymers *89*: 338-344, 2008.

14 Mizuno S and Glowacki J: Low oxygen tension enhances chondroinduction by demineralized bone matrix in human dermal fibroblasts *in vitro*. Cells Tissues Organs *180*: 151-158, 2005.

15 Mueller SM, Mizuno S, Gerstenfeld LC and Glowacki J: Medium perfusion enhances osteogenesis by murine osteosarcoma cells in three-dimensional collagen sponges. J Bone Miner Res *14*: 2118-2126, 1999.

16 Navarro FA, Mizuno S, Huertas JC, Glowacki J and Orgill DP: Perfusion of medium improves growth of human oral neomucosal tissue constructs. Wound Repair Regen *9*: 507-512, 2001.

17 Glowacki J, Mizuno S and Greenberger JS: Perfusion enhances functions of bone marrow stromal cells in three-dimensional culture. Cell Transplant *7*: 319-326, 1998.

18 Mizuno S, Allemann F and Glowacki J: Effects of medium perfusion on matrix production by bovine chondrocytes in three-dimensional collagen sponges. J Biomed Mater Res *56*: 368-375, 2001.

19 Mizuno S, Tateishi T, Ushida T and Glowacki J: Hydrostatic fluid pressure enhances matrix synthesis and accumulation by bovine chondrocytes in three-dimensional culture. J Cell Physiol *193*: 319-327, 2002.
20 Mizuno S: A novel method for assessing effects of hydrostatic fluid pressure on intracellular calcium: a study with bovine articular chondrocytes. Am J Physiol Cell Physiol *288*: C329-337, 2005.
21 Manome Y, Saeki N, Yoshinaga H, Watanabe M and Mizuno S: A culture device demonstrates that hydrostatic pressure increases mRNA of RGS5 in neuroblastoma and CHC1-L in lymphocytic cells. Cells Tissues Organs *174*: 155-161, 2003.
22 Stein GH: T98G: an anchorage-independent human tumor cell line that exhibits stationary phase G_1 arrest *in vitro*. J Cell Physiol *99*: 43-54, 1979.
23 Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H and Parks WP: *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst *51*: 1417-1423, 1973.
24 Ponten J and Macintyre EH: Long term culture of normal and neoplastic human glia. Acta Pathol Microbiol Scand *74*: 465-486, 1968.
25 Takeshita I, Takaki T, Kuramitsu M, Nagasaka S, Machi T, Ogawa H, Egami H, Mannoji H, Fukui M and Kitamura K: Characteristics of an established human glioma cell line, KNS-42. Neurol Med Chir *27*: 581-587, 1987.
26 Corcoran A, De Ridder LI, Del Duca D, Kalala OJ, Lah T, Pilkington GJ and Del Maestro RF: Evolution of the brain tumour spheroid model: transcending current model limitations. Acta Neurochir *145*: 819-824, 2003.
27 Grobben B, De Deyn PP and Slegers H: Rat C6 glioma as experimental model system for the study of glioblastoma growth and invasion. Cell Tissue Res *310*: 257-270, 2002.
28 Manome Y, Furuhata H, Hashimoto A, Funamizu N, Suzuki R, Ishizawa S, Akiyama N, Kobayashi T and Watanabe M: Application of therapeutic insonation to malignant glioma cells and facilitation by echo-contrast microbubbles of levovist. Anticancer Res *29*: 235-242, 2009.
29 Friedl P and Wolf K: Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer *3*: 362-374, 2003.
30 Hegedüs B, Marga F, Jakab K, Sharpe-Timms KL and Forgacs G: The interplay of cell-cell and cell-matrix interactions in the invasive properties of brain tumors. Biophys J *91*: 2708-2716, 2006.
31 Kaufman LJ, Brangwynne CP, Kasza KE, Filippidi E, Gordon VD, Deisboeck TS and Weitz DA: Glioma expansion in collagen I matrices: analyzing collagen concentration-dependent growth and motility patterns. Biophys J *89*: 635-650, 2005.
32 Stein AM, Demuth T, Mobley D, Berens M and Sander LM: A mathematical model of glioblastoma tumor spheroid invasion in a three-dimensional *in vitro* experiment. Biophys J *92*: 356–365, 2007.
33 Werbowetski T, Bjerkvig R and Del Maestro RF: Evidence for a secreted chemorepellent that directs glioma cell invasion. J Neurobiol *60*: 71-88, 2004.

Received August 25, 2009
Revised January 19, 2010
Accepted January 21, 2010