Three-dimensional Cellular Spheroid Formation Provides Human Prostate Tumor Cells with Tissue-like Features

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Abstract. *Background: Alterations in the cellular biological responses were examined in a series of LNCaP human prostate tumor cells growing under different conditions. Materials and Methods: LNCaP cells were grown in two-dimensional monolayer cultures, three-dimensional spheroids, or as solid tumors in immune-deprived mice. Results: As compared with the growth in the monolayers, cell growth in the spheroids was reduced, while VEGF production was increased. Immunohistochemical analysis of the spheroids revealed that cells showing Ki-67 up-regulation were localized in the peripheral layer, and that the central core was necrotic. The gene expression profile in the solid tumor tissue was obviously different from that in the monolayers; however, it was similar to that in the spheroids. The prostate-specific antigen levels in the culture supernatants of spheroids increased with time and decreased with anticancer agent treatment. Conclusion: Spheroid formation from human prostate tumor cells exhibits tissue-like features.*

The development and growth of prostate cancer are initially androgen-dependent processes, but prostate cancer cells often escape from primary hormonal control. Therefore, patients with hormone-refractory prostate cancer (HRPC) must be treated with a non-hormonal mediator. The effects of several anticancer agents have been evaluated in patients with prostate cancer; however, most of these anticancer agents had little or no impact on the survival of patients with HRPC and metastatic prostate cancer (1-4). The progression of prostate cancer is characterized by the dissemination of malignant prostatic epithelial cells and small clusters in the marrow (5). These disordered arrangements of tumor cells in the tumor tissue differ from the arrangement of tumor cells in monolayer cultures *in vitro*. A pre-clinical experimental model simulating the clinical profile of prostate cancer is necessary to explore the progression of chemoresistant prostate cancer.

Regarding the morphology of cultured tumor cells *in vitro*, multicellular aggregates are more useful than two-dimensional monolayer cultures from an experimental perspective, because preservation of the three-dimensional structure is important for cell-to-cell and cell-to-matrix interactions. Multicellular tumor spheroids, one type of three-dimensional culture, are a well-studied *in vitro* tumor tissue model. These spheroids have been applied in various studies, including experiments involving immunotherapy (6-10). Spheroid culture systems can mimic some of the *in vivo* microenvironmental characteristics of solid tumors, including anchorage-independent growth, that are fundamental to tumor progression. These morphological changes can cause intrinsic resistance to radiation and chemotherapeutic agents (11-13). Since multicellular tumor spheroids reproduce the tumoral microenvironment more accurately than conventional monolayer culture systems, they might act as better models for the biological and biochemical characteristics of solid tumors.

The tumor microenvironment may play a key role in responses to environmental stress, including responses to drugs. The spheroid model has been shown to represent a tissue-like architecture that can form cell-to-cell junctions and invoke signaling cascades, which are important *in vivo* features. Anoxic cells were shown to up-regulate vascular

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endothelial growth factor (14). Hypoxic conditions have long been known to reduce the sensitivity of tumor cells to radiation and chemotherapeutic drugs and have been shown to modulate apoptotic cell death (15, 16). The importance of tumor cell interactions with the extracellular matrix during the development of drug resistance has also been shown using three-dimensional culture systems (17). Therefore, spheroids seem to manifest various means of cell survival and may help to better understanding prostate cancer biology.

In this study, biological alterations were examined, in a series of LNCaP prostate tumor cells isolated under different growing conditions, to evaluate differences between *in vivo* and *in vitro* cellular responses.

Materials and Methods

Cell cultures. The human prostate cancer cell line LNCaP was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in RPMI1640 medium supplemented with 10% fetal calf serum, in 5% CO_2 humidified air at 37˚C. A trypsinized single cell suspension was seeded into a 96-well plate on culture day 0 (2,500 cells/well; 100 μL/well). Half the volume of the culture medium was renewed on days 3, 5 and 7. For the monolayer cultures, the cells were seeded onto a flat-bottom plate. Spheroids were cultured using a liquid overlay method (18, 19). Briefly, round-bottom plates were coated twice with 10 mg/mL of poly(2-hydroxyethyl methacrylate) (poly-HEMA; Sigma, Inc., St. Louis, MO, USA) in 95% ethanol and washed once with PBS before cell seeding.

Measurement of growth parameters. Cultured cells were trypsinized and pooled from 24 wells. The number of cells was counted using a hematocytometer and the growth rate was estimated using the ratio of the fold-increase in cell number compared with the initial number of seeded cells. To calculate the mean volumes, the diameters of 16 spheroids were measured using a micrometer under a microscope. The volume of each spheroid was estimated from the major (a) and minor (b) axes using the following formula: $a \times b^2/2$.

Establishment of in vivo tumors. A single cell suspension ($1x10^7$ cells/mouse) in Matrigel (BD Biosciences, Bedford, MA, USA) was subcutaneously inoculated into the flanks of six-week old male SCID mice. The resulting tumors were over 1 cm in diameter at the time of dissection. Each tumor was divided into two pieces and immediately immersed in formalin or RNAlator (Ambion, Inc., Austin, TX, USA).

Immunohistochemical analysis. The spheroids were harvested and fixed in 4% formalin. After paraffin embedding, the spheroids were sectioned and stained with hematoxylin and eosin (H&E). For immunostaining, the sections were stained with either anti-Ki-67, anti-$p27^{kip1}$ or anti-Bcl-2 monoclonal antibody (mAb) (clones MIB-1, SX53G8, and 124, respectively; all from Dako, Carpinteria, CA, USA). To measure the oxygen gradient, the spheroids were incubated with pimonidazole hydrochloride (Hypoxyprobe™-1; Chemicon International, Inc., Temecula, CA, USA) before fixation, and the paraffin sections were then stained with a specific antibody directed against pimonidazole protein adducts, according to the manufacturer's protocol.

VEGF assay. Culture supernatants were collected from monolayers or spheroids and stored at –80˚C until assay. The amount of VEGF in each sample was determined using a human VEGF ELISA system (R&D Systems, Inc., Minneapolis, MN, USA). The detected values were corrected according to the growth rate.

Microarray analysis. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to a microarray analysis using a Human Whole Genome Oligo Microarray chip, a Low RNA Fluorescent Linear Amplification Kit, an *In situ* Hybridization Plus kit, and an Agilent dual-laser DNA micro array scanner (all from Agilent Technologies, Palo Alto, CA, USA), according to the manufacturer's instructions. After scanning the array's features, noise-contributing genes were removed and the array was normalized using Feature Extraction software (Agilent Technologies); a filter was then applied to the remaining 32,217 features. Annotation of the filtered gene list and gene functionally categorization according to the Gene Ontology consortium were performed using GeneSpring 6.0 (Silicon Genetics, Redwood City, CA, USA). A comparative analysis of the expression profiles for monolayer cultures, spheroids and tumor samples was performed by comparing expression levels that differed by more than 2-fold (up-regulation) or less than 1/2-fold (down-regulation), compared with expression in the monolayer culture.

Cytotoxicity assay. Cells were seeded on day 0 and exposed to an anticancer agent for 48 h on days 1 to 3 for the monolayer cultures and on days 5 to 7 for the spheroid cultures. The number of viable cells was determined before and after exposure to the anticancer agent to estimate cytotoxic parameter following a general assay protocol (20) using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). The following anticancer agents were tested: cisplatin (CDDP), oxaliplatin (L-OHP), 5-fluorouracil (5-FU), gemcitabine (GEM), SN-38, mitoxantrone (MXT), paclitaxel (TXL) and docetaxel (DTX; all from Sigma except for SN-38 from Yakult Honsha, Co., Ltd., Tokyo, Japan).

Prostate-specific antigen (PSA) assay. Culture supernatants of spheroids, collected on days 5 and 7, were stored at –80˚C until assay. Spheroids were also exposed to L-OHP from day 5 to day 7 to monitor the changes in the PSA levels. The PSA levels were determined using a human KLK3/PSA ELISA system (R&D Systems).

Results

Growth parameter analysis. The formation of spheroids suppressed cell proliferation in LNCaP cells (Figure 1a). The growth rate increased over time in both monolayers and spheroids but the rate was slower in spheroids than in the monolayer culture. In the monolayers, cells reached confluency on day 5 and numerous nonadherent cells were collected on day 7. Prolonging the number of culture days increased the spheroid volume (Figure 1b). H&E-stained sections of LNCaP spheroids were morphometrically

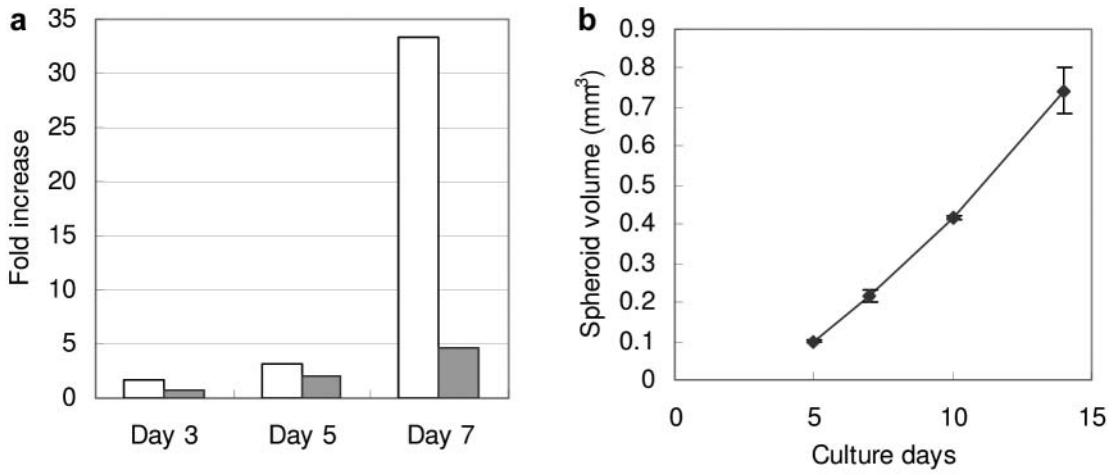

Figure 1. *Growth parameters of LNCaP prostate tumor cells. a) Growth rates of monolayer cultures (open squares) and spheroids (closed squares). Cells were seeded on day 0 and the number of cells was counted on the indicated culture days. The growth rate was represented by the ratio of the fold increase in the number of cells compared with the initial cell number. b) Volume of the LNCaP spheroids. Bars: SD.*

measured and found to be disordered with tight cell-to-cell contacts (Figure 2a). Central necrosis was observed on day 7, indicating that the prolongation of the culture period led to the starvation of the LNCaP cells, especially the cells located in the central regions of the spheroids. Immunostaining demonstrated that the expression of Ki-67 and Bcl-2 was up-regulated in LNCaP cells in the peripheral layers of the spheroids (Figure 2b). $P27^{kip1}$-positive cells were detected between the outer layer and the necrotic center (Figure 2c). These data showed that the characteristics of the LNCaP cells differed according to their location within the spheroid, particularly as to whether they were within viable or quiescent cell layers.

Structurally-induced enhancement in VEGF production. The cells within the central region of the spheroid were thought to be hypoxic because hypoxic cells were detected in the central rim of the spheroid (Figure 3a). Hypoxic environments are known to induce various cell responses, such as the up-regulation of VEGF production. As shown in Figure 3b, the amount of VEGF in the culture supernatant increased with time in both monolayer cultures and spheroids, but the degree of the increase was much higher in the spheroids than in the monolayer cultures. Hence, spheroid formation accelerated VEGF production in LNCaP cells.

Global gene expression profiles. The change of global gene expression profiles in spheroids and solid tumor tissues were compared with monolayer cultures. After categorizing the genes according to their functions, the gene expression profiles were expressed as the number of genes that were either up-regulated or down-regulated by spheroid or tumor formation as a percentage of the total number of genes in a specific functional category (Figure 4). The expression patterns of a wide range of genes were altered by the growing conditions, even in the same tumor cell line. The gene expression profiles of LNCaP cells grown in monolayer cultures and solid tumors were drastically different. The gene expression profiles of the spheroid and solid tumor tissues were similar, except for the expression patterns of angiogenesis-related and hypoxia-related genes. The characteristics of the LNCaP cells grown in spheroids seem to resemble those of *in vivo* tumor tissue.

Chemosensitivities. The responses of the LNCaP cells to anticancer agents were compared between monolayers and spheroids (Figure 5). Among the platinum drugs tested, oxaliplatin was more effective than cisplatin. The activity of oxaliplatin was slightly lower in monolayers than in spheroids, but this activity level was still more effective than that of cisplatin in the monolayer cultures. Among the antimetabolites that were tested, similar anticancer activity levels were observed in monolayers and spheroids. Topoisomerase inhibitors were effective in the monolayer cultures. The cytotoxicity of mitoxantrone, but not SN-38 - an active metabolite of CPT-11, was lower in the spheroids than in the monolayers. Tubulin effectors were highly active in the monolayer cultures, but their cytotoxicity was drastically lower in spheroids. Many of the anticancer agents were effective when the LNCaP cells were grown as monolayers, but when the LNCaP cells were grown as spheroids, they acquired resistance to most, but not all, of the anticancer agents tested.

PSA response. Prostate cells produce PSA and the PSA level in the serum is known as a biomarker of prostate cancer growth. The PSA levels were measured in the culture

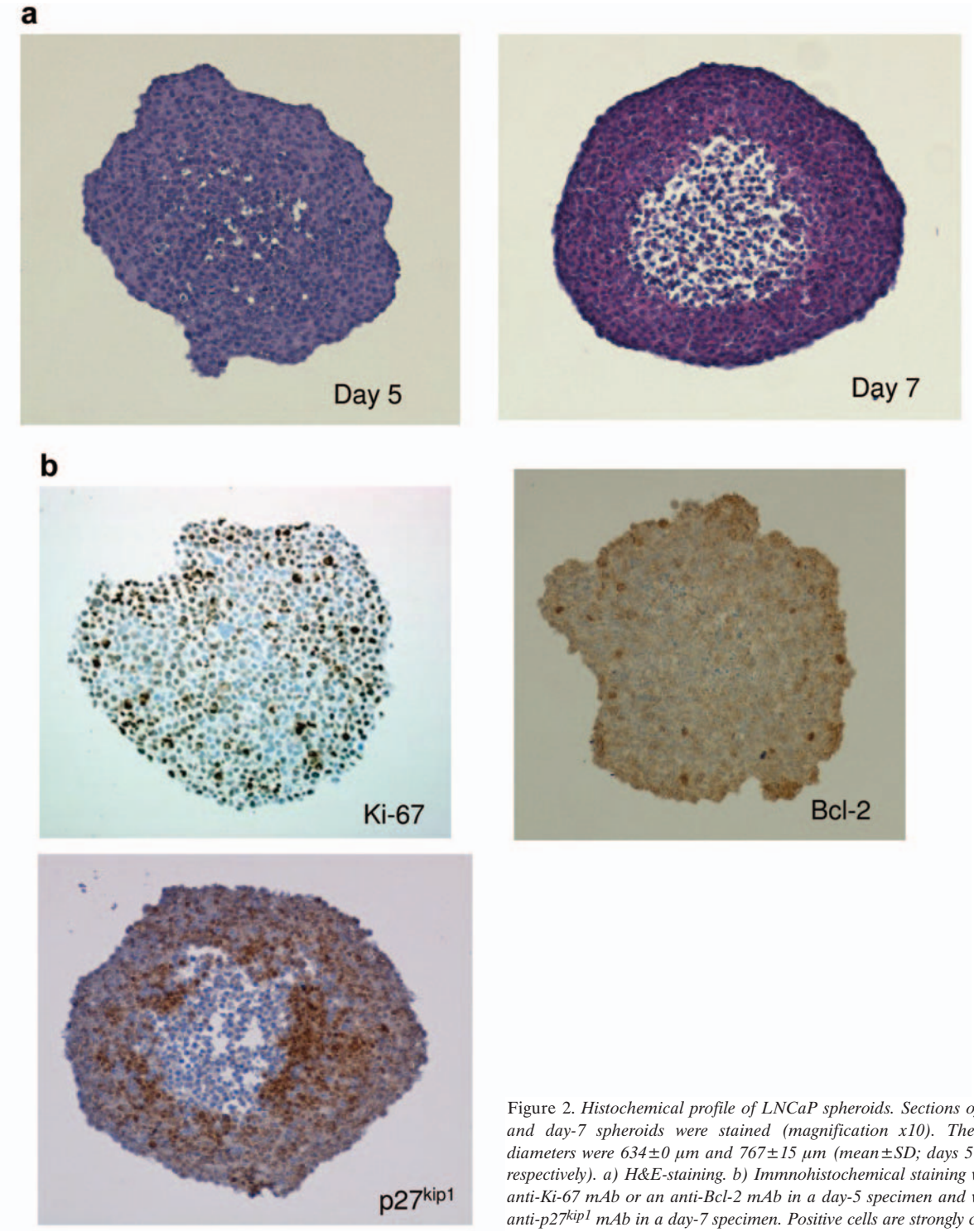

Figure 2. *Histochemical profile of LNCaP spheroids. Sections of day-5 and day-7 spheroids were stained (magnification x10). The mean diameters were 634±0 μm and 767±15 μm (mean±SD; days 5 and 7, respectively). a) H&E-staining. b) Immnohistochemical staining with an anti-Ki-67 mAb or an anti-Bcl-2 mAb in a day-5 specimen and with an anti-$p27^{kip1}$ mAb in a day-7 specimen. Positive cells are strongly colored.*

supernatants of spheroids (Figure 6). The PSA level in the culture supernatant on day 7 was higher than that on day 5, corresponding to growth progression of the spheroids with time. The PSA level was reduced by L-OHP treatment. It was demonstrated that this PSA response corresponded to the survival of the spheroids.

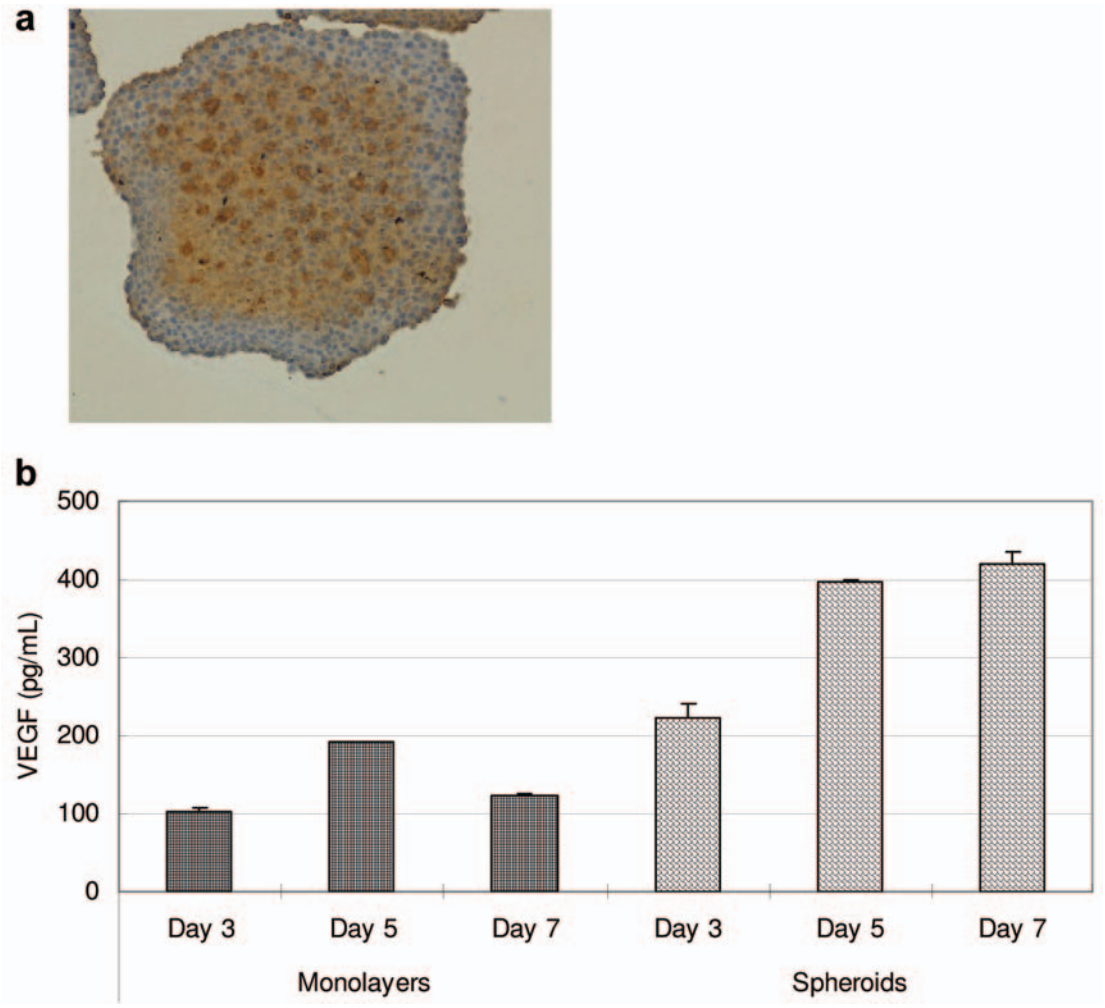

Figure 3. *Microenvironment of LNCaP spheroids and VEGF production. a) Immunohistochemical detection of hypoxic cells using pimonidazole as a hypoxic probe. Day-5 spheroids were treated with the probe before preparing the specimens, and the specimens were then treated with a specific mAb against the probe (magnification x10). b) Amount of VEGF in culture supernatants from monolayer cultures or spheroids. Culture supernatant was collected on the indicated culture days, and the VEGF concentration was measured using ELISA. Bars: SD.*

Discussion

In the microenvironment of internal spheroids, the gradients for metabolites, glucose, lactate, pH and oxygen are much larger than those obtained during two-dimensional growth, where a larger surface area-to-volume ratio exists and all the cells have equal contact with the medium (6). Only the outer layer of cells in spheroids has direct contact with the medium, simulating *in vivo* conditions where the majority of tumor cells are located at some distance from a blood vessel. In this study, Ki-67-expressing cells were localized in the peripheral layer of the spheroids. This unequal distribution was consistent with that of doxorubicin-treated DU145 prostate tumor cell spheroids (21). In addition, $p27^{kip1}$-positive cells were localized between the outer layer and the necrotic center. These data suggest that spheroid formation generates proliferative heterogeneity consisting of viable, quiescent and necrotic cells.

The peripheral layer in LNCaP spheroids was also observed to up-regulate the Bcl-2 antigen. Overexpression of the Bcl-2 gene was detected in several tumor tissues, including prostate cancer (22). The overexpression of Bcl-2 is known to protect cells from anoikis (19). Moreover, Bcl-2 expression has been associated with the progression of prostate cancer from androgen-dependence to androgen-independence (23). Therefore, the up-regulation of Bcl-2 in LNCaP spheroids is regarded as an important feature of *in vitro* tumor models.

Compounds that are present outside of a spheroid may have poor access to the internal cells of the spheroid. In an

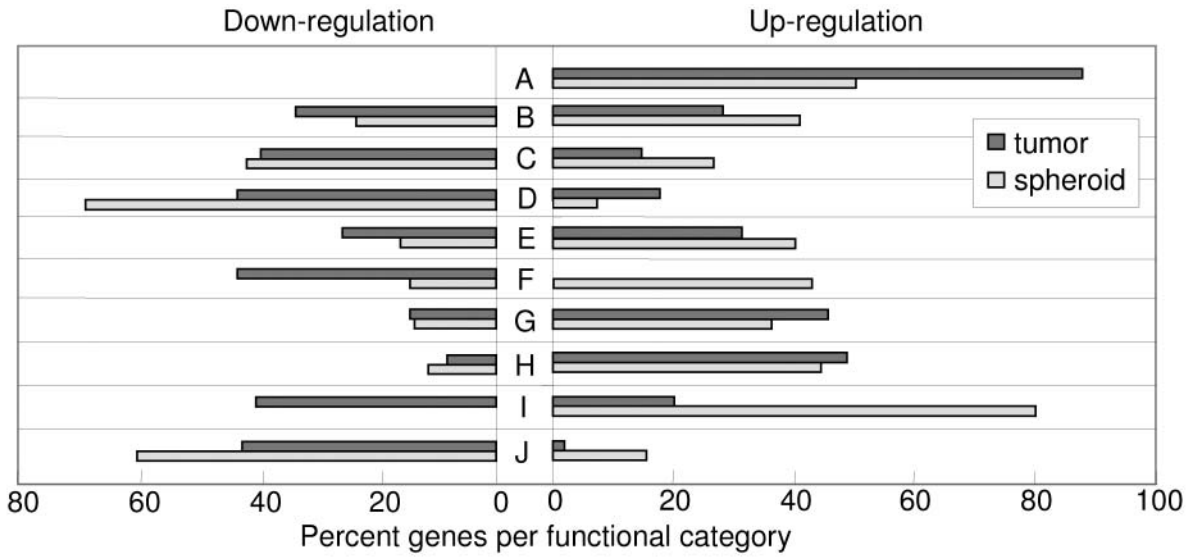

Figure 4. *Global gene expression profiles in spheroids and solid tumor tissues composed of LNCaP cells, compared with monolayer cultures. The gene expression profiles are shown according to functional gene categories. The percentages of up-regulated (more than 2-fold) and down-regulated (less than 1/2-fold) genes when compared with monolayer cultures are shown for each functional category. Gene functions: A, angiogenesis; B, cell death; C, cell cycle; D, cell division; E, differentiation; F, invasion; G, cell adhesion; H, immune response; I, hypoxia; and J, DNA repair.*

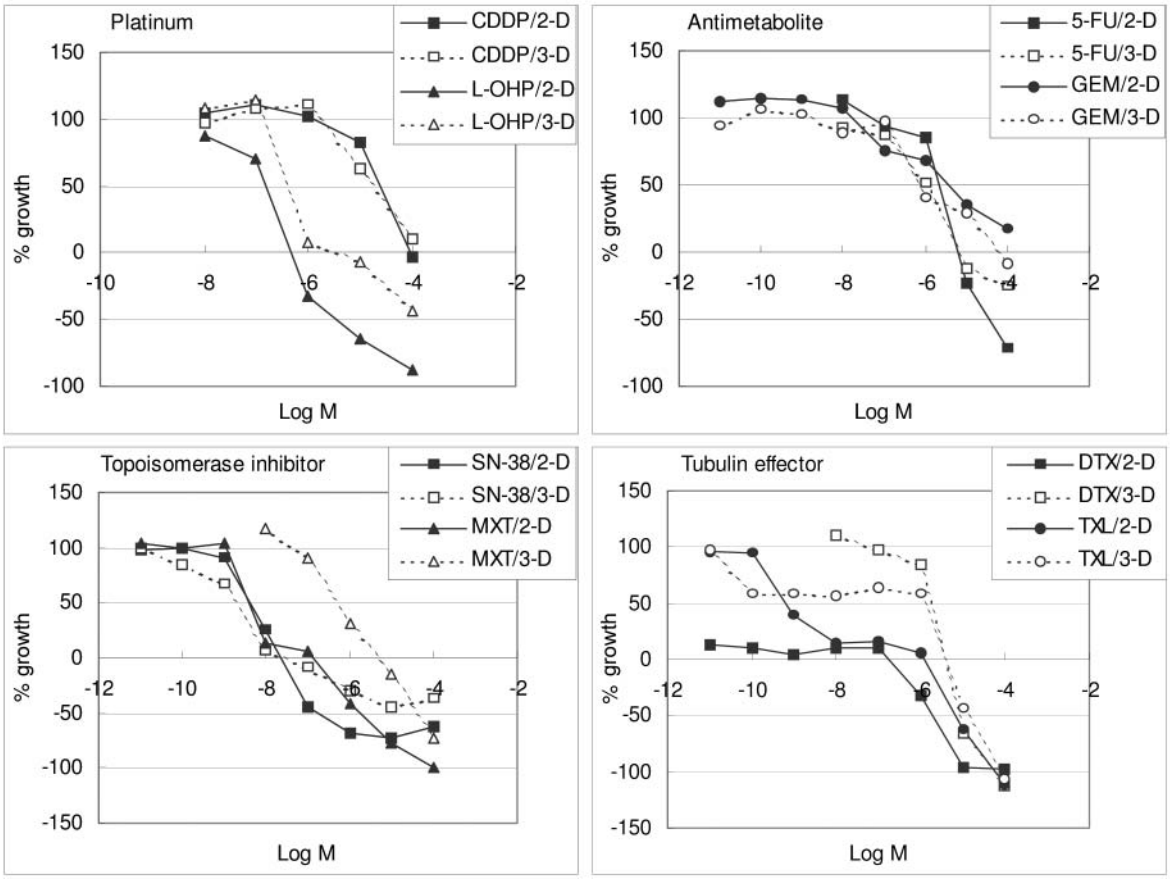

Figure 5. *Comparison of cytotoxicity in monolayer cultures (solid line, 2-D) and spheroids (broken line, 3-D) of LNCaP cells. The responses of the LNCaP cells against anticancer agents were determined using an MTT assay after 48 h of dosing the cells with the agent. Abbreviations: CDDP, cisplatin; L-OHP, oxaliplatin; 5-FU, 5-fluorouracil; GEM, gemcitabine; MXT, mitoxanetrone, TXL, paclitaxel; DTX, docetaxel.*

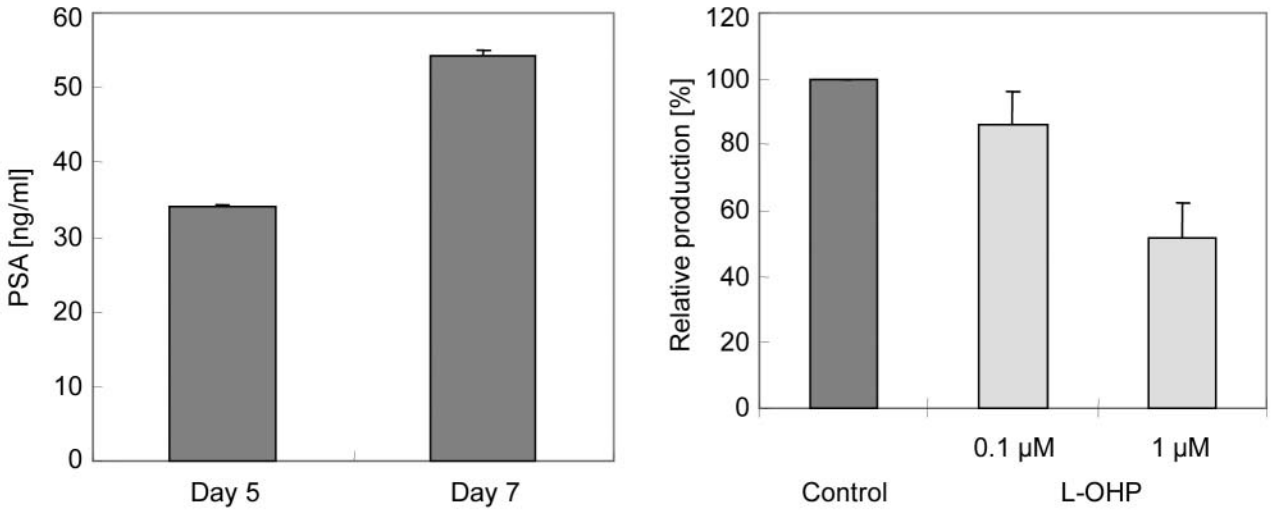

Figure 6. *Prostate-specific antigen (PSA) response of LNCaP spheroids. The PSA levels in the culture supernatants were determined by ELISA. a) PSA level on day 5 and day 7. b) Relative production of PSA following treatment with an anticancer agent. Spheroids were exposed to 0.1 or 1.0 μM of L-OHP from day 5 to day 7. The amount of PSA released from day 5 to day 7 was compared with that in the non-treated control. Bars: SD.*

oxygen gradient experiment, however, we found that a low-molecular weight compound, pimonidazole hydrochloride, could be delivered from outside of the spheroid into its central region. An oxygen gradient was visualized inside the LNCaP spheroids before developing a central necrosis. Thus, the hypoxic cells might have succumbed to necrosis. Since necrosis occurred in the poorly-vascularized regions of the solid tumors, but was difficult to generate in the monolayer cultures, necrosis might be an important characteristic of spheroids.

The enhanced VEGF production observed in the LNCaP spheroids might depend on the hypoxic conditions within the spheroids. Hypoxia is common in human cancer tissue and stabilizes the expression of hypoxia-inducible transcriptional mediator HIF-1α, which regulates VEGF transcription (24). VEGF might be consumed to enable the survival of the spheroid *via* an autocrine mechanism and might also act as an angiogenetic factor when LNCaP cells are grown *in vivo*. Although compensatory pathways to induce angiogenesis independent of HIF-1α have been demonstrated (25), whether such alternative pathways are evoked in spheroids remains to be verified.

The effects of two platinum agents, cisplatin and oxaliplatin, have been evaluated in prostate cancer patients (26, 27). In this study, oxaliplatin was more effective against LNCaP cells than cisplatin. Deficiencies in DNA mismatch repair have been reported to contribute to microsatellite instability in the genome and to cisplatin resistance, but not to oxaliplatin resistance (28, 29). Defects in DNA mismatch repair were reported in human prostate tumor specimens and cell lines, including LNCaP cells (30), suggesting that oxaliplatin may be superior to cisplatin against tumors with microsatellite instability. Defective DNA mismatch repair and microsatellite instability might also contribute to fluorouracil-based chemotherapy resistance (31, 32). Interestingly, the effectiveness of the antimetabolites, including 5-FU, was similar in both monolayer cultures and spheroids. The down-regulation of DNA mismatch repair has been reported in some tumor spheroids (33); however, whether these down-regulations might depend on depressed cell proliferation or DNA synthesis was not clarified. Regarding the topoisomerase inhibitors, the cytotoxicity of mitoxantrone, but not of SN-38, was lower in the spheroids than in the monolayer cultures. It has corresponded to the spheroids of Chinese hamster cells, because a lower activity of topoisomerase II inhibitors, but not of topoisomerase I inhibitors, was reported (34). Spheroids have also been shown to acquire a resistance to microtubulin effectors. It was reported that a Bcl-2 antisense oligonucleotide increased the efficacy of docetaxel in HRPC patients and that the up-regulation of $p27^{kip1}$ was correlated with the taxol resistance in ovarian cancer spheroids (35, 36). In this study, a reduction in the growth rate and the up-regulation of Bcl-2 and $p27^{kip1}$ was observed in LNCaP spheroids, and these processes were suggested to contribute to chemoresistance (37-39). The cytotoxicity of almost all the anticancer agents that were tested was lower in the spheroids than in the monolayers, but the degrees of acquired resistance differed; thus, the mechanism responsible for the lower chemosensitivity of spheroids may not be common to all anticancer agents and specific mechanisms present in spheroids are likely responsible for resistance to different anticancer agents.

The growing conditions affected the expressions of numerous genes in LNCaP cells. It was found that the global gene expression profiles altered by the growing conditions were similar in *in vivo* tumor tissue and spheroids of LNCaP cells. Spheroids may be useful as an *in vitro* avascular tumor model, as supported by our results showing that angiogenesis-related genes were not down-regulated. The percentage of up-regulated genes in angiogenesis was higher *in vivo* than that of the spheroids. In addition, the down-regulation of hypoxia-related genes was observed in solid tumor tissue, but not in the spheroids. These differences might have depended on the neovasculature of the solid tumor tissue. Though these results were obtained using LNCaP cells, we are presently examining specific genes and validating the association of these findings with those for human tumor specimens.

In conclusion, the microenvironment of spheroids – including proliferative heterogeneity and hypoxic and necrotic conditions – causes the expression of numerous genes to be altered in LNCaP cells; these morphological changes influence responses to environmental stress. The present results strongly support the use of spheroids as an appropriate pre-clinical study model for *in vivo* tumors. It was also supported by the secretory response of PSA, used as a clinical marker of prostate tumor growth. The PSA level in the culture supernatant reflected the survival of the spheroids, since the PSA levels increased with time and decreased with anticancer agent treatment.

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