The Role of the 3D Environment in Hypoxia-induced Drug and Apoptosis Resistance

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Abstract. *Background: 3D tumors express different adhesion receptors from those expressed in monolayers, leading to a distinct microenvironment. The third dimension also brings mass transport into relevance, as inadequate diffusion of oxygen produces hypoxia. This study investigates the effects of distinct 3D environments on hypoxia-associated apoptosis and drug resistance. Materials and Methods: Under hypoxia and normoxia, U251 glioma cells and U87 astrocytoma cells were grown as spheroids on flat substrates, scaffolds seeded with dispersed cells, and spheroid-seeded scaffolds. The samples were subsequently treated with doxorubicin and resveratrol, known inducers of apoptosis. Results: All 3D environments induced increased but distinct resistance to apoptosis, as evident by lower caspase-3 activity, and higher production of anti-apoptotic proteins BCL-2 and survivin. Hypoxic monolayers also exhibited higher resistance to doxorubicin and higher production of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), but lower production of BCL-2 and survivin. Conclusion: These findings suggest that in vitro, 3D models acquire greater apoptosis resistance via up-regulation of anti-apoptotic proteins, and that the precise mechanism depends on the individual 3D microenvironment.*

The hypoxic core of cells within *in vivo* tumors and cells in three-dimensional (3D) cultures have been shown to exhibit resistance toward anticancer drugs and radiotherapy (1). Hypoxic cells are known to have higher apoptosis resistance than normoxic cells through various mechanisms, including manipulating regulations over apoptosis effectors (2-4). Many apoptotic pathways converge at mitochondria, and release cytochrome *c* almost universally (5). The mechanism behind cytochrome *c* exit from mitochondria is known to be intimately related to BCL-2 protein family members that spend most of their time on the cytoplasmic face of the mitochondrial outer membrane. Previous studies have shown that BCL-2, an anti-apoptotic member of the BCL-2 family, can be up-regulated in hypoxia and 3D cultures and suggested that BCL-2 may have an important role in apoptosis resistance induced by hypoxia in tumor models (3-4, 6).

Some apoptotic pathways, such as those induced by death receptors, can be insensitive to BCL-2 regulated cytochrome *c* release and still activate downstream apoptosis effectors (5). In mammals, these pathways can be regulated by a family of proteins known as inhibitor of apoptosis (IAP) proteins, which can inhibit activated downstream apoptosis effectors called caspases. Survivin is a member of the IAP family that exhibits up-regulation by hypoxia and in the extracellular matrix (2, 7). Therefore, exploring expression of survivin and BCL-2 may provide insights into the potential role of both pathways on hypoxia-induced apoptosis resistance in 3D tumor models.

Cells in 3D scaffolds and 3D spherical aggregates (spheroids) exhibit similar responses to chemo- or radiotherapy as cells *in vivo* and may be used as an important tool in cancer biology (1, 8). 3D scaffolds provide signaling and physical support to the attached cells and 3D spheroids provide avascular tumor nodules that are very similar to microregions of tumors *in situ*. Previous studies have shown that pro- and anti-apoptotic proteins are regulated differently in 3D models (1). 3D spheroids and 3D scaffolds represent *in vivo* tumors better than do 2D monolayers, and the two 3D environments present unique characteristics and advantages. Spheroids are completely cell-based and represent a different 3D microenvironment from that of 3D scaffolds (9). However, culturing cells in 3D matrices may not capture the cell-to-cell interaction present in aggregated tumors (10).

We previously developed an *in vitro* system by incorporating 3D multicellular spheroids of a glioma cell line, U251, into a 3D biocompatible scaffold made with poly(lactic-co-glycolic acid) (PLGA) (11). This combination of 3D

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environment increased drug resistance substantially compared to other 2D and 3D culture environments. In the present study, we investigate the role of different 3D microenvironments on hypoxia-induced apoptosis and drug resistance.

Materials and Methods

Cell culture. The U251 cell line was generously donated by Dr. Robert Prins and Dr. Linda Liau from the Department of Neurosurgery at the University of California Los Angeles. The U87 cell line was generously donated by Dr. Daniel T. Kamei from the Department of Bioengineering at the University of California Los Angeles. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5g/l glucose containing 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37˚C, 5% CO_2 in humidified incubators. Specimens for hypoxic culture were transferred to sealed microincubators (Billups-Rothenberg, Del Mar, CA, USA) that are placed within standard incubators. The sealed microincubators contained a small dish of water for humidity, were purged for 5 min at 20 l/min and saturated with medical-grade gases comprising 1% oxygen, 5% CO_2, and balanced with nitrogen. At each time point, the sealed microincubators were opened briefly to remove well plates to be assayed and the chambers re-purged and re-calibrated to 1% oxygen. To prevent reperfusion injury to the hypoxic cells, media were prepared 24 hours in advance in the chamber before adding them to hypoxic cells.

Fabrication of polymeric scaffolds. 3D scaffolds were fabricated using solvent casting and the particulate leaching technique (12). Polymer solution (20%, w/w) was made by dissolving PLGA (Birmingham Polymers, Birmingham, AL, USA) in a mixture of methanol and chloroform (33/67, w/w; Sigma-Aldrich, St. Louis, MO, USA), then 500-1000 μm salt and 50-100 μm sucrose particles were added, and the resulting mixture (PLGA/particle ratio 5/95, w/w; sucrose/salt ratio 12/88, w/w) was cast into Teflon molds with an inner diameter of 8.5 mm and thickness of 2 mm (McMaster-Carr, Atlanta, GA, USA). The solvents were evaporated in a fume hood overnight. Salt and sucrose particles were removed by immersing the scaffolds in deionized water overnight. Scaffolds were subsequently sterilized in 70% ethanol for 30 minutes and rinsed three times with phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA). Surfaces of the scaffolds were coated with collagen by immersing scaffolds in 1 ml of 0.025% (w/v) neutralized PureCol (Allergan, Irvine, CA, USA) in PBS overnight at 4˚C.

Generation of spheroids. The method developed by Ivascu and Kubbies was modified to create spheroids of desired size (13). Cells from monolayer culture were trypsinized (Invitrogen, Carlsbad, CA, USA) and re-suspended to 1×10^4 cells/ml (2,000 cells/spheroid). To prevent attachment to the bottom of the wells and promote spheroid formation, 96-well conical plates were coated with poly(2-hydroxyethyl methacrylate), or poly-HEMA (Polysciences, Warrington, PA, USA). Wells with 50 μl of 5 mg/ml solution of poly-HEMA dissolved in anhydrous ethanol were allowed to dry for 48 hours at 37˚C. Cell solution (200 μl) was pipetted into each of the poly-HEMA coated wells and the plates were subsequently centrifuged at 1,000×g for 10 minutes to initiate spheroid formation. Spheroids were cultured at 37˚C, 5% CO_2 in humidified incubators.

Incorporation of spheroids into scaffolds. Based on preliminary studies, the combination of spheroids of 2,000 cells and scaffolds with 500-1000 μm diameter pores allowed proper seeding. After 48 hours of culture, spheroids were seeded onto scaffolds placed on sterile filter paper (Whatman, Florham Park, NJ, USA) to absorb excess culture medium through capillary action. One hundred spheroids were pipetted onto each scaffold using specialty pipette tips with large orifices (Fisher Scientific, Pittsburgh, PA, USA). The seeded scaffolds were moved onto poly-HEMA-coated wells of 12-well non-tissue culture-treated plates (Fisher Scientific, Fremont, CA, USA) (500 μl of 5 mg/ml polyHEMA in anhydrous ethanol in each well dried for 48 hours at 37˚C), and 50 μl of culture medium was added to each scaffold. The spheroids were left to attach for 4 hours in humidified incubators, and 2 ml of culture medium was subsequently added to each well. After 24 hours of culture, the plates were placed on an orbital shaker. Successful incorporation of spheroids into scaffolds was confirmed with microscopic imaging. 3D spheroids and 2D monolayers on 3D scaffolds are referred to as '3D_3D' and '2D_3D', respectively. 3D spheroids and 2D monolayers on 2D tissue culture plates are referred to as '3D_2D' and '2D_2D', respectively.

Caspase-3 assay. Doxorubicin and resveratrol are known inducers of apoptosis. The samples were treated with 100 μM resveratrol (Sigma-Aldrich, St Louis, MO, USA) for 48 hours and 1 μM doxorubicin (Sigma-Aldrich, St Louis, MO, USA) for 24 hours. Apoptosis was then quantified with a colorimetric assay (Biovision, Mountain View, CA, USA) that utilizes Aspartate-Glutamine-Valine-Aspartate-p-Nitroanilide (DEVD-pNA), a substrate molecule that changes color when cleaved by an active caspase-3. For each sample, the amount of caspase-3 activity was divided by the number of cells. The resulting values were then normalized to the respective non-treated values.

ELISA for quantifying survivin, BCL-2, vascular endothelial growth factor, and basic fibroblast growth factor production. To quantify survivin, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) production, aliquots of culture medium of cells in monolayer, spheroids, 2D_2D, and 3D_3D were analyzed with Quantikine Human Survivin, Quantikine Human VEGF, and Quantikine Human bFGF basic enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA), respectively. To quantify BCL-2 production, aliquots of culture medium of cells in monolayer, spheroids, 2D_2D, and 3D_3D were analyzed with BCL-2 ELISA kit (Calbiochem, La Jolla, CA, USA). For each sample, the amounts of survivin, BCL-2, VEGF, and bFGF were first divided by the number of cells. The resulting values were then normalized to the respective 2D values.

Drug resistance assay. For the 2D_2D system, cells were seeded at $10\times10^3/cm^2$ and were cultured for one day. Samples were subsequently incubated in solutions of different concentrations (10 μM, 3.14 μM, 1 μM, 0.314 μM, 0.1 μM, 0.0314 μM and 0.01 μM) of doxorubicin (Sigma-Aldrich, St. Louis, MO, USA). The cells were left to grow for 2 more days, and the bicichoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA), which is a colorimetric assay that quantifies proteins, was performed with Infinite® 200 Microplate Reader (Tecan Systems, San Jose, CA, USA). BCA cell viability at each drug concentration was quantified relative to that of wells without drugs.

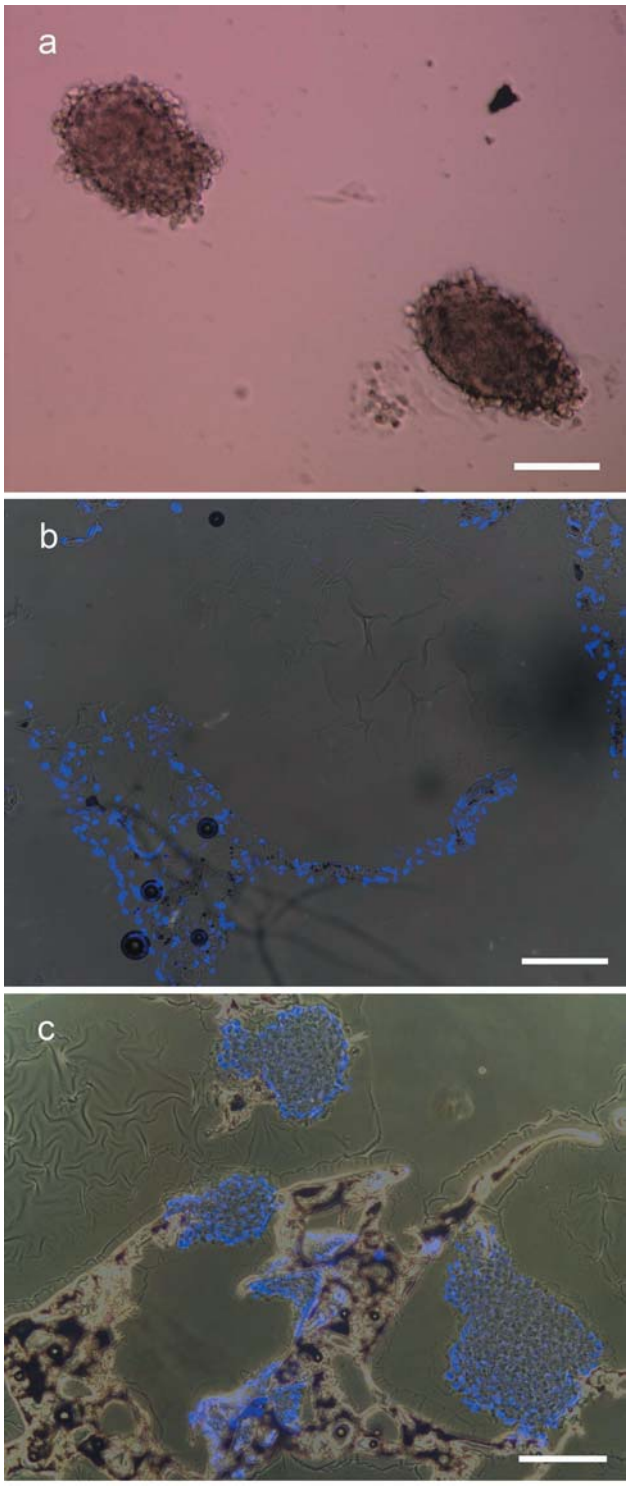

Figure 1. *Apoptosis resistance of different 3D microenvironments were compared. a: 3D spheroids on 2D tissue culture plate (3D_2D); b: 2D monolayers on 3D scaffold (2D_3D), and c: 3D spheroids on 3D scaffolds (3D_3D). Monolayer-seeded scaffolds and spheroid-seeded scaffolds were frozen in optimal cutting temperature medium and cryosectioned. The slices were mounted onto slides and cell nuclei were stained with DAPI. Scale bar, 100 μm.*

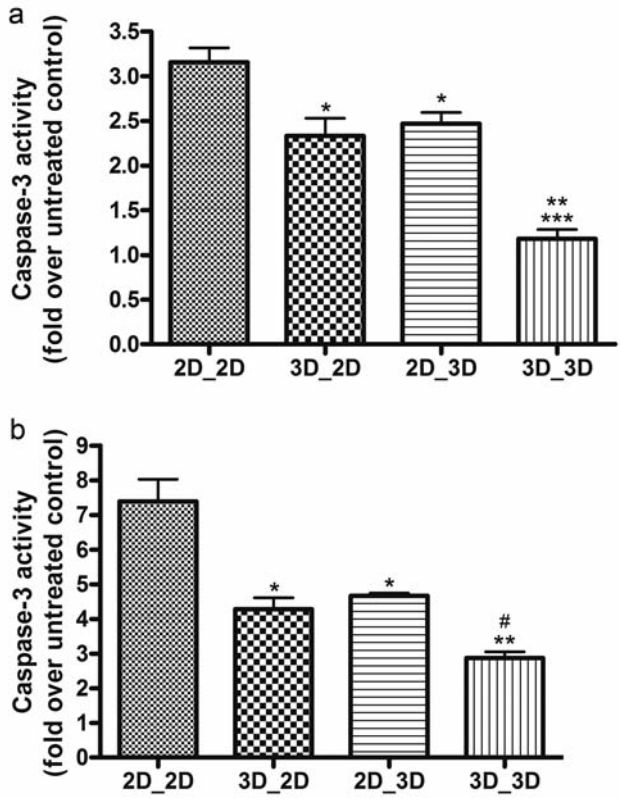

Figure 2. *Spheroid-seeded scaffolds exhibit the highest apoptosis resistance. a: Fold increase of caspase-3 activity after treatment of U251 cell in 2D_2D, 3D_2D, 2D_3D, and 3D_3D with 1 μM of doxorubicin for 24 hours was quantified. *P<0.05 (2D_2D vs. 3D_2D and 2D_2D vs. 2D_3D); ***p<0.001 (2D_2D vs. 3D_3D); **p<0.01 (3D_2D vs. 3D_3D and 2D_3D vs. 3D_3D). b: Fold increase of caspase-3 activity after treatment of U251 cell in each model with 100 μM of resveratrol for 48 hours was quantified. *p<0.05 (2D_2D vs. 3D_2D and 2D_2D vs. 2D_3D); **p<0.01 (2D_2D vs. 3D_3D); #p<0.05 (3D_2D vs. 3D_3D and 2D_3D vs. 3D_3D). All data (mean+standard deviation, n=3) are normalized to their untreated controls.*

Microscopic imaging of spheroids and image analysis. Cell-seeded scaffold samples were frozen in Tissue-Tek® optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) and were cryosectioned (20 μm-thick) onto superfrost plus® microslides (VWR International, West Chester, PA, USA). PLGA scaffold structure was visualized in bright field imaging and cells stained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) were imaged with fluorescent microscopy at 360 nm/460 nm excitation/emission (ex/em) wavelengths.

Statistical analysis. To assess the statistical difference between two groups, a two-tailed, unpaired *t*-test assuming equal variance was selected since no predictions about the changes in the means were made and a Gaussian distribution for each population was assumed. As for the statistical difference among three or more groups, a one-way analysis of variance (ANOVA) was used.

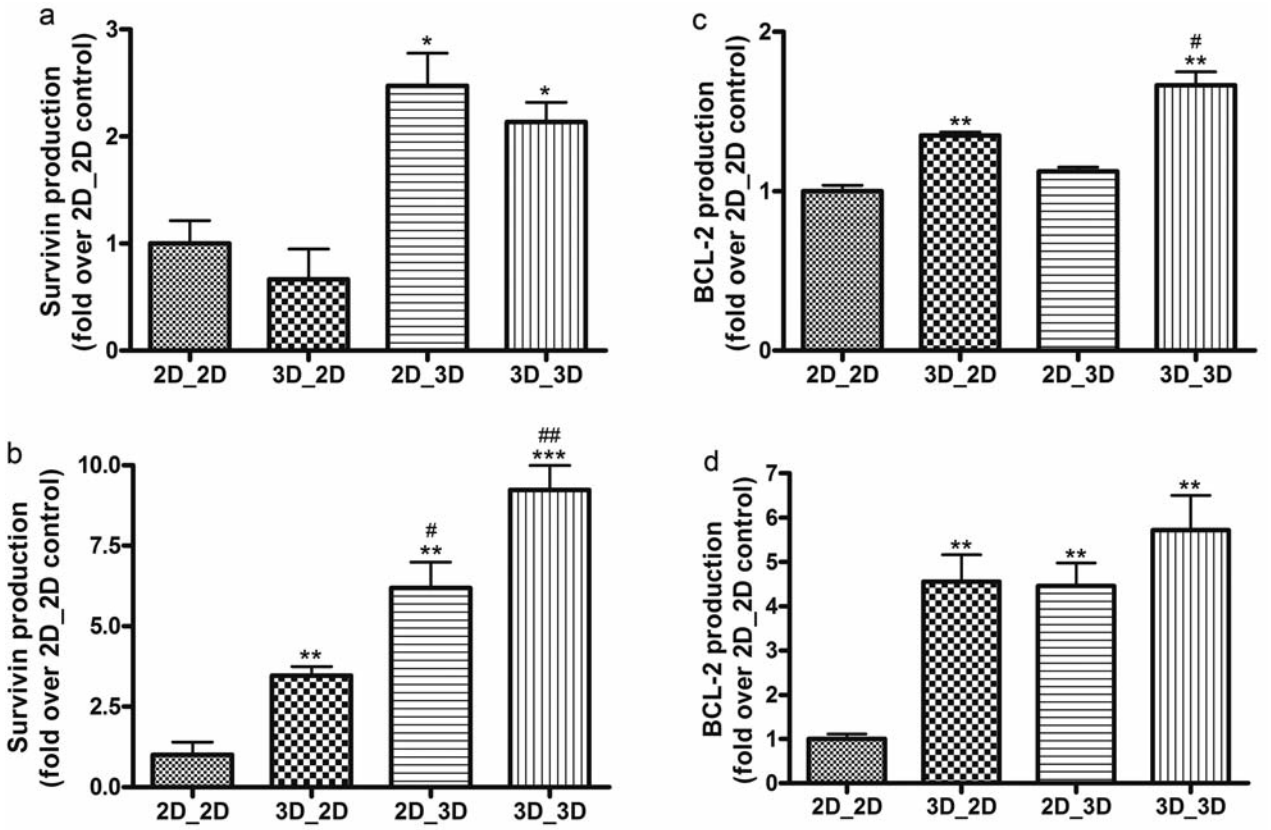

Figure 3. *Spheroid-seeded scaffolds have up-regulated production of survivin and BCL-2. Each of the cell lines, U251 and U87, were cultured as 2D_2D, 3D_2D, 2D_3D, and 3D_3D for 24 hours. Then production of survivin and BCL-2 was quantified with ELISA kit. a: Production of survivin in U251 cells. *p<0.05 (vs. 2D_2D). b: Production of survivin in U87 cells. **p<0.01 and ***p<0.001 (vs. 2D_2D); #p<0.05 and ##p<0.01 (vs. 3D_2D). c: Production of BCL-2 in U251 cells. **p<0.01 (vs. 2D_2D); #p<0.05 (vs. 3D_2D). d: Production of BCL-2 in U87 cells. **p<0.01 (vs. 2D_2D). All data (mean±standard deviation, n=3) are normalized to their respective monolayer values.*

Results

Spheroid-seeded scaffold (3D_3D) exhibited higher apoptosis resistance. The cryosectioned images confirmed that monolayer and spheroids were seeded inside the scaffolds and were attached to the pores (Figure 1). The models were treated with 1 μM doxorubicin, a DNA intercalator that is known to ultimately induce apoptosis by activating caspase-3 (14). As expected, the monolayer cells (2D_2D) exhibited the lowest resistance as indicated by the highest caspase-3 activity (Figure 2a). In contrast, combining spheroids into scaffolds (3D_3D) produced the greatest apoptosis resistance and lowest caspase-3 activity ($p<0.05$). 3D_2D and 2D_3D produced intermediate resistance ($p<0.05$). These findings were verified with another caspase-3 activating molecule, resveratrol (Figure 2b) (15).

Production of anti-apoptotic proteins. Production of survivin was enhanced in 2D_3D and 3D_3D but not in 2D_2D or 3D_2D, suggesting the 3D porous scaffold environment had more significant influence on survivin production than did the 3D spheroid environment ($p<0.05$, Figure 3a). When the experiment was repeated with another glioma cell line, U87, survivin production was again higher in 2D_3D and 3D_3D (Figure 3b). Since the dimension for mass transport is significantly higher in 3D scaffolds (~1 mm) than spheroids (~0.1 mm), survivin production may be more sensitive to the diffusion length-scale of the culture system and hypoxia. Clinical analysis of irradiated human cancer tissues revealed correlation between survivin production and anemia (16).

Production of BCL-2 was enhanced in 3D_2D and 3D_3D, but not in 2D_2D or 2D_3D, suggesting the 3D spheroid environment had more significant influence on BCL-2 production than did the 3D porous scaffold environment ($p<0.05$, Figure 3c). BCL-2 is known to be up-regulated by cell-to-cell and cell-to-matrix interaction mediated by proteins of the membrane such as cadherins and integrins (17-19). When the experiment was repeated with another glioma cell line, U87, all three 3D culture systems (2D_3D, 3D_2D, 3D_3D) produced more BCL-2 protein than the monolayer controls (Figure 3d). Therefore, although

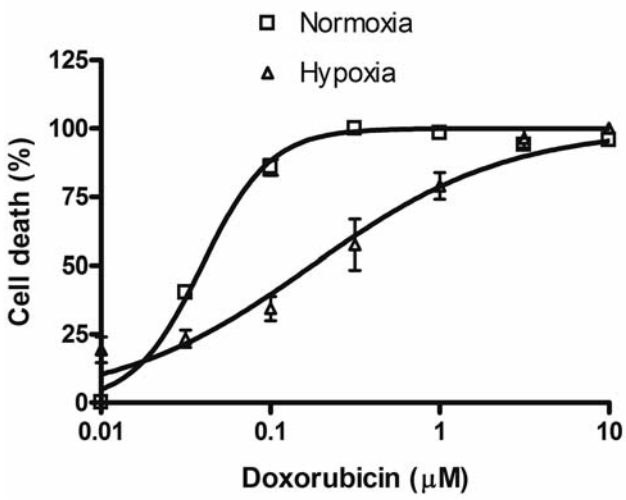

Figure 4. *Hypoxic cells possess higher resistance to doxorubicin. Cellular drug resistance of normoxic and hypoxic U251 cells was evaluated by quantifying cell death after consistent exposure to anticancer drugs. Cell cultures were subjected to different concentrations of doxorubicin, and cell survival with respect to the negative control (cell samples without the addition of drugs) was assessed using BCA assay. Treatment of monolayer cells with hypoxia resulted in rightward shift of the curve or higher IC_{50} value when compared to normoxic cells. Mean % cell deaths and standard deviation are plotted over drug concentrations (n=3).*

U251 showed that spheroids seem to have greater effect than scaffolds in promoting BCL-2 production, this difference may depend on specific cellular physiology, as U87 and U251 cells may elaborate different cell-to-cell and cell-to-matrix interaction in each culture environment.

Regardless of the cell line, the consistently high production of BCL-2 and survivin in 3D_3D suggests that effects induced by spheroids sans 3D scaffolds, and *vice versa*, can be captured by incorporating spheroids in 3D scaffold (3D_3D). In all cases, 3D_3D consistently produced the highest drug resistance, apoptosis resistance, and production of survivin and BCL-2 compared to the remaining culture environments.

Hypoxia induces higher drug resistance for monolayers, but less than 3D_3D. To identify the effect of hypoxia in spheroids in 3D scaffolds on the drug resistance, U251 cells in all four culture conditions were exposed to a range of doxorubicin concentration in hypoxic microenvironmental chambers (4). A sigmoidal drug – responsive curve was generated (Figure 4) to interpolate the drug concentration at which 50% of the cells remain viable (IC_{50}). Table I shows the IC_{50} values for each condition under hypoxia and normoxia. In 2D_2D, cells grown in hypoxia had significantly higher IC_{50} values than their normoxic counterparts.

Table I. *IC_{50} of systems assayed for drug resistance.*

Culture system	Doxorubicin (μM) (95% confidence interval)
2D (10,000/cm^2)	0.03967 (0.03576-0.04357)
2D (10,000/cm^2) hypoxia	0.1862 (0.1250-0.2474)
2D_3D hypoxia	1.654 (0.8193-2.489)
3D_2D hypoxia	4.571 (2.154-6.987)
3D_3D hypoxia	19.97 (9.362-30.57)

Compared to hypoxic 2D_2D, the hypoxic 3D culture systems exhibited higher IC_{50} in the following ascending order of IC_{50}: 2D_3D (1.4 μM), 3D_2D (3.9 μM), and 3D_3D (16.9 μM). This trend is consistent with our previous findings (11), and suggests that hypoxia can increase drug resistance, but it alone cannot explain the increased drug resistance shown in 3D_3D. When U251 3D_2D, 2D_3D, and 3D_3D are cultured under hypoxia, no significant change in drug resistance was observed when compared to the values under normoxia (11). Therefore, the effect of hypoxia on increasing drug resistance may already be saturated in these models and further hypoxia does not have any influence.

Hypoxia up-regulates the production of VEGF and bFGF but down-regulates that of survivin and BCL-2. It is known that hypoxia can up-regulate survivin and BCL-2 (2-3). To verify whether hypoxia, which may be present in 3D_3D, up-regulates the production of both anti-apoptotic proteins in U251, ELISA assay kits were used to quantify survivin and BCL-2 in U251 cultured under hypoxia (11). Contrary to our expectations, production of survivin and BCL-2 was lower under hypoxia than under normoxia (Figure 5a and b). In contrast, VEGF and bFGF were up-regulated by hypoxia (Figure 5c and d) as others have suggested (4, 9). Along with another study, our results show that hypoxia may not always up-regulate the production of BCL-2 and survivin (20). Additionally, the higher drug resistance shown by hypoxic cells may be more closely related to pathways that involve production of VEGF and bFGF but not necessarily of survivin and BCL-2 (21).

Discussion

The role of hypoxia in apoptosis resistance in spheroid-seeded scaffolds. Hypoxia in tumors can induce resistance to chemotherapeutic agents through mechanisms involving apoptosis resistance due to up-regulation of anti-apoptotic proteins such as BCL-2 and survivin (2-3). The major function of BCL-2 is to inhibit the release of cytochrome *c*, preventing initiation of apoptosis (5). Survivin inhibits activation of caspases, a set of cysteine proteases that are

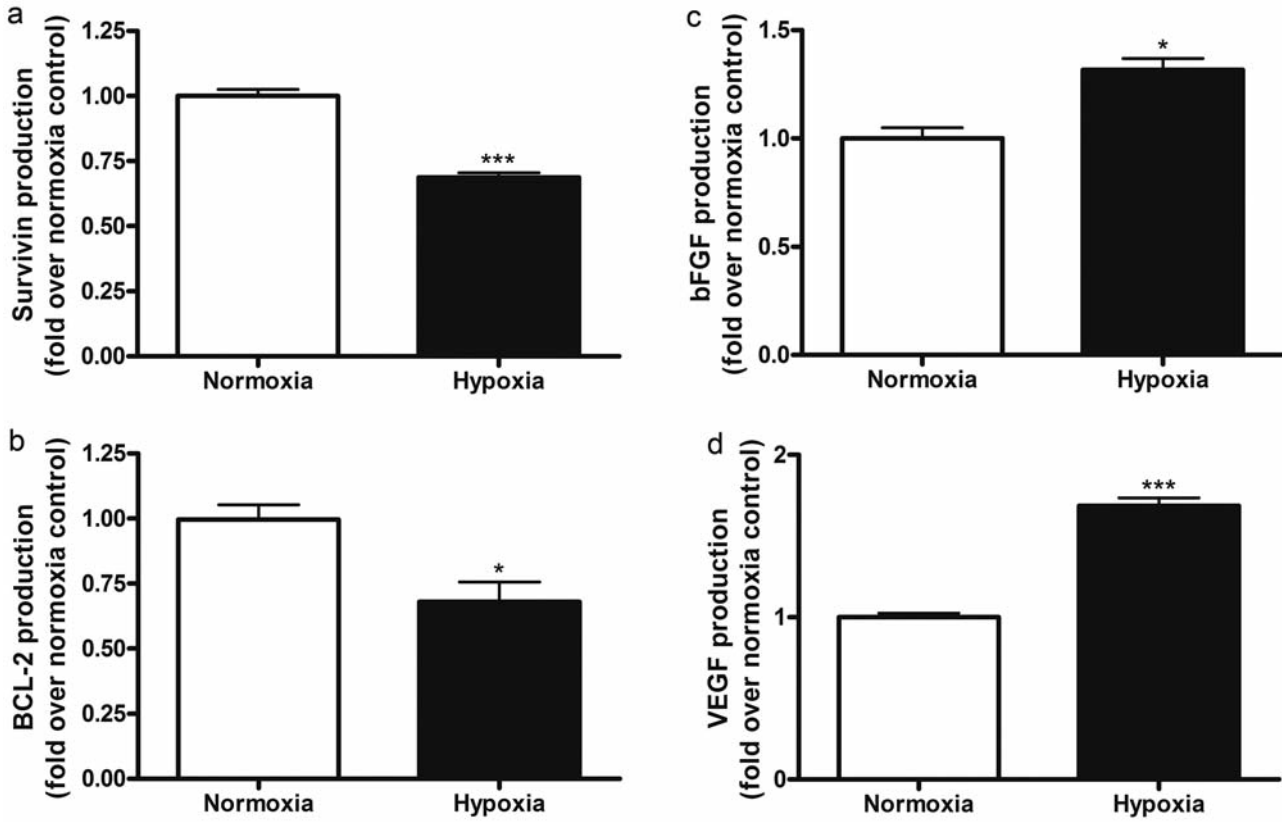

Figure 5. *Hypoxia up-regulated bFGF and VEGF expression but down-regulated that of survivin and BCL-2. U251 cells were cultured under hypoxia for 24 hours. Then the culture media and ELISA were used to quantify production of survivin, BCL-2, bFGF and VEGF. a: Production of survivin under hypoxia. ***p<0.001. b: Production of BCL-2 under hypoxia. *p<0.05. c: Production of bFGF under hypoxia. *p<0.05. d: Production of VEGF under hypoxia. ***p<0.001. All data (mean+standard deviation, n=3) are normalized to their respective normoxia values.*

activated specifically in apoptotic cells (5). While their functions are well established, such up-regulation of BCL-2 and survivin under hypoxia may not be universal in all tumor types as shown in this study.

Under hypoxia, U251 cells exhibited higher resistance to doxorubicin. However, in the process of confirming that hypoxia up-regulates production of survivin and BCL-2, we found that hypoxia reduced the production of both proteins, which seemed to disagree with previous studies (2-3). Kardosh *et al*. distinguished acute (<24 h) and chronic hypoxia (>24 h), and despite the increased survivin production under acute hypoxia, production decreased in chronic hypoxia (20). However, their result showed no increase in survivin at different time points even within a 24-hr frame, and in this regard, it may be more likely that the production of survivin under hypoxia varies depending on the cell line. Production of BCL-2 and survivin is also known to be up-regulated through receptors that mediate cell-to-cell and cell-to-matrix adhesion (7, 17-19). Clearly, these factors are significantly impacted by the three-dimensionality of the cultures.

While studies have shown that the expression of BCL-2 and survivin can both be related to hypoxia and adhesion, our results suggested that they may influence tumor cells in separate ways. Fornaro *et al*. and Tran *et al*. also demonstrated that cell adhesion *via* integrins and cadherins alone, without introducing hypoxia, increased survivin expression (7, 18). Others have shown that although cell-to-cell adhesion may increase BCL-2 expression, hypoxia may reduce the expression (17, 22). However, further studies are required to elucidate the possible crosstalk between the pathways.

3D cultures provide a hypoxic environment for the spheroids and the scaffolds. Hypoxic cells are resistant to apoptosis regardless of lower production of BCL-2 and survivin compared to normoxic cells. Thus, 3D cultures may acquire the resistance to apoptosis *via* a hypoxic environment, but independent of BCL-2 or survivin expression. The up-regulated production of bFGF and VEGF in apoptosis-resistant cells under hypoxia suggests that a pathway involving VEGF and bFGF but independent of BCL-2 and survivin may induce higher drug resistance in tumor cells. RAF-1 has been studied as an important

regulator of apoptosis, and it is activated differentially by bFGF and VEGF. bFGF activates RAF-1 *via* p21-activated protein kinase-1 (PAK) phosphorylation of serines 338 and 339, which leads to RAF-1 mitochondrial translocation and protection from apoptosis (21). This pathway is BCL-2 dependent, and therefore it is unlikely to explain the observations made in this study (23). VEGF activates RAF-1 *via* Src kinase, resulting in phosphorylation of tyrosines 340 and 341. The subsequent activation of the mitogen-activated protein kinase (MEK) and the extracellular signal-regulated kinase (ERK) inhibit activation of caspase-3, even after the release of cytochrome *c* (24). Furthermore, it is suggested that ERK may phosphorylate and inhibit caspase-9 (25). Since these events are downstream of BCL-2 and survivin (26), the RAF/MEK/ERK pathway activation induced by VEGF may be a future direction in further studying the mechanism behind hypoxia-induced apoptosis resistance in 3D cultures.

The role of anti-apoptotic proteins in drug resistance of spheroid-seeded scaffolds. In vivo tumors and 3D tumor models are known to have higher apoptosis resistance compared to monolayer cells due to multiple ways including anchorage dependence, cell-to-cell signaling, cell-to-matrix signaling, cellular quiescence and hypoxia (1, 17). The overproduction of both BCL-2 and survivin is probably involved in the higher drug resistance in 3D_3D when compared to 2D_2D, 3D_2D, and 2D_3D. However, other anti- and pro-apoptotic proteins can have important relationships with the 3D morphology of tumors. This study has shown that apoptosis resistance due to up-regulated production of BCL-2 and survivin may vary depending on the kind of 3D culture system.

ELISA data shows that the monolayer cells in 3D scaffold environment (2D_3D) produce more survivin than the 3D spheroids on the 2D surface (3D_2D), suggesting a potential contribution of diffusion length scale to survivin production. It has been shown, mathematically and experimentally, that thick 3D porous scaffold presents diffusional transport limitations to cells within its inner core (27-29). On the other hand, the spheroid environment (3D_2D) exert slightly greater influence on BCL-2 production than the scaffold (2D_3D) environment ($p<0.05$, Figure 3c). Since BCL-2 is up-regulated by receptor mediated cell-to-cell and cell-to-matrix interactions, the spheroid environment may promote interactions that favor BCL-2 production. Saito *et al*. and Timmer *et al*. provided independent evidence that extracellular matrix architecture may influence BCL-2 and survivin expressions (30-31). Although in our case the scaffold was made with a hydrophobic synthetic polymer coated with collagen, incorporating other biomaterials may produce different cellular responses. Interestingly, we have also found that seeding spheroids inside a scaffold sustained the increased production of BCL-2 and survivin. It is possible that the production of these proteins can be manipulated by combining different models, which can be a useful method for developing *in vitro* models that more closely mimics the *in vivo* behavior. Furthermore, our results suggest that growing cells in 3D *in vitro* models may regulate apoptosis in a way that cannot be mimicked by treating monolayer cells with hypoxia alone, as other morphological factors, such as proteins involved in cell-to-cell or cell-to-matrix interactions should be considered for drug resistance shown by 3D_3D.

Targeting survivin and BCL-2 in therapeutics for 3D tumors. Currently, various cancer therapeutic strategies including antisense oligonucleotides, siRNA, and small molecular weight molecules that target BCL-2 and survivin are under investigation for clinical use (32-33). Since incorporating spheroids into scaffolds has demonstrated an increase in production of both BCL-2 and survivin, with higher drug resistance, spheroid-seeded scaffolds may be utilized to screen for therapeutics that target those proteins. Furthermore, because they are highly up-regulated in 3D_3D, which we believe is a better representation of *in vivo* tumors than the other models we have tested, targeting these two proteins may be especially effective in treating tumors. Indeed, some have already shown that blocking BCL-2 removed multicellular resistance of tumor spheroids (6). Therefore, the next step in characterizing the spheroid-seeded scaffold may involve assaying its resistance to drugs that target BCL-2 and survivin. Finding other anti-apoptotic and pro-apoptotic proteins that are associated with 3D tumor morphology may also make such an approach more effective.

Limitations and future direction. There are several limitations to our study. First of all, as we have shown with U251 and U87 cells, the choice of cell lines may change the production of BCL-2 and survivin. Thus, although the drug-resistance in 2D and various 3D cultures is remarkably consistent across multiple cell lines, the cellular apoptotic response to induced hypoxia needs to be investigated in multiple cell lines with a range of tolerance to hypoxia. Secondly, there are multiple members in the BCL-2 protein family and IAP family that may exert varying anti-apoptotic and pro-apoptotic cues, contributing to the overall drug resistance *via* effects on apoptotic mechanisms. Thirdly, this study has explored only one aspect of 3D tumor environment, hypoxia, while other factors, such as the production of cadherins and integrins, may play important roles. Furthermore, cytochrome c exit regulated by the BCL-2 family is only one of many ways in which mitochondria can affect apoptosis. Mitochondria can also release apoptosis-inducing factor, Smac/DIABLO, and procaspase-2, -3, and -9 (5). IAP-2, another member of the IAP has also been shown to be up-

regulated in hypoxia (34). Therefore future exploration of other proteins will be necessary to definitively attribute their roles in normoxic and hypoxic 2D and 3D environments. While this study simply does not intend to explore every major pathway or proteins associated with apoptosis in cancer physiology, it has effectively demonstrated that specific cellular characteristics in the 3D environments are not necessarily universal and that their effects may be additive towards drug resistance specific to the model. Therefore, future studies involving a targeted incorporation of biological characteristics specific to a tumor of interest, contrary to developing a generalized tumor model, may make way towards enhanced clinical applicability and significance of engineered *in vitro* models.

Contributions of the Authors

All authors have contributed significantly to the manuscript and took active roles in the design, development, and refinement of all experiments. Jun Woo Kim performed all the experiments. Benjamin M. Wu supervised the study. All authors provided analyses and conceptual advices. Jun Woo Kim prepared the manuscript. Won Jin Ho and Benjamin M. Wu, revised the manuscript. All authors are in agreement with the content of the manuscript.

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