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Degradation of endothelial network in disordered tumor-containing cell sheet

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Tumor angiogenesis is an important event in tumor malignancy; and the vasculature formed in tumor region is typically dysfunctional. Multiple factors are associated with tumor vessel abnormalities, but the precise mechanism has not been fully understood. In the present study, a tumor-containing cell sheet was prepared by mixing a small population of human embryonal rhabdomyosarcoma (RMS) cells (RDs) with human skeletal muscle myoblasts (HSMMs) to mimic muscle tissue invaded by RMS cells. Sheet fluidity and the extracellular matrix (ECM) meshwork of the tumor-containing cell sheet were found to be elevated and disordered, demonstrating the disruptive effect of tumor cells on sheet structure. When green fluorescent protein expressing human umbilical vein endothelial cells (GFP-HUVECs) were co-cultured with the tumor-containing cell sheet, an endothelial network was formed, but degraded faster as a result of activated migration of endothelial cells in the tumor-containing cell sheet. This study suggested that disorganized tissue structure facilitate tumor angiogenesis by activation of endothelial cell migration.

[**Key words:** Cell sheet; Embryonal rhabdomyosarcoma; Tumor angiogenesis; Extracellular matrix; Tissue remodeling; Endothelial network]

Tumor angiogenesis, the process of new blood vessel formation induced by a tumor mass, is an important process in tumor malignancy (1). Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma occurring in children. In the tumor region of RMS, increased angiogenesis was found (2). Over-expression of vascular endothelial growth factors (VEGFs), basic fibroblast growth factors (bFGFs) and interleukin-8 have been detected in serum and urine samples from RMS patients (3), demonstrating an important role of tumor angiogenesis in the disease malignancy. Tumor angiogenesis has already been identified as a therapeutic target for cancer treatment (4). However, tumor neovascularization is typically disorganized compared with its healthy counterpart, characterized by a defective endothelial monolayer with abnormal sprouts and large intercellular openings (5). Tumor neovascularization is thought to impair drug delivery to the tumor core and induce radioresistance due to a hypoxic microenvironment, thereby hampering the effectiveness of chemotherapy and radiotherapy.

Disruption of tumor blood vessel formation is a complex process associated with many factors; however, the exact mechanism has not been fully understood. Recently, overexpression of VEGFs by tumor cells has been considered as one cause of tumor vessel abnormality (6). Targeted therapy against VEGF expression has been performed in an attempt to normalize tumor vasculature prior to subsequent chemotherapy or radiotherapy (7). Other studies reported decreased endothelial cell networks along with increasing extracellular matrix (ECM) stiffness (8,9). However, these studies failed to demonstrate a direct role for tumor cells in the disorganization of the tumor blood vessels. Three-dimensional (3D) co-culture of tumor cells and endothelial cells has typically been conducted in a spheroid system, with limited success. Timmins et al. (10) observed that a 3D branched endothelial network formed when single endothelial cells were seeded onto HCT116 spheroids. However, quantitative evaluation of endothelial network formation is difficult in a spheroid aggregate.

Intact cell sheets can be harvested on temperature-responsive poly-*N*-isopropylacrylamide (PNIPAAm) grafted surfaces by lowering the temperature to less than 32°C (11,12). Cell sheets preserve both the cells and the naturally formed ECM, thus closely mimicking the true *in vivo* conditions. Using this 3D model, observation of cell behaviors can be divided into the X–Y plane and the Z-axis enabling temporal and spatial interpretation (13). In our previous study, we observed active and rapid migration of malignant embryonal rhabdomyosarcoma (ERMS) cells (RDs) in the human skeletal muscle myoblast (HSMM) sheet (14). Disruption of sheet structure was observed in the heterogeneous cell sheets when co-cultured with 10%–50% tumor cells. In the present study, a tumor-containing cell sheet was prepared by mixing a small population of RDs with HSMMs and co-cultured with green fluorescent protein expressing human umbilical vein endothelial cells (GFP-HUVECs). The effect of this small population of tumor cells on the behavior of endothelial cells and network formation was investigated.

MATERIALS AND METHODS

Cell preparation Human skeletal muscle myoblasts (HSMMs, Lot. No. 4F1619; Lonza Walkersville Inc., Walkersville, MD, USA), a human embryonal RMS cell line (RD, Cat. No. EC85111502, American Type Culture Collection, VA, USA) and green fluorescence expressing human umbilical vein endothelial cells (GFP-HUVECs, Lot. No.20100201001, Angio-Proteomie, MA, USA) were used in this study. Using

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previously reported procedures (14), HSMMs were subcultured on laminin-coated surfaces at 37°C in an atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich, MO, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA) and antibiotics (100 U/cm^3 penicillin G, 0.1 mg/cm^3 streptomycin, and 0.25 mg/cm^3 amphotericin B; Invitrogen, CA, USA). RDs were grown in the same medium as HSMMs without laminin coating. GFP-HUVECs were cultured in a commercially available medium (EGM-2; Lonza Walkersville Inc.). The medium depth was set to 2 mm throughout the experiments. All cells were harvested until 70%–80% confluency.

Incubation of five-layered tumor-containing cell sheet with GFP-HUVECs RDs and HSMMs harvested from subcultures were stained using a fluorescent reagent (CellTracker Orange, Invitrogen), according to the commercial instruction. A monolayer tumor-containing cell sheet was prepared by mixing 8% RDs with HSMMs at a final seeding density of 3.5×10^5 cells/cm^2 then seeded on the temperature-responsive 24-well Upcell plate (CellSeed, Tokyo). The seeded cells were incubated for 24 h at 37°C in a 5% CO_2 atmosphere to form a monolayer sheet. A five-layered tumor-containing cell sheet was constructed according to previously reported method and transferred to a 35-mm culture dish (ibidi GmbH, Planegg, Germany) with pre-cultured GFP-HUVECs (15). A five-layered HSMM sheet without RDs was used as a control sheet. GFP-HUVECs were seeded at a density of 5.0×10^3 cells/cm^2 in EGM-2 supplemented with 20% (v/v) FBS and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. A commercially available medium (SkGM-2; Lonza Walkersville Inc.) supplemented by 10 ng/ml bFGF (ReproCell Inc., NJ, USA) was adapted for co-incubation of the tumor-containing cell sheet with GFP-HUVECs. During the incubation period, the medium was renewed every 24 h. The morphology of GFP-HUVECs was observed using a fluorescence microscope (IN Cell Analyzer 2000, GE Healthcare, BUX, UK) with a 10× objective lens every 24 h. Time-lapse observation of the endothelial network formation was conducted by obtaining images every hour at several positions with a 10× objective lens using a confocal laser scanning microscope (FV10i; Olympus, Tokyo).

Evaluation of endothelial network formation inside the cell sheet Evaluation of the endothelial network formation was based on a previously described method (15). Briefly, the images were captured using a confocal laser scanning microscope (FV10i; Olympus) with a 10× objective lens at more than eight positions in each sample. Each image was 8-bit gray scale with a size of 256 × 256 pixels and covered an area of 1.27 × 1.27 mm. Image processing was performed using commercialized software (Image-Pro Plus, Media Cybernetics Inc., MD, USA). The total network length per image area (L; mm^{-1}), and the number of total tips of the network (N_T; tip/mm^2) were measured to calculate the extent of network (L/N_T; mm/tip).

Immunofluorescence staining of fibronectin in monolayer tumor-containing cell sheet Fibronectin structure was measured in the monolayer tumor-containing cell sheet. The monolayer tumor-containing cell sheet was cultured in SkGM-2 medium supplemented with 10 ng/ml bFGF for 96 h. The samples were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka) in PBS for 15 min at room temperature and rinsed twice with PBS. The samples were then incubated in PBS containing 0.5% polyoxyethylene octylphenyl ether (Wako Pure Chemical Industries) for 5 min and then blocked in 1% (w/v) bovine serum albumin (BSA; Wako Pure Chemical Industries) in PBS for 90 min at room temperature. The specimens were incubated with primary antibody against human fibronectin (F7387, Sigma–Aldrich) at 4°C overnight. The samples were rinsed in PBS for three times and reacted with secondary antibodies (Alexa Flour 594-conjugated anti-mouse IgG; Life Technologies) for 1 h at room temperature. Images were captured using a confocal laser scanning microscope (FV1000; Olympus) with 60× objective lens.

Evaluation of sheet fluidity in tumor-containing cell sheet Evaluation of sheet fluidity in the tumor-containing cell sheets and control sheets was based on a previously reported method (13,14). Briefly, a five-layered cell sheet with a basal layer (stained using CellTracker Green) and upper layers (stained using CellTracker Orange) was prepared for spatial distribution observation using a confocal laser scanning microscope (FV1000; Olympus) with a 60× objective lens at 9 random positions in each sample. Original 8-bit images (256 × 256 pixels) of both colors in each slice were converted into binary images after identifying intensity threshold values. The ratio of green pixels to the sum of green pixels in each slice was normalized to determine the distribution of green pixels by dividing into five layers. The normalized distribution of green pixels was assumed to be equivalent to the green cell distribution in the sheet, which was considered to be the frequency of green cells, f_G, in each layer. To quantify sheet fluidity, the diffusivity (D, μm^2/h) was applied to measure vertical distribution of target cells based on Fick's second law, $\frac{\partial f_G}{\partial t} = D\frac{\partial^2 f_G}{\partial h^2}$, in which f_G, t and h represent the green cell frequency, incubation time, and sheet thickness, respectively. The Crank-Nicolson finite difference method and least squares method were applied to calculate the diffusivity using customized software designed by LabVIEW (National Instruments, Austin, TX, USA).

Vertical distribution of GFP-HUVECs in five-layered tumor-containing cell sheet Five-layered tumor-containing cell sheets and control sheets were stained with a fluorescent reagent (CellTracker Orange, Invitrogen) and co-cultured with GFP-HUVECs at a seeding density of 2.0 x 10^3 cells/cm^2 for early individual cell migration (fixed at $t = 12$ h) and 5.0×10^3 cells/cm^2 for final localization of GFP-HUVECs with network formation at $t = 96$ h. Vertical distribution of GFP-HUVECs inside the tumor-containing cell sheets and control sheets was observed by a confocal laser scanning microscope (FV1000; Olympus) at 60× magnification. The frequency of green cells (f_G) in each layer was analyzed by using the same method described above.

Transwell experiment The effect of cytokines secreted from tumor cells on endothelial network formation was investigated by using the polyester membrane transwell-clear inserts with the 0.4 μm pore size (Cat. No. 3450, Corning, NY, USA). 2.6 ml of DMEM medium with 10% FBS was firstly added to the lower chamber. Then 1.5 ml RDs suspension containing the same number as RDs in five-layered tumor-containing cell sheet was added to the upper chamber of the transwell and incubated at 37°C in a 5 % CO_2 atmosphere for 24 h in advance. Five-layered HSMM sheets with or without 8% RDs were constructed as described above and transferred to a 6-well culture plate (Cat. No. 3450, Corning) on which GFP-HUVECs were seeded at a density of 5.0×10^3 cells/cm^2. The transwell inserts with RDs were transferred on the top of HSMM sheet and incubated in the SkGM-2+bFGF (10 ng/ml) medium with the total volume of 4.1 ml according to the commercial instruction. The same volume of medium was added for the co-culture of tumor-containing cell sheet or the control sheet with GFP-HUVECs. The medium was renewed every 24 h. All samples were fixed at $t = 96$ h for analyzing the final localization of GFP-HUVECs. Vertical distribution of GFP-HUVECs in cell sheets was observed by a confocal laser scanning microscope (FV1000; Olympus) at 60× magnification. The frequency of green cells (f_G) in each layer was analyzed by using the same method described above.

Statistical analysis Data presented in this study were obtained from three independent cultures and expressed as means with standard deviations (SDs). Student's t-test was used to determine the statistical significance of differences among data sets, and values of $P < 0.05$ were considered significant.

RESULTS

Endothelial network formation inside the tumor-containing cell sheet Tumor-containing cell sheets were prepared by mixing 8% RDs with HSMMs. The behavior of HUVECs in the five-layered tumor-containing cell sheet was observed for 96 h to estimate network formation according to the parameters L, N_T, and L/N_T. Endothelial network formation in a five-layered HSMM sheet without RDs was used as a control sheet. The initial density of HUVECs was $1.60 \pm 0.03 \times 10^4$ cells/cm^2 and $1.56 \pm 0.12 \times 10^4$ cells/cm^2 in the tumor-containing cell sheet and control sheet, respectively. As shown in Fig. 1A, GFP-HUVECs were observed to be single and round-shaped with early elongation in the tumor-containing cell sheet as well as in the control sheet at the beginning of the incubation period. Fig. 1B shows the quantitative analysis of L, N_T, and L/N_T. At $t = 24$ h, HUVECs were found to elongate and connect with each other, resulting in an increase in L and decrease in N_T. Less L/N_T was found in the tumor-containing cell sheet compared to the HSMM sheet. After 24 h, HUVECs elongated with overlapping each other to form a thick network, and L started to decrease correspondingly. At $t = 48$ h, the value of L/N_T in the tumor-containing cell sheet and HSMM sheet was 0.29 and 0.30 mm/tip, respectively. Degradation of the endothelial network in the tumor-containing cell sheet was observed, and the value of L/N_T decreased to 0.21 mm/tip at $t = 96$ h, while the value of L/N_T increased to 0.34 mm/tips in HSMM sheet. From time-lapse observations (Movie S1), endothelial network was formed by dynamic connections (white arrows) and disconnections (red arrows) among endothelial cells. From 24 h to 48 h, the frequency of connection and disconnection is balanced in both cases. From 48 h to 72 h, more red arrows against white arrows was observed in tumor-containing cell sheet, indicating the degradation of endothelial network. This trend is continuously observed from 72 h to 96 h, resulting in small and branched network.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2017.01.017.

Disordered fibronectin meshwork in monolayer tumor-containing cell sheet Fibronectin assembly was observed by immunofluorescence staining of fibronectin in the monolayer sheet at $t = 96$ h (Fig. 2). Initially, the fibronectin structure was