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The radiosensitivity of endothelial cells isolated from human breast cancer and normal tissue *in vitro*

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ABSTRACT

We developed a novel method for harvesting endothelial cells from blood vessels of freshly obtained cancer and adjacent normal tissue of human breast, and compared the response of the cancer-derived endothelial cells (CECs) and normal tissue-derived endothelial cells (NECs) to ionizing radiation. In brief, when tissues were embedded in Matrigel and cultured in endothelial cell culture medium (ECM) containing growth factors, endothelial cells grew out of the tissues. The endothelial cells were harvested and cultured as monolayer cells in plates coated with gelatin, and the cells of 2nd–5th passages were used for experiments. Both CECs and NECs expressed almost the same levels of surface markers CD31, CD105 and TEM-8 (tumor endothelial marker-8), which are known to be expressed in angiogenic endothelial cells, *i.e.*, mitotically active endothelial cells. Furthermore, both CECs and NECs were able to migrate into experimental wound in the monolayer culture, and also to form capillary-like tubes on Matrigel-coated plates. However, the radiation-induced suppressions of migration and capillary-like tube formations were greater for CECs than NECs from the same patients. In addition, *in vitro* clonogenic survival assays demonstrated that CECs were far more radiosensitive than NECs. In summary, we have developed a simple and efficient new method for isolating endothelial cells from cancer and normal tissue, and demonstrated for the first time that endothelial cells of human breast cancer are significantly more radiosensitive than their normal counterparts from the same patients.

Introduction

In human and animal tumors, nutrients including oxygen must be supplied through vascular networks in order for tumor cells to survive and proliferate (Folkman, 1995; Hida et al., 2004). Tumor blood vessels are formed by three processes: angiogenesis, growth of new blood vessels from pre-existing vessels; vasculogenesis, formation of blood vessels from endothelial progenitors and other stem-like cells found in the blood and bone marrow, and intussusception, the splitting of new vessels off existing vessels (Ahn and Brown, 2009; Folkman, 1995; Hida et al., 2004; Kim et al., 2010). In contrast to the vascular beds of normal tissues which are hierarchically organized microarchitectures formed of mature vessels, tumor vasculature is structurally tortuous, and the vessels are often formed from only a single layer of endothelial cells frequently separated by gaps and lack underlying basement membrane and smooth muscle layers. These immature tumor vessels are hyper-permeable leading to an increase in extravasation of plasma protein (Konerding et al., 2001; Song and Levitt, 1971). Compressive stresses generated by cancer cells and other stromal components can cause collapse of microvasculature in the tumor (Boucher and Jain, 1992; Padera et al., 2004). Consequently, the intratumor microenvironment is hypoxic, nutritionally deprived and acidic (Choi et al., 2004; Lunt et al., 2009; Park et al., 2000; Vaupel et al., 1987), which greatly affects the response of tumor cells to various treatments such as radiotherapy or certain chemotherapies. Because of the importance of the functional integrity of blood vessels for the survival, proliferation and radioresponse of tumor cells, the changes in vascular functions in human tumors after irradiation have been studied by a

Abbreviations: EC, endothelial cell; HUVEC, human umbilical-vein endothelial cell; CECs, cancer-derived endothelial cells; NECs, normal tissue-derived endothelial cells; HDMEC, human dermal microvascular endothelial cell; TEM, tumor endothelial marker; SMA, α-smooth muscle actin; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; FACS, fluorescence-activated cell sorting; ECM, endothelial cell medium; PBS, phosphate buffered saline.

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number of investigators. Since it is highly likely that the radiation-induced morphological and functional damage in blood vessels are caused by damage or death of endothelial cells, the effect of radiation on tumor endothelial cells has also attracted considerable interest in the radiation community (Folkman and Camphausen, 2001; Moeller et al., 2004). An interesting and potentially important observation is that radiation caused rapid apoptosis in endothelial cells by promoting ASMase-mediated generation of ceramide, a pro-apoptotic second messenger, and that radiosensitivity of endothelial cells, rather than radiosensitivity of tumor cells, determined the outcomes of radiotherapy (Fuks and Kolesnick, 2005; Garcia-Barros et al., 2003). This conclusion was, however, questioned by other investigators (Brown et al., 2003; Gerweck et al., 2006). In order to define the precise role of tumor endothelial cells in the response of tumors to radiotherapy, it is highly desirable to further delineate various radiobiological changes in tumor endothelial cells.

Despite the strong possibility that endothelial cells of tumors and normal tissues may be different in various aspects (Hida et al., 2004, 2008; St Croix et al., 2000), most of the previous studies on endothelial cells have been conducted with normal endothelial cells largely due to the lack of an appropriate technique for obtaining sufficiently pure populations of endothelial cells from tumor tissues. Previously, endothelial cells were obtained from tumors and normal tissues using tedious multi-step procedures. For example, tissues were mechanically disrupted and enzymatically digested, and the endothelial cells were isolated with immunomagnetic cell sorting methods (Hida et al., 2004, 2008; Manconi et al., 2000; Miebach et al., 2006; van Beijnum et al., 2008). In this report, a novel simple method is described for isolating large numbers of pure endothelial cells from human tumor and normal tissue, and the cells' radiobiological properties are characterized.

Materials and methods

Reagents

Matrigel was obtained from BD Biosciences (Bedford, MA). Antibodies against Alexa Fluor 647-conjugated CD31 and Alexa Fluor 488-conjugated CD105 were obtained from Biolegend (San Diego, CA). Antibodies against TEM-8 and α-smooth muscle actin (SMA) were obtained from Abcam (Cambridge, UK). Texas Red-conjugated goat anti-rabbit IgG and FITC-conjugated anti-goat IgG were obtained from Gibco Invitrogen Corporation (Paisley, UK).

Patients and specimens

All patients who were to undergo breast resection for breast cancer were considered eligible for the study. Written informed consent was obtained from all patients, using a form approved by the Institutional Review Board of the Inha University Hospital's Clinical Trial Center (IRB No. 2009–1983; Incheon, Korea). Tumor and adjacent normal tissue of breasts were removed from the gross specimens at the time of resection. Initial diagnosis, histological type and tumor percentages were determined from hematoxylin–eosin-stained slides by an experienced pathologist.

Isolation of endothelial cells from cancer and normal tissues

Our novel procedure for obtaining endothelial cells from cancer and normal tissues is illustrated in Fig. 1. The resected tissue specimens were immediately transferred to culture dishes containing ice-cold PBS (Gibco Invitrogen Corporation, Paisley, UK), and then minced into about 0.2 cm^3 pieces with a sterile surgical knife and forceps. After being transferred to a 60 mm culture dishes, the minced tissues were embedded in 50 μl of Matrigel containing 20 ng/ml of bFGF (Sigma-Aldrich Co., St. Louis, MO), and kept for 30 min at room temperature. The tissue explants embedded in the solidified Matrigel were incubated with 5 ml of ECM (Endothelial cell culture medium) (Sciencell Research Laboratories, San Diego, CA) supplemented with 5% fetal bovine serum, 1% antibiotics and 1% endothelial cell growth supplement (Sciencell Research Laboratories, San Diego, CA) in a humidified 5% CO_2 incubator at 37 °C. After incubation for 2 week, the Matrigel and endothelial cells, which grew out from the explants in to the surrounding Matrigel, were carefully removed from the explants with a sterile surgical knife and forceps, and transferred to a sterile 1.5 ml tube. The Matrigel was dissolved by maintaining the tubes in ice-water for 30 min, mixed with 200 μl of trypsin-EDTA (Gibco Invitrogen Corp., Paisley, UK) and agitated gently for 3 min. The endothelial cells were dispersed to single cells by repeated pipetting, collected by centrifugation at 1500 rpm for 3 min at 4 °C, and then resuspended in 500 μl of ECM. The cells were then transferred to a gelatin-coated 60 mm culture dish, covered with 4 ml of complete ECM, and cultured in a humidified 5% CO_2 incubator at 37 °C. Cells from passages 2–5 were used for experiments. As normal control endothelial cells, human dermal microvascular endothelial cell (HDMEC) obtained from Sciencell Research Laboratories (San Diego, CA) was used. Like NECs and CECs, HDMECs were cultured in complete ECM.

Flow cytometry

Single cells were detached from the culture dishes with trypsin-EDTA, counted, about 1×10^5 cells were centrifuged at 2000 rpm for 5 min and then resuspended in PBS containing 5% FBS and 0.1% NaN_3 (Sigma-Aldrich Co., St. Louis, MO). The cells were incubated at 4 °C with Alexa Fluor 647-conjugated anti-CD31, Alexa Fluor 488-conjugated anti-CD105, anti-SMA or anti-TEM-8 antibody for 1 h. The cells incubated with antibodies against SMA or TEM-8 were washed with PBS and incubated with Texas Red- or FITC-conjugated secondary antibody, (Gibco Invitrogen Corporation, Carlsbad, CA), respectively, for 1 h at 4 °C. Anti-rabbit IgG and anti-goat IgG were used as isotypic controls. The labeled cells were washed twice with PBS, resuspended in PBS, and then analyzed by flow cytometry (Becton Dickinson, Mountain View, CA).

Confocal microscopy

Cells were seeded on gelatin-coated coverslips, about 2×10^4 each, incubated for 16 h, washed twice with ice-cold PBS and fixed with ice-cold methanol. After blocking with 2% bovine serum albumin in PBS containing 0.2% Triton X-100, the cells were incubated for 1 h with Alexa Fluor 647-conjugated anti-CD31, Alexa Fluor 488-conjugated anti-CD105 or anti-TEM-8 antibody. The cells incubated with anti-TEM-8 antibody were washed three times with blocking solution and then incubated with FITC-conjugated secondary antibody for 1 h. All cells were washed twice with PBS and the cell nuclei were stained with DAPI (Molecular Probes, Eugene, OR) for 10 min. The coverslips were washed three times with PBS and mounted onto microscopic slides using the mounting reagent (Molecular Probes, Eugene, OR). The cells were analyzed by confocal laser-scanning microscopy (Nikon TE-2000E; Nikon, Tokyo, Japan).

Irradiation

Cells were exposed to γ-rays with a ^{137}Cs irradiation source (Model 68; J.L. Shepherd and Associates, San Fernando, CA) at a dose rate of 200–300 cGy/min.

Clonogenic survival assay

Appropriate numbers of endothelial cells were plated onto gelatin-coated 60 mm dishes with ECM, incubated overnight, irradiated with various doses of γ-ray, and then cultured for 14 days in a 5% CO_2 incubator at 37 °C. The formed colonies were fixed with 95% methanol and stained with 0.5% crystal violet, and the colonies containing more than 50 cells were counted. The averages of triplicate dishes were obtained for each sample, the results were normalized with respect to the plating