Glioblastoma cells block radiation-induced programmed cell death of endothelial cells

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Abstract We demonstrate that human umbilical vein endothelial cells (HUVEC) grown in co-culture (CC) with U87 glioblastoma cells transfected with green fluorescent protein (GFP-U87) exhibit resistance to radiation-mediated apoptosis. cDNA macroarray analysis reveals increases in the accumulation of RNAs for HUVEC genes encoding cell adhesion molecules, growth factor-related proteins, and cell cycle regulatory/DNA repair proteins. An increase in protein expression of integrin α_v, integrin β_1, MAPK(p42), Rad51, DNA-PK$_{CS}$, and ataxia telangiectasia gene (ATM) was detected in HUVEC grown in CC with GFP-U87 cells compared with HUVEC grown in mono-culture. Treatment with anti-VEGF antibody decreases the expression of integrin α_v, integrin β_1, DNA-PK$_{CS}$ and ATM with a corresponding increase in ionizing radiation (IR)-induced apoptosis. These data support the concept that endothelial cells growing in the tumor microenvironment may develop resistance to cytotoxic therapies due to the up-regulation by tumor cells of endothelial cells genes associated with survival.

Keywords: Co-culture; Endothelial cell survival; DNA repair and checkpoint gene; VEGF; DNA array

1. Introduction

Numerous studies have shown that the tumor vasculature is a potential target for therapy [1]. Although the precise role of angiogenesis inhibitors as a sole treatment for cancer is controversial, experimental evidence suggests that anti-angiogenic compounds enhance the anti-tumor effects of ionizing radiation (IR) [2–4]. Anti-tumor effects have also been reported when IR is combined with neutralizing antibodies to vascular endothelial growth factor (VEGF) [5,6], VEGF receptor 2 antibodies [7,8], blocking peptides [9,10], tyrosine kinase inhibitors [11], and dominant-negative mutant receptor constructs [12]. We investigated whether tumor cells influence the expression of endothelial cell genes by growing human umbilical vein endothelial cells (HUVEC) in co-culture (CC) with U87 glioblastoma tumor cells transfected with green fluorescent protein (GFP-U87). We report that HUVEC exhibit increased resistance to IR-mediated apoptosis following CC with GFP-U87 cells. Using cDNA macroarray analysis, we identified 8 HUVEC genes whose RNAs are accumulated >10-fold following CC with GFP-U87 cells. These genes encode integrin proteins and proteins involved in critical pathways of DNA repair, recombination and checkpoint control. Furthermore, our results indicate that VEGF secretion by GFP-U87 cells alters the expression of these endothelial cell genes. We propose that tumor cell up-regulation of endothelial cell genes associated with survival following IR exposure may mediate tumor-associated endothelial cell radioresistance and, therefore, contribute to the overall resistance of tumors to radiotherapy.

2. Materials and methods

2.1. Cell culture and transfection

Human umbilical vein endothelial cells were purchased from Clonetics (Walkerville, MD) and maintained in EGM-2 medium (Clonetics). EGM-2 medium contains EBM-2 medium (serum-free, growth factor-free), supplemented with 2% fetal bovine serum (FBS), human fibroblast growth factor-B (hFGF-B), human epidermal growth factor (hEGF), human vascular endothelial cell growth factor (hVEGF), long R insulin-like growth factor-1 (R^3-IGF-1), ascorbic acid, hydrocortisone and heparin. All HUVEC used in these experiments were passage 4 or less. GFP-U87 cells are stable transformants of U87MG glioblastoma cells (a gift from the UCSF Brain Tumor Tissue Bank, San Francisco, CA) transfected with GFP to aid in the discrimination of these cells as previously described [13]. Transfection was performed by standard methods using LIPOFECTIN® reagent (Invitrogen, Carlsbad, CA) and the pEGFP-C1 vector system (Clontech, Palo Alto, CA). GFP-U87 tumor cells were maintained in low glucose Dulbecco's Modified Eagle's Medium (Gibco-Invitrogen, Grand Island, NY) supplemented with 10% FBS (Serologicals Corp., Norcross, GA), 1% non-essential amino acids (Gibco-Invitrogen), and 400 μg/ml G418 (Sigma, St. Louis, MO). Monolayer cells were disaggregated into single cells and counted using a fluorescent microscope to discriminate GFP-fluorescent U87 tumor cells from the non-fluorescent HUVEC.

2.2. CC of HUVEC with GFP-U87 cells

CC was performed in serum-free, growth factor-free EBM-2 medium. Before CC was initiated, both cell types were washed three times with EBM-2 medium. Two CC conditions were employed in the current studies: (1) "physically combined" CC in which GFP-U87 cells were cultured in the same flasks with HUVEC cells and (2) "physically separated" CC employing TransWell® chambers (0.4 μm pores, Corning Costar, Cambridge, MA) with GFP-U87 cells loaded in the bottom wells and HUVEC loaded in the inserts. Our previous published data demonstrate that an "activated" HUVEC phenotype is achieved in both CC conditions [13].

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2.3. Cell culture irradiation

HUVEC cultures were grown in EGM-2 medium overnight and the next day washed three times with EBM-2 medium. GFP-U87 cells were disaggregated into a single cell suspension using trypsin:EDTA, washed three times with EBM-2 medium and added to HUVEC cultures growing in EBM-2 medium to establish "physically separated" CC employing TransWell® chambers. 3 h later, cultures were treated with a single IR dose of 300 cGy using a Pantak PMC 1000 X-ray generator operating at 250 kVp, 15 mA with a 0.5 mm copper filter. Following irradiation, cells were incubated for 24 h.

2.4. HUVEC purification

HUVEC grown in mono-culture (MC) or CC with GFP-U87 cells were disaggregated into single cell suspensions, washed two times with PBS containing 0.1% BSA, and isolated using superparamagnetic polystyrene beads coated with anti-CD31 monoclonal antibody (Dynabeads® CD31, Dynal Biotech, Oslo, Norway) according to the manufacturer's protocol. Efficacy of HUVEC separation from GFP-U87 cells in CC was determined by counting the number of fluorescent GFP-U87 cells and non-fluorescent HUVEC cells using a hemocytometer. In independent experiments efficacy ranged from 97% to 99%.

2.5. Apoptosis detection

Apoptosis was evaluated using "physically separated" CCs. HUVEC growing in EGM-2 medium were plated in TransWell® inserts on day 1 and 24 h later HUVEC or GFP-U87 cells in EBM-2 medium were plated in TransWell® chambers at the ratio of 1:10 insert:chamber. Three hours later, cultures were treated with a single IR dose of 300 cGy as described above. HUVEC apoptosis was scored 24 h after IR exposure (27 h after establishment of CC). Floating and attached HUVEC were harvested by centrifugation at 1500 rpm for 5 min. Membranes were cut from the inserts and mounted on glass slides. After applying Vectashield containing DAPI (Vector Labs, Burlingame, CA), coverslips were placed on membranes and slides were incubated for 30 min in the dark. HUVEC were photographed at 10× magnification and apoptotic bodies were evaluated in 10 fields from each of duplicate membranes.

2.6. cDNA macroarray

HUVEC were grown to 70–80% confluence in EGM-2 medium. GFP-U87 cells were added to HUVEC at a ratio of 1:20 to establish "physically combined" CCs. 48 h later, HUVEC were isolated from GFP-U87 cells using anti-CD31 coated superparamagnetic polystyrene beads as described above. RNA preparation, cDNA generation and hybridization of the Atlas™ cDNA expression arrays were performed according to the manufacturer's recommendations (Clontech, Palo Alto, CA) as previously described [14]. ImageQuant® image analysis software (Molecular Dynamics, Inc., Sunnyvale, CA) was used for data acquisition of intensities using "volume" and "local background" options. Microsoft Excel was employed for data manipulation. Background was determined from the average signal of 6 previously determined non-data-bearing areas on the array. This background value was subtracted from all signal intensities and the resulting values were then normalized by dividing by the average mean value of all signal intensities on each filter (normalization by global means) [15]. Signals for individual genes on CC arrays were compared to those on MC arrays to estimate "relative transcriptional ratio". Those ratios representing a 2-fold or greater signal change were considered to be "up-regulated" or "down-regulated" genes.

2.7. Western blot analysis

Total cell extracts were prepared by homogenizing purified HUVEC in cold RIPA buffer for 15 min (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100; 1 mM 2-mercaptoethanol, 1 mM Na_3VO_4; 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Samples were centrifuged at 14 000 rpm for 15 min at 4 °C. The supernatant was collected and protein concentration determined using BioRad Standard Protein Assay Reagent (Hercules, CA). Lysates (25 μg per well) were subjected to SDS–PAGE and transferred to Immobilon-P membranes (Millipore Corp. Burlington, MA). The membranes were immunoblotted with the following primary antibodies: anti-integrin α_v monoclonal antibody (SC-9969); anti-integrin β_1 polyclonal antibody (SC-6622); anti-MAPK(p42) monoclonal antibody (SC-1647); anti-Rad51 polyclonal antibody (SC-6862); and anti-DNA-PK_{CS} polyclonal antibody (SC-1552) (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-goat secondary antibodies and the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) system were used to visualize bands. Detection of β-actin as an internal control was performed on membranes using an anti-actin-HRP goat polyclonal antibody (SC-1616, Santa Cruz Biotechnology). Nuclear rather than total cell extracts were employed for Western blot analysis of ataxia telangiectasia gene (ATM) expression. Bead-isolated HUVEC were placed in lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) and incubated for 5 min on ice. The nuclear fraction was collected by centrifugation at 2000 × g for 5 min at 4 °C and then processed as above for total cell extracts. Immunoblot detection of ATM was performed using a goat polyclonal anti-ATM antibody (SC-7128, Santa Cruz Biotechnology, Santa Cruz, CA). Autoradiographs were scanned using a Canon CanoScan FB1200S scanner and quantified using Scion Image software.

2.8. Neutralizing antibody against VEGF

HUVEC were plated in EGM-2 medium on Transwell® inserts (Corning Costar) for 16 h. HUVEC and trypsinized GFP-U87 cells were washed two times with EBM-2 medium. GFP-U87 cells were plated in the wells at a 1:10 HUVEC:GFP-U87 ratio to establish "physically separated" CCs. VEGF inhibition was performed by adding 100 ng/ml anti-human VEGF-165 monoclonal antibody (R&D Systems, Inc., Minneapolis, MN) to the wells. Mouse IgG (Sigma, St. Louis, MO) was used as the negative control. Cells were trypsinized and viability of HUVEC was determined by trypan blue exclusion.

2.9. Neutralizing antibody against αVβ3 integrins (vitronectin receptor)

HUVEC were plated in EGM-2 medium on Transwell® inserts as described above. Mouse anti-human integrin αVβ3 (vitronectin receptor) monoclonal antibody (MAB1976, Chemicon International, Temecula, CA) at a concentration of 10 μg/ml was added to the wells. Mouse IgG (Sigma, St. Louis, MO) was used as the negative control. Cells were trypsinized and viability of HUVEC was determined by trypan blue exclusion.

3. Results

3.1. CC with GFP-U87 cells protects HUVEC from radiation-induced apoptosis

IR-induced apoptosis of HUVEC grown in "physically separated" CC with GFP-U87 cells was compared with IR-induced apoptosis of HUVEC grown in MC. We measured HUVEC apoptosis at time 0 just prior to loading GFP-U87 cells in the bottom wells of TransWell® chambers. Baseline HUVEC apoptosis in EGM-2 growth medium was $2.0 \pm 1.32\%$ and is represented as the control (CTL) in Fig. 1. By 24 h of CC, there was no difference in apoptosis between non-irradiated MC ($8.41 \pm 0.99\%$) and non-irradiated CC ($7.60 \pm 2.52\%$). However, following exposure to 300 cGy, a significant increase in apoptosis was detected in HUVEC grown in MC (MC + IR, $36.27 \pm 12.36\%$) compared with HUVEC grown in CC with GFP-U87 cells (CC + IR, $8.72 \pm 2.38\%$, $P = 0.0012$, t test). These data demonstrate that CC with GFP-U87 cells protects HUVEC from radiation-induced apoptosis and illustrate that the tumor cells and endothelial cells do not have to be in physical contact to establish the protective effect.

3.2. Differences in transcriptional profiles of HUVEC grown in MC versus CC

DNA array analysis was employed to investigate if HUVEC resistance to IR-mediated apoptosis was associated with differential gene expression and/or RNA accumulation. Total RNA was isolated from HUVEC and ratios of the hybrid-

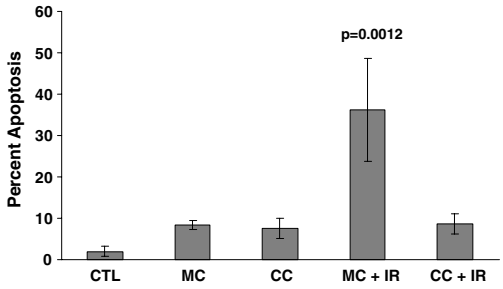

Fig. 1. CC with GFP-U87 cells protects HUVEC from radiation-induced apoptosis. The effects of IR on apoptosis were evaluated in HUVEC grown in MC compared with HUVEC grown in CC with GFP-U87 cells. Attached and floating HUVEC were centrifuged, and membranes were stained with DAPI. Apoptotic bodies from 10 fields from duplicated membranes were counted. Following 300 cGy, a significant increase ($P = 0.0012$) in HUVEC apoptosis was detected in MC ($36.27 \pm 12.36\%$) compared with CC ($8.72 \pm 2.38\%$).

ization signal intensity (CC signal relative to MC signal) were calculated. CC of HUVEC with GFP-U87 cells was associated with an increase in HUVEC mRNA of 82 genes. These genes were then placed into functional groups. Genes encoding proteins that regulate cell cycle, growth factor related proteins and cell adhesion molecules represented the most common functional groups that were up-regulated by CC. Eight selected genes with RNA expression ratios greater than 10-fold are presented in Table 1. These genes encode integrins α_v, α_6, and β_1, cell cycle regulatory/DNA repair proteins cyclin A, ATM, the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{CS}), Rad51, and MAPK(p42). These findings demonstrate that tumor cells modify the transcriptional patterns of endothelial cells, including genes which participate in the response to the genotoxic stress.

3.3. Increases in HUVEC gene expression induced by GFP-U87 cells result in an increase in protein production

Western blot analysis was performed on HUVEC extracts to determine if the relative RNA expression ratios detected by cDNA array analysis reflect changes in protein expression. The relative protein levels of the 8 genes with RNA expression ratios greater than 10-fold were determined. The protein levels of all 8 genes including integrin α_v, integrin β_1, MAPK(p42), Rad51, DNA-PK_{CS}, MAPK(p42) and ATM were elevated in HUVEC grown in CC with GFP-U87 cells compared with HUVEC grown in MC (MC, Fig. 2). Specifically, a 2-fold increase in α_v and β_1 integrin was detected following CC (1.97-fold and 2.11-fold, respectively). Protein levels of MAPK(p42) and Rad51 were also increased (1.40-fold and 1.67-fold, respectively) with ATM (2.51-fold) and DNA-PK_{CS} (3.38-fold) exhibiting the greatest increase.

Table 1
HUVEC genes exhibiting a 10-fold increase in transcription following CC with GFP-U87 cells

Gene	Ratio
Integrin α_v; vitronectin receptor α subunit	22.1
Cyclin A	17.6
ATM gene	17.3
DNA-PK_{CS}	15.1
Integrin α_6 subunit	11.8
Integrin β_1; fibronectin receptor β subunit	11.2
DNA repair protein RAD51	10.9
MAPK(p42)	10.2

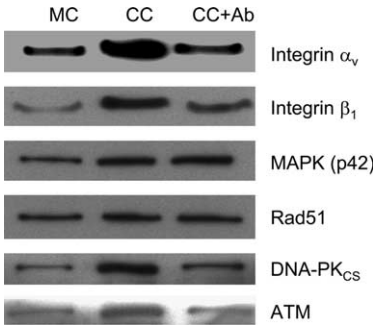

Fig. 2. Representative western blot of selected up-regulated HUVEC gene products and the effects of treatment with anti-VEGF antibody. Relative ratios of gene products of integrin α_v, integrin β_1, MAPK(p42), Rad51, DNA-PK_{CS}, and ATM from cell extracts of HUVEC grown in MC and CC are shown. The elevation in protein levels coincides with the increases in gene expression detected by DNA array analyses. Treatment with anti-VEGF antibody in CC (CC + Ab) decreases the expression of integrin α_v, integrin β_1, DNA-PK_{CS}, and ATM, but not Rad51 or MAPK(p42).

3.4. Growth stimulation and up-regulation of gene expression in HUVEC is due in part to VEGF production by GFP-U87 cells

We have previously reported that conditioned medium from U87 glioblastoma cells contains 12.7 ng of VEGF. [5] In our current CC model, we detected 20 ng/ml VEGF in GFP-U87 conditioned medium to which HUVEC are exposed. To evaluate the effects of VEGF neutralization, we treated HUVEC MCs and CCs with anti-VEGF neutralizing antibody (Ab). We previously reported that treatment with anti-VEGF antibody potentiates IR-mediated HUVEC cytotoxicity. [5] A significant reduction in HUVEC cell number was observed in CC ($CC = 24.45 \pm 4.4 \times 10^3$, $CC + Ab = 5.63 \pm 0.53 \times 10^3$, $P < 0.001$) but not in MC ($MC = 12.75 \pm 0.79 \times 10^3$, $MC + Ab = 15.53 \pm 0.48 \times 10^3$, Fig. 3A) following treatment with anti-VEGF antibody. These findings indicate that VEGF released by GFP-U87 cells promotes the growth of HUVEC in CC.

We also evaluated the effects of VEGF neutralization on HUVEC protein expression. Using Western blot analysis, we determined that treatment with anti-VEGF antibody decreased the expression of integrin α_v ($CC = 1.97$, $CC + Ab = 1.05$), integrin β_1 ($CC = 2.11$, $CC + Ab = 1.46$), DNA-PK_{CS} ($CC = 3.38$, $CC + Ab = 1.59$), and ATM ($CC = 2.51$, $CC + Ab = 1.24$), but not Rad51 ($CC = 1.67$, $CC + Ab = 1.64$) or MAPK(p42) ($CC = 1.4$, $CC + Ab = 1.32$, Fig. 2). Taken together, these findings suggest that HUVEC growth stimulation and up-regulation of HUVEC proteins, including integrin α_v, integrin β_1, DNA-PK_{CS}, and ATM, are mediated, in part, by GFP-U87-derived VEGF.

3.5. Genes other than VEGF participate in the survival of HUVEC grown in CC

To validate the functional significance of our data, we employed neutralizing antibodies to the vitronectin receptor (the $\alpha V\beta 3$ integrin complex). We selected anti-integrin antibody because the HUVEC gene profiling data (Table 1) demonstrated that the vitronectin receptor α subunit exhibited the greatest up-regulation (22.1 expression ratio) following CC with GFP-U87 cells. HUVEC MCs and HUVEC + GFP-U87 CCs were

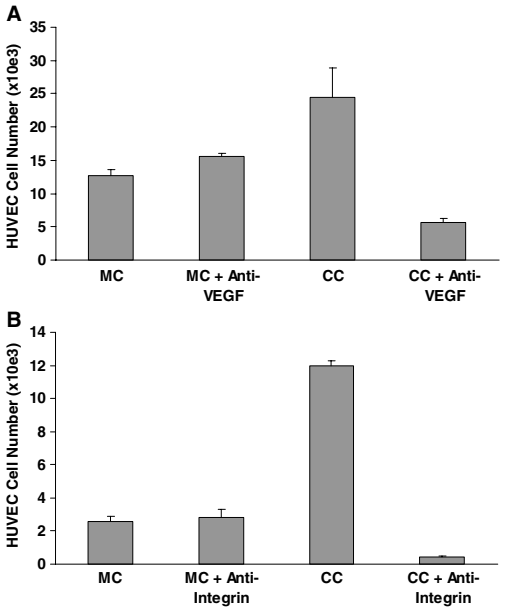

Fig. 3. Effect of neutralizing antibodies on HUVEC growth. HUVEC were grown in MC or in CC with GFP-U87 cells. Cultures were treated with anti-human VEGF-165 (A) or anti-human integrin αVβ3 (vitronectin receptor) (B) monoclonal antibody. Viability was determined by trypan blue exclusion. Neither anti-VEGF nor anti-integrin inhibited the growth of HUVEC MCs (MC versus MC + Ab). A significant decrease ($P < 0.001$) in HUVEC cell number was observed in CCs treated with neutralizing antibodies to VEGF and αVβ3 integrin (CC versus CC + Ab).

exposed to anti-integrin antibody and HUVEC viability was evaluated 48 h later. A significant reduction in HUVEC cell number was observed in CC ($CC = 12.0 \pm 0.3 \times 10^3$, $CC + Ab = 0.40 \pm 0.10 \times 10^3$, $P < 0.001$) but not in MC ($MC = 2.6 \pm 0.30 \times 10^3$, $MC + Ab = 2.8 \pm 0.5 \times 10^3$, Fig. 3B) following treatment with anti-integrin antibody. These data demonstrate that up-regulation of αV integrin gene expression by HUVEC following CC with GFP-U87 cells stimulates the growth and survival of HUVEC.

4. Discussion

In the present studies we report that CC of HUVEC with GFP-U87 tumor cells protects HUVEC from IR-mediated apoptosis. These findings support our previous observations that tumor cells influence the phenotype of endothelial cells [13]. Employing cDNA macroarray analysis, we investigated the transcriptional profiles of HUVEC grown in CC and found a >10-fold increase in the accumulation of RNAs for genes encoding cell adhesion molecules and proteins that regulate the cell cycle and DNA repair. Importantly, we detected an increase in the expression of ATM, Rad51, DNA-PK_{CS} and cyclin A which may be associated with the decrease in radiation-induced apoptosis of HUVEC grown in CC. One limitation of our current studies is that kinases, such as ATM and DNA-PK_{CS}, are regulated by post-translational modifications. Our findings do not confirm the production of functional proteins but do suggest that tumor cells can alter endothelial cell gene transcription thereby promoting survival and radioresistance.

The current studies raise questions regarding how genes with differential basal expression respond to IR treatment. To address these questions we performed expressional profiling of HUVEC MCs and CCs using GeneFilter® arrays (Research Genetics, Invitrogen, Life Technologies, Carlsbad, CA). We found that the expression of 4 genes in Table 1 (ATM, MAPK(p42), Rad51 and integrin β_1) was up-regulated in CC during the first 24 h following exposure to 3 Gy (data not shown). Complete analysis of these data is underway. In separate studies mock-transfected U87 cells (U87-Lux8) treated with 3 Gy demonstrated up-regulation of several genes associated with positive regulation of angiogenesis, including TGF-α, neuregulin (heregulin-β3) and VEGF. These findings may also explain the increase in endothelial survival observed in irradiated CCs comparing with irradiated MCs [16].

Tumor cells develop resistance to anti-cancer therapy as a result of the mutations that generate resistant phenotypes from an inherently unstable genome. The notion that the vascular components of tumors are attractive targets for anti-cancer therapy stems from the belief that endothelial cells are derived from 'normal' host cells and, therefore, are less likely to develop resistance to anti-cancer regimens. However, our data challenge this conventional view in that while tumor cells develop resistance to therapy, the interaction between tumor cells and endothelial cells may also render 'normal cells' resistant to anti-neoplastic agents. We further demonstrate using cDNA array analysis that interactions between tumor cells and endothelial cells lead to the activation of endothelial cell genes whose products mediate cell growth and decreased susceptibility to IR-induced apoptosis. Additionally, our findings indicate that VEGF secretion by tumor cells augments the expression of these endothelial cell genes. We hypothesize that the paracrine up-regulation of genes, which mediate survival following IR exposure, may alter the radioresistance of tumor-associated endothelial cells and, therefore, may contribute to the overall resistance of tumors to conventional treatments.

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