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Tumor Endothelial Cells Acquire Drug Resistance by MDR1 Up-Regulation via VEGF Signaling in Tumor Microenvironment

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Tumor endothelial cells (TECs) are therapeutic targets in anti-angiogenic therapy. Contrary to the traditional assumption, TECs can be genetically abnormal and might also acquire drug resistance. In this study, mouse TECs and normal ECs were isolated to investigate the drug resistance of TECs and the mechanism by which it is acquired. TECs were more resistant to paclitaxel with the up-regulation of multidrug resistance (MDR) 1 mRNA, which encodes the P-glycoprotein, compared with normal ECs. Normal human microvascular ECs were cultured in tumor-conditioned medium (CM) and became more resistant to paclitaxel through MDR1 mRNA up-regulation and nuclear translocation of Y-box-binding protein 1, which is an MDR1 transcription factor. Vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) and Akt were activated in human microvascular ECs by tumor CM. We observed that tumor CM contained a significantly high level of VEGF. A VEGFR kinase inhibitor, Ki8751, and a phosphatidylinositol 3-kinase-Akt inhibitor, LY294002, blocked tumor CM-induced MDR1 up-regulation. MDR1 up-regulation, via the VEGF-VEGFR pathway in the tumor microenvironment, is one of the mechanisms of drug resistance acquired by TECs. We observed that VEGF secreted from tumors up-regulated MDR1 through the activation of VEGFR2 and Akt. This process is a novel mechanism of the acquisition of drug resistance by TECs in the tumor microenvironment. (*Am J Pathol* 2012, 180: 1283–1293; DOI: 10.1016/j.ajpath.2011.11.029)

Tumor angiogenesis is necessary for solid tumor progression and metastasis.¹ Tumor blood vessels promote tumor progression by providing nutrition and oxygen² and also function as metastasis gatekeepers for tumor cells.² In addition, tumor endothelial cells (TECs) are genetically stable and do not acquire drug resistance.^{3,4} Therefore, inhibition of tumor angiogenesis is an attractive strategy for cancer treatment.

Low-dose anti-angiogenic or metronomic chemotherapy has been developed to target tumor angiogenesis.^{5,6} Adjuvant chemotherapy with uracil-tegafur, which is considered a form of metronomic chemotherapy, improves survival among patients with completely resected pathological stage I lung adenocarcinoma.⁷ Furthermore, low-dose metronomic paclitaxel has been proposed for use in breast cancer treatment.⁸ The theoretical advantage of targeting ECs instead of tumor cells is that, unlike tumor cells, ECs are genetically stable and do not develop drug resistance.^{3,4}

However, such therapeutic treatments lose their effect over time, possibly because of drug resistance caused by the compensatory response of tumor cells. Long-term suppression of the expression of one angiogenic protein can lead to the emergence of the expression of other angiogenic proteins. Another reason might be the acquisition of drug resistance by ECs. Several studies have reported that tumor blood vessels differ from their normal counterparts with respect to changes in morphological characteristics,⁹ such as altered blood flow, enhanced leakiness,¹⁰ structural abnormalities in the basement membrane, and abnormal pericytes.¹¹ Recent studies^{12–14} have reported differences between TECs and

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normal ECs (NECs) at the molecular level. We have reported that TECs have several abnormalities,^{15,16} such as differences in responsiveness to growth factors [eg, epidermal growth factor (EGF),¹⁷ adrenomedullin,¹⁸ and vascular endothelial growth factor (VEGF)]¹⁹ and to inhibitors (eg, epidermal growth factor receptor¹⁷ and epigallocatechin-3-gallate),²⁰ compared with NECs. In addition, TECs have cytogenetic abnormalities, such as aneuploidy or abnormal centrosomes in mouse tumors¹⁵ and human renal carcinomas.²¹ Other studies²² have also reported cytogenetically abnormal TECs in malignant tumors, such as lymphoma. Because aneuploidy in TECs suggests the presence of genetic instability, the question arises whether TECs are drug resistant.

Some TECs are resistant to certain drugs.^{23–25} Renal carcinoma ECs are resistant to vincristine,²³ whereas hepatocellular carcinoma ECs are resistant to 5-fluorouracil and doxorubicin (Adriamycin).²⁵ However, the mechanism of drug resistance in TECs remains unknown. In the present study, we demonstrated that TECs are drug resistant and investigated the mechanism of acquisition of drug resistance. We treated human microvascular ECs (HMVECs), a type of NECs, with tumor-conditioned medium (CM) and analyzed changes in drug sensitivity and gene expression related to drug resistance.

Materials and Methods

Cell Line and Culture Conditions

HMVECs were obtained from Lonza (Tokyo, Japan) and cultured in endothelial cell growth medium for microvascular cells (EGM-2MV) (Lonza, Basel, Switzerland). A375SM cells (supermetastatic human melanoma cells) were kindly provided by Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center, Houston, TX) and were cultured as previously described.²⁶

Antibodies

The following antibodies were used: rat anti-mouse CD31 (eBioscience, San Diego, CA), fluorescein isothiocyanate (FITC)–*Bandeiraea simplicifolia* lectin isolectin B4 (BS1-B4; Vector Laboratories, Burlingame, CA), FITC–anti-mouse CD31, anti-mouse CD105, anti-mouse CD144 (BD Pharmingen, San Diego), APC–anti-mouse CD11b, APC–anti-mouse CD45 (Biolegend, San Diego), Alexa Fluor 488 goat anti-rat IgG, Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Tokyo), anti-Y-box-binding protein 1 (YB-1), anti-phosphorylated Akt (Ser473), anti-pan-Akt (11E7), anti-VEGF receptor 2 (VEGFR2; 55B11), anti-phosphorylated VEGFR2 (Tyr 951; 15D2) (Cell Signaling Technology, Danvers, MA), MFP488 phalloidin (F-actin) (MoBiTec, Göttingen, Germany), anti-P-glycoprotein (P-gp; C219) (Abcam, Cambridge, UK), monoclonal anti- β -actin (AC-15; Sigma-Aldrich, St Louis, MO), anti-heterogeneous nuclear ribonucleoproteins (hnRNP) A1 (E-17; Santa Cruz Biotechnology, Santa Cruz, CA), and anti- β -tubulin (Millipore, Billerica, MA).

Human Tissue Samples for Immunohistochemistry

Tissues from two cases of renal tumor clinically diagnosed as renal cell carcinoma were surgically resected. All protocols were approved by the Institutional Ethics Committee, and written informed consent was obtained from each patient before surgery. Samples were surgically excised from the tumor tissues and from the corresponding normal renal tissues 5 to 10 cm from the tumor when possible. The samples were immediately snap frozen in liquid nitrogen and stored at -80°C for immunohistological analysis. The final diagnosis of renal cell carcinoma was confirmed by pathological examination of formalin-fixed surgical specimens.

Isolation of TECs and NECs

TECs were isolated from melanoma xenografted in nude mice, and NECs were isolated from mouse dermis, as previously described.^{15,20,21,27} All animal experimentation procedures were approved by the local animal research authorities, and animal care was in accordance with the institutional guidelines. TECs and NECs were isolated using a magnetic activated cell sorting system (Miltenyi Biotec, Tokyo) with FITC–anti-CD31. CD31-positive cells were sorted and plated on 1.5% gelatin-coated culture plates and grown in EGM-2MV medium containing 15% fetal bovine serum (FBS). Diphtheria toxin (DT; 500 ng/mL; Calbiochem, San Diego) was added to the TEC subcultures to kill any remaining human tumor cells and to the NEC subcultures for technical consistency.¹⁵ Human cells express a heparin-binding EGF-like growth factor (HB-EGF), which is a DT receptor.²⁸ However, murine ECs survive DT treatment because DT does not interact with mouse HB-EGF. Isolated ECs were purified with a second purification round using FITC–BS1-B4.^{15,20,27} All experiments in this study were performed at three different times. Three experiments were performed to verify data reproducibility.

Flow Cytometry

TECs and NECs were incubated with FITC–BS1-B4, APC–CD11b, APC–CD45, primary antibodies against CD31, CD105, and CD144, and Alexa Fluor 488 goat anti-rat IgG, as previously described,^{19,20} and analyzed by FACSAria II (Becton Dickinson, San Jose, CA).

Tube Formation Assay

Tube formation of ECs on Matrigel (BD Biosciences, San Jose, CA) was observed with an inverted microscope (CKX41; Olympus, Tokyo), as previously described.²⁹

Survival Assay

Cells were plated in triplicate at a density of 2×10^3 cells for the proliferation assay and 5×10^3 cells for the survival assay in a 96-well plate containing EGM-2MV and

were washed after 6 hours. The cells were incubated in EGM-2MV for the proliferation assay and were treated with paclitaxel (Taxol; Enzo Life Sciences, Plymouth Meeting, PA) for 72 hours in EGM2-MV or each CM containing paclitaxel at the indicated concentrations for the survival assay. Cell viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay (Promega, Tokyo).

Cell Fractionation

Cell fractionation was performed by separating cells into cytoplasmic and nuclear fractions, as previously described.²⁹ The cells were harvested and resuspended in a fractionation buffer [10 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.5% Nonidet P-40 (Sigma-Aldrich), and a protease inhibitor cocktail], followed by vigorous shaking for 5 minutes and centrifugation at $13,200 \times g$ for 30 seconds. The supernatant was used as the cytoplasmic fraction. The cell fractionation accuracy was confirmed by immunoblotting using cytoplasmic protein, β -tubulin, and nuclear protein, hnRNP.

RT-PCR and Quantitative Real-Time RT-PCR

Total RNA was extracted using the RNeasy Micro kit (Qiagen, Valencia, CA), and cDNA was synthesized using ReverTra-Plus (Toyobo, Osaka, Japan), as previously described.²⁹ cDNA was amplified by PCR. PCR products were visualized by ethidium bromide staining and UV transillumination. Real-time RT-PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). Cycling conditions followed the manufacturer's instructions based on the use of CFX Manager (Bio-Rad). The relative expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase. The following primers were used: mouse glyceraldehyde-3-phosphate dehydrogenase, 5'-TCTGACGTGCCGCTGGAG-3' (forward) and 5'-TCGAGGAGACAACCTGGTC-3' (reverse); mouse multidrug resistance (MDR) 1, 5'-ATCCGGGAGCAGAAGTTTGA-3' (forward) and 5'-GCACCAAAGACAACAGCAGA-3' (reverse); VEGFR1, 5'-GAGGAGGATGAGGGTGTCTATAGGT-3' (forward) and 5'-GTGATCAGCTCCAGGTTGACTT-3' (reverse); VEGFR2, 5'-GCCCTGCTGTGGTCTCACTAC-3' (forward) and 5'-CAAAGCATTGCCCATTCGAT-3' (reverse); CD11b, 5'-CCAAGAAAGTAGCAAGGAGTGTG-3' (forward) and 5'-AGGGTCTAAGCCAGGTCATAAG-3' (reverse); CD45, 5'-CCTCAAACCTCGACGGAGAG-3' (forward) and 5'-CACTTGACCATCAGACACC-3' (reverse); human HB-EGF, 5'-CGGCCGGGACCGGAAA-3' (forward) and 5'-CCTGTTTGGTGTGG-3' (reverse); human glyceraldehyde-3-phosphate dehydrogenase, 5'-ACAGTCAGCCGCATCTTCTT-3' (forward) and 5'-GCCCAATACGACCAAATCC-3' (reverse); and human MDR1, 5'-GTGGGGCAAGTCAGTTCATT-3' (forward) and 5'-TCTTCACCTCCAGGCTCAGT-3' (reverse).

Western Blot Analysis

Cells were lysed as previously described.^{20,29} Total protein was measured using a BCA Protein Assay kit (Pierce, Rockford, IL). Western blot analysis was performed using antibodies specific to P-gp, YB-1, Akt, phosphorylated Akt, VEGFR2, phosphorylated VEGFR2, β -actin, and horseradish peroxidase-conjugated secondary antibody, as previously described.²⁰ The cell fractionation accuracy was confirmed by immunoblotting using the cytoplasmic protein, β -tubulin, and the nuclear protein, hnRNP.²⁹ P-gp and YB-1 levels were normalized to β -actin levels, and phosphorylated Akt was normalized to total Akt by scanning densitometry using ImageJ software version 1.43u (NIH, Bethesda, MD).

Preparation of Tumor CM

A375SM cells were grown to 70% to 80% confluency in minimal essential medium (MEM) with 10% FBS (10% MEM; culture medium for tumor cells). The culture medium was replaced with fresh medium, and the cells were incubated for 18 to 20 hours. CM was collected from these cells and passed through a 0.22- μ m filter (Millipore). HMVECs were exposed to fresh CM, and the medium was changed after 2 days of the 5-day incubation period. HMVECs were also incubated for 18 to 20 hours in 10% MEM. HMVEC CM was collected in the same way as A375SM CM and was used as the control. HMVECs treated with control and tumor CM are indicated as HMVECs with control CM and HMVECs with tumor CM, respectively, in figures. CM containing 10% FBS was replaced with CM without FBS to analyze cell apoptosis under serum starvation conditions.

Cell Proliferation Assay

After HMVECs were cultured in HMVEC CM and tumor CM for 5 days, 2×10^4 cells per well were seeded in a 24-well plate to analyze cell proliferation. The number of cells was counted after 48 hours. The experiment was repeated three times. Data are presented as the average of three well counts under the respective conditions.

Flow Cytometry Analysis of Apoptotic-Necrotic Cell Death

HMVECs were cultured under their respective conditions for 5 days, after which 1.5×10^5 cells per well were seeded in a six-well plate to analyze apoptotic-necrotic cell death. HMVECs were cultured in CM without FBS for 24 hours for the starvation assay. In addition, HMVECs were treated with paclitaxel and verapamil (Taiyo, Osaka) for 48 hours to test for drug resistance. The cells were stained with FITC-annexin V and propidium iodide using the annexin V-FLUOS staining kit (Roche, Basel, Switzerland) and were analyzed by FACSaria II.

Immunocytochemistry

The cells were cultured on coverslips under their respective conditions in a six-well plate, fixed in 4% paraformaldehyde for 10 minutes, washed with PBS for 5 minutes, and then permeabilized with Triton X-100 (Sigma-Aldrich). The cells were incubated with anti-YB-1 overnight at 4°C, followed by incubation with secondary antibody and phalloidin (MoBiTec) at room temperature for 2 hours. Nuclei were stained with DAPI (Roche Diagnostics, Penzberg, Germany). Stained samples were visualized using a confocal microscope (FluoView FV1000; Olympus). The nuclear intensity ratio of YB-1 immunoreactivity in cells was quantified with ImageJ software. Quantitative analysis was performed using 30 separate images. Human samples were embedded in a cryocompound (Tissue-Tek; Miles, Elkhart, IN) and immediately immersed in liquid nitrogen. Frozen sections were double stained using Alexa Fluor 647–anti-CD31 and anti-P-gp and Alexa Fluor 594 goat anti-mouse IgG antibodies to reveal colocalization of CD31 and P-gp in ECs. All immunohistochemical samples were counterstained with DAPI.

Heat Inactivation of Tumor CM

Each CM was heated to 95°C for 60 minutes and then cooled to room temperature. HMVECs were incubated with control, tumor, or heat-treated CM for 1 day. RNA was then extracted, and the relative MDR1 mRNA expression levels under the respective conditions were analyzed by real-time RT-PCR.

Determination of VEGF Using ELISA

An enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was used to determine VEGF protein release, according to the manufacturer's instructions. CM was normalized by cell number per well.

Kinase Inhibitor Assay

HMVECs were pretreated with the indicated phosphatidylinositol 3-kinase (PI3K)–Akt kinase inhibitor, LY294002, or the VEGFR kinase inhibitor, Ki8751 (Sigma-Aldrich), for 2 hours. HMVECs were then treated with LY294002 or Ki8751 in each CM. HMVECs were treated

Figure 1. Characterization of NECs and TECs isolated from mice. **A:** Representative flow cytometric analysis of ECs showing white expression area of the endothelial markers CD31, CD105, CD144, and BS1-B4 lectin (mouse endothelial marker). The isotype control is shown in the black area. BS1-B4 lectin binding and CD31, CD105, and CD144 expression revealed the high purity of the isolated NECs and TECs by FACS. NECs and TECs were negative for non-EC markers, such as CD11b and CD45. **B:** All ECs were negative for the monocyte marker CD11b and the hematopoietic marker CD45, unlike non-ECs (CD31-negative tumor stromal cells). Human HB-EGF, which is expressed by human tumor cells, was not expressed in TECs, indicating that TECs were not contaminated with human tumor cells. mGAPDH, mouse glyceraldehyde-3-phosphate dehydrogenase. **C:** The mRNA expression levels of VEGFR1 and VEGFR2 were higher in TECs than in NECs, as analyzed by real-time PCR. **D:** Isolated and cultured ECs formed tubes on Matrigel-coated plates. Scale bar = 50 μ m. **E:** Proliferation of isolated and cultured ECs was determined by the MTS assay. TECs proliferated faster than NECs in 72 hours. ** $P < 0.01$.

for 1 day to analyze the effect of LY294002 or Ki8751 on MDR1 mRNA expression and for 30 minutes to reveal VEGF signaling.

Statistical Analysis

Differences between groups were evaluated using the Student's *t*-test. $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant.

Results

Isolation and Characterization of TECs and NECs

Mouse TECs were isolated from human tumor xenografts grown s.c. in nude mice, and NECs were isolated from normal dermal tissue. TECs and NECs were characterized as previously described.^{15,20,27} TECs and NECs were observed to be positive for EC markers (CD31, CD105, CD144, and BS1-B4) and negative for the monocyte marker CD11b and the hematopoietic marker CD45 by fluorescence-activated cell sorter (FACS) analysis (Figure 1A). All ECs were observed to be negative for CD11b and CD45 by RT-PCR, indicating that they were highly purified ECs. We have demonstrated that TECs were purified without any contamination of tumor cells by using DT, which binds to human tumor cells expressing HB-EGF (a DT receptor²⁸) but not to mouse cells. By using this method, we obtained TECs that were not contaminated with any human tumor cells (data not shown). Human HB-EGF, which is expressed by human tumor cells, was negative in TECs, indicating that they were not contaminated with human tumor cells (Figure 1B). VEGFR1 and VEGFR2 expression was analyzed by real-time PCR. TECs expressed higher levels of VEGFR1 and VEGFR2 compared with NECs (Figure 1C). Cultured ECs formed tubes on Matrigel-coated plates (Figure 1D). Furthermore, TECs proliferated faster than NECs ($P < 0.01$, Figure 1E). High levels of VEGFRs might relate to high proliferation activity in TECs.

TECs Are Resistant to Anticancer Drugs, MDR1/P-gp Is Up-Regulated, and YB-1 Accumulates in TEC Nuclei

TEC and NEC responses to paclitaxel were compared to analyze sensitivity to the anticancer drug. TEC and NEC proliferation was inhibited by paclitaxel in a concentration-dependent manner after 72 hours. NECs were sensitive to paclitaxel (inhibitory concentration of 50%, 11 nmol/L), whereas TECs were resistant to it (inhibitory concentration of 50%, 39 nmol/L) ($P < 0.01$, Figure 2A). We measured the MDR1 mRNA expression levels and P-gp protein levels in TECs and NECs because the *MDR1* gene and P-gp, the *MDR1* gene product, are major drug-resistance-related molecules and MDR1/P-gp is up-regulated in drug-resistant cells. The MDR1 mRNA expression level was approximately 2.2-fold higher in TECs than in NECs ($P < 0.01$, Figure 2B). In addition, the P-gp

Figure 2. TECs were resistant to the anti-cancer drug paclitaxel. **A:** Sensitivity to the cytotoxic drug, paclitaxel, was determined by the MTS assay after 72 hours of exposure. TECs were more resistant to paclitaxel than NECs. $**P < 0.01$. **B:** MDR1 mRNA expression was measured by real-time PCR. The MDR1 mRNA expression level in TECs was significantly higher than that in NECs. $**P < 0.01$. **C:** The P-gp expression level in TECs was up-regulated compared with that in NECs. $**P < 0.01$. **D:** Paclitaxel sensitivity under verapamil treatment was determined by the MTS assay after 72-hour exposure. TECs were resensitized to paclitaxel by verapamil. $**P < 0.01$ versus TECs without verapamil. **E:** The YB-1 expression level in NECs and TECs was analyzed under low and high magnification. The broken line indicates the nuclear area. YB-1 accumulation in TEC nuclei was greater compared with that in control NEC nuclei. Scale bar = 10 μm. **F:** The brightness value of YB-1 in the nucleus was quantified using ImageJ software. $*P < 0.05$.

expression level was higher in TECs than in NECs ($P < 0.01$, Figure 2C). Furthermore, to determine whether paclitaxel resistance was due to MDR1/P-gp, we treated TECs with the P-gp inhibitor, verapamil, and confirmed that TECs were resensitized to paclitaxel by verapamil at 50 μmol/L (Figure 2D). Verapamil treatment was not cytotoxic to NECs or TECs at 50 μmol/L (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). We then analyzed the localization of YB-1, which is a transcription factor of the *MDR1* gene. Nuclear localization of YB-1 predicts drug resistance in some tumors.^{30–32} Nuclear expression of YB-1 was hardly detected in NECs, whereas nuclear accumulation of YB-1 was detected in TECs by immunocytochemical staining using confocal microscopy (Figure 2E). A quantitative analysis revealed that nuclear expression of YB-1 increased significantly in TECs ($P < 0.05$, Figure 2F). These results suggest that MDR1/P-gp up-regulation may be a mechanism of drug resistance in TECs and that nuclear translocation of YB-1, which was

Figure 3. Tumor CM stimulated HMVEC proliferation and inhibited apoptosis. **A:** After HMVECs were cultured under their respective conditions for 5 days, cell numbers were counted after 48 hours of culture in each CM. Tumor CM significantly stimulated HMVEC proliferation. ** $P < 0.01$. **B:** Tumor CM enhanced resistance to serum starvation. HMVECs were cultured under their respective conditions for 5 days and starved for 24 hours in serum-free medium. The apoptotic cell number was determined by flow cytometry with annexin V–FLUOS and propidium iodide (PI). The apoptotic and dead cell numbers significantly decreased in tumor CM–treated HMVECs than in HMVECs treated with control CM under a serum starvation condition.

observed in tumor cells, contributes to MDR1/P-gp up-regulation in TECs.

Tumor CM Stimulates Human NEC Proliferation and Enhances Resistance to Serum Starvation

CM was collected from tumor cells and human NECs; HMVECs were used to analyze the effects of tumor cell factors on the drug-resistant EC phenotypes. HMVECs were cultured with or without tumor CM. Controls were treated with HMVEC CM collected after culturing HMVECs in 10% MEM. All HMVECs were treated for 5 days under each condition. To determine whether HMVEC CM was cytotoxic or cytostatic, we counted the dead cells after each medium treatment. HMVEC CM did not increase the number of dead HMVECs compared with EC medium (see Supplemental Figure S2 at <http://ajp.amjpathol.org>), indicating that HMVEC viability is not affected by culture in HMVEC CM. HMVEC proliferation was significantly higher in tumor CM than in control CM ($P < 0.01$, Figure 3A). In addition, apoptotic and dead cells from HMVEC cultures under serum and growth factor–free conditions were analyzed by FACS. The number of apoptotic and dead cells in tumor CM decreased significantly compared with that in the control (Figure 3B), suggesting that tumor CM confers resistance to serum starvation in HMVECs within 5 days.

Tumor CM Induces MDR1 Up-Regulation and Nuclear Translocation of YB-1 in HMVECs

To evaluate the effect of tumor CM on HMVEC sensitivity to anticancer drugs, we used real-time PCR for analyzing the MDR1 mRNA expression levels under the respective conditions. The MDR1 mRNA expression levels were two-fold higher in HMVECs exposed to tumor CM for 5 days than those in control CM ($P < 0.01$). Furthermore, the MDR1 mRNA expression levels in HMVECs were elevated even after a single day of tumor CM treatment ($P < 0.01$, Figure 4A).

We then analyzed the localization of YB-1 in HMVECs after CM treatment. Nuclear expression of YB-1 was hardly detected in the control, whereas nuclear accumulation of YB-1 was detected in HMVECs after 1 day of tumor CM treatment (Figure 4B). To confirm nuclear localization of YB-1 in HMVECs after CM treatment, the extracted HMVEC protein was separated into cytoplasmic and nuclear fractions. We confirmed that the nuclear and cytoplasmic extracts were pure in each fraction using the cytoplasmic protein, β -tubulin, and the nuclear protein, hnRNP. YB-1 in each fraction was detected by using Western blot analysis. The amount of YB-1 in the nuclear fraction from tumor CM-treated HMVECs was significantly higher than that in the fraction from control HMVECs ($P < 0.01$, Figure 4C), suggesting that nuclear translocation of YB-1 contributes to MDR1 mRNA up-regulation through tumor CM treatment in HMVECs.

Tumor CM Induces Anticancer Drug Resistance in HMVECs

We analyzed the sensitivity of tumor CM-treated HMVECs to the anticancer drug paclitaxel. Because paclitaxel is exported by P-gp, we surmised that drug sensitivity may be altered by tumor CM treatment. HMVECs were treated with respective CM for 5 days, followed by paclitaxel treatment for 48 hours. Paclitaxel-induced cell apoptosis was analyzed by FACS. The apoptotic cell number increased in a concentration-dependent manner with paclitaxel treatment. However, the apoptotic cell number in tumor CM-treated HMVECs was less than that in the control ($P < 0.05$, Figure 4D). To determine whether paclitaxel resistance was due to MDR1/P-gp, we treated HMVECs with the P-gp inhibitor verapamil (Figure 4E). The number of paclitaxel-induced apoptotic cells was analyzed with or without verapamil (50 μ mol/L) by FACS. Verapamil treatment was not cytotoxic to HMVECs at 50 μ mol/L, and it resensitized HMVECs to paclitaxel even under tumor CM treatment ($P < 0.01$, Figure 4E), sug-

Figure 4. Tumor CM up-regulated MDR1 mRNA expression and YB-1 expression in HMVEC nuclei. **A:** HMVECs were cultured for 1 or 5 days in CM, and the MDR1 mRNA expression level was measured by real-time PCR. After 1- or 5-day tumor CM treatment, MDR1 mRNA expression in HMVECs was significantly up-regulated compared with that in the control. ** $P < 0.01$. **B:** The YB-1 expression level in HMVECs cultured in control and tumor CM is shown under low and high magnification. The broken line shows the nuclear area. After 1 day of culture in tumor CM, YB-1 accumulation was greater in HMVEC nuclei than in control HMVECs. Scale bar = 20 μ m. **C:** HMVECs were separated into cytoplasmic and nuclear fractions. The cell fractionation accuracy was confirmed by immunoblotting using the cytoplasmic protein, β -tubulin, and the nuclear protein, hnRNP. The amounts of YB-1 in the nuclear fraction from tumor CM-treated HMVECs were much higher than those in the fraction from control HMVECs. ** $P < 0.01$. **D:** Tumor CM-treated HMVECs acquired paclitaxel resistance. The apoptotic cell number, as measured by FACS analysis with annexin V and propidium iodide, increased in a concentration-dependent manner. * $P < 0.05$. **E:** Verapamil was not cytotoxic to HMVECs at 50 μ mol/L. The apoptotic cell number in HMVECs was restored by verapamil under each CM treatment condition. ** $P < 0.01$.

gesting that MDR1/P-gp up-regulation is a mechanism of tumor CM-induced paclitaxel resistance in HMVECs.

Akt Is Activated by Tumor CM, and Tumor CM-Induced MDR1 Up-Regulation Is Blocked by a PI3K Inhibitor in HMVECs

Because the MDR1 mRNA expression levels are regulated by YB-1³³ or microRNA,³⁴ we hypothesized that MDR1 mRNA up-regulation may be caused by soluble factors, such as growth factors, cytokines, or microRNA, in tumor CM. The MDR1 mRNA expression levels did not change on exposure to HMVEC CM. In addition, resistance was not induced by CM from human melanocytes (melanocyte CM; see Supplemental Figure S3, A and B, at <http://ajp.amjpathol.org>). Thus, it was assumed that the factors responsible for MDR1 up-regulation and paclitaxel resistance may be tumor specific and contained in tumor CM. We analyzed MDR1 mRNA expression using preheated tumor CM to determine the factors in tumor CM that caused drug resistance in HMVECs. MDR1 mRNA expression did not change on treating HMVECs with preheated CM (Figure 5A), suggesting that proteins, such as cytokines or growth factors, in tumor CM may be responsible for MDR1 mRNA up-regulation in HMVECs. Akt activation was greater in tumor CM-treated HMVECs than in the control ($P < 0.01$, Figure 5B). However, tumor CM did not elevate the MDR1 mRNA expression level when HMVECs were treated with the PI3K inhibitor, LY294002 (Figure 5C). These results suggest that Akt activation may be a mechanism of MDR1 mRNA up-regulation by tumor CM.

VEGFR Kinase Inhibitor Blocks Tumor CM-Induced MDR1 mRNA Up-Regulation

We compared the cytokine levels released from control and tumor cells by cytokine array analysis. Higher levels of several cytokines were observed in tumor CM than in control CM. Among these, the VEGF level in tumor CM was significantly higher than that in control CM (data not shown). ELISA also revealed that tumor CM contained significantly higher VEGF levels than control CM ($P < 0.01$, Figure 6A). Furthermore, VEGFR2 was phosphorylated and Akt was activated by tumor CM. However, the VEGFR kinase inhibitor, Ki8751, blocked Akt and VEGFR2 activation, even under tumor CM treatment (Figure 6B). To investigate the relationship between VEGF and MDR1 mRNA up-regulation, HMVECs were treated with Ki8751. Tumor CM did not increase the MDR1 mRNA expression level when HMVECs were treated with Ki8751 (Figure 6C). In summary, the results suggest that VEGF in tumor CM up-regulated the MDR1 mRNA expression level via the PI3K-Akt pathway.

Discussion

In this study, we report several new findings. i) TECs were more resistant to the anticancer drug paclitaxel after nuclear translocation of YB-1 and MDR1/P-gp up-regulation

Figure 5. Tumor CM-induced MDR1 mRNA up-regulation in HMVECs was blocked by heat inactivation of tumor CM and LY294002. **A:** The MDR1 mRNA expression level was evaluated using tumor CM and heat-inactivated (95°C for 1 hour before use) tumor CM. HMVECs were treated with CM for 1 day, and the MDR1 mRNA expression level was measured by real-time PCR. The MDR1 mRNA level did not change with exposure to heat-inactivated tumor CM. ** $P < 0.01$. **B:** Akt was activated by tumor CM, whereas the total Akt level remained unchanged. ** $P < 0.01$. The phosphorylated Akt (P-Akt) level was normalized to the total Akt (T-Akt) level and analyzed by scanning densitometry using ImageJ software. **C:** HMVECs were treated with LY294002 and each CM for 1 day. The MDR1 mRNA expression level was measured by real-time PCR. LY294002 inhibited tumor CM-induced MDR1 up-regulation. ** $P < 0.01$.

compared with NECs. ii) Tumor CM stimulated cell proliferation in HMVECs (human NECs) and conferred resistance to serum starvation. iii) The MDR1 mRNA expression levels in HMVECs were up-regulated by tumor CM. The MDR1 transcription factor YB-1 was translocated into the nucleus, and HMVECs acquired paclitaxel resistance on tumor CM treatment. iv) Akt and VEGFR2 were activated in HMVECs by tumor CM. The PI3K-Akt inhibitor, LY294002, and the VEGFR kinase inhibitor, Ki8751, blocked tumor CM-induced MDR1 up-regulation, suggesting that MDR1 up-regulation via Akt activation by VEGF secreted from tumor cells may be one of the mechanisms of acquisition of drug resistance by TECs (Figure

7). We observed that TECs isolated from melanomas were more resistant to paclitaxel, suggesting that even ECs can acquire drug resistance in the tumor microenvironment, contrary to the previous concept that TECs are genetically stable and do not develop drug resistance. Regarding the previous concept, low-dose anti-angiogenic chemotherapy or metronomic chemotherapy has been developed to target TECs that do not acquire drug resistance. Several studies^{35,36} have shown an inhibitory effect of metronomic therapy using mouse tumor models. Paclitaxel is often used in these studies, and a range of its dose is from 10 to 20 nmol/L. However, recent studies^{37–40} report that drug resistance develops to even this

Figure 6. Tumor CM contained a high level of VEGF, and tumor CM-induced MDR1 mRNA up-regulation was blocked by a VEGFR kinase inhibitor. **A:** VEGF concentrations in CM were analyzed using ELISA and normalized by cell number per well. The VEGF level in tumor CM was higher than that in control CM. ** $P < 0.01$. **B:** VEGFR2 was phosphorylated and Akt was activated by tumor CM. Ki8751 blocked Akt and VEGFR2 activation under tumor CM treatment. p, phosphorylated; t, total. **C:** HMVECs were treated with Ki8751 and each CM for 1 day. The MDR1 mRNA expression levels were measured by real-time PCR. Ki8751 inhibited tumor CM-induced MDR1 up-regulation. * $P < 0.05$. NS, not significant.

Figure 7. Possible mechanism of TEC drug resistance. TECs may acquire drug resistance through MDR1 mRNA up-regulation and nuclear translocation of YB-1 via Akt activation, caused by VEGF secreted from tumor cells in the tumor microenvironment.

anti-angiogenic therapy. In our study, the inhibitory concentration of 50% of paclitaxel was 11 nmol/L in NECs and 39 nmol/L in TECs, showing that TECs are resistant to paclitaxel. Taken together, it was suggested that the dose used in low-dose metronomic paclitaxel may be effective for NECs but may not be effective anymore in TECs that have acquired drug resistance in the tumor microenvironment. These findings suggested that drug resistance against long-term anti-angiogenic chemotherapy may occur because of TEC resistance.

Many molecules participate in the drug-resistance mechanism. P-gp, which is encoded by the *MDR1* gene, is a transmembrane glycoprotein that belongs to the ATP-binding cassette superfamily and functions as a multi-drug transporter. Thus, MDR1/P-gp plays a major role in drug resistance.⁴¹ P-gp overexpression causes resistance to several anti-cancer drugs.^{42–44} Paclitaxel is a substrate transported by P-gp that is used together with an anti-angiogenic drug in treating several types of carcinomas.^{45,46} The transcriptional factor YB-1 is a multifunctional protein that participates in the transcriptional regulation of MDR1 mRNA, drug resistance, cell growth, and survival in tumor cells.^{33,47} Many studies^{30,32,48} have shown that YB-1 is highly expressed in various solid tumors, such as those in breast, lung, colon, and ovarian cancers. It was recently reported that YB-1 is highly expressed in tumor vessels compared with normal vessels.⁴⁹ However, the mechanism of expression remains unknown.

In this study, we observed that mouse TECs were resistant to paclitaxel by nuclear translocation of YB-1 and MDR1/P-gp up-regulation. We, therefore, surmised that TECs may acquire drug resistance in the tumor microenvironment. To analyze P-gp expression in human tumor blood vessels *in vivo*, we performed immunofluorescent double staining with anti-CD31 and anti-P-gp antibodies in the same frozen sections of human renal tumors and normal kidney tissues. P-gp was strongly expressed in tumor blood vessels but was hardly expressed in normal blood vessels, thereby suggesting that P-gp was up-regulated in human and mouse TECs *in vivo* (see Supplemental Figure S4 at <http://ajp.amjpathol.org>).

Thus, we speculated that TECs acquire drug-resistance characteristics through the effects of tumor-derived factors. In fact, cross talk between tumor cells and stromal cells is important for tumor progression or acceleration of tumor malignancy.⁵⁰ However, the mechanism by which TECs are affected by tumor-derived factors remains unclear.

To determine the mechanism of drug resistance in TECs, we analyzed the effect of tumor-derived factors on NECs using tumor CM. Tumor CM stimulated HMVEC proliferation and conferred resistance to serum starvation, suggesting that a factor from tumor cells could change HMVEC characteristics, even in a period as short as 5 days. Surprisingly, MDR1 mRNA was up-regulated from day 1 of tumor CM treatment. We observed that tumor CM induced nuclear translocation of YB-1 in HMVECs, suggesting that MDR1 mRNA up-regulation was induced by nuclear translocation of YB-1 and that drug resistance occurred in HMVECs through tumor CM, similar to that observed in tumor cells. Similarly, tumor CM up-regulated MDR1 expression and caused an anti-apoptotic phenotype in mouse NECs, as shown in HMVECs (see Supplemental Figure S5 at <http://ajp.amjpathol.org>), suggesting that the effects of tumor-derived factors may cause drug resistance in both human and mouse NECs.

Furthermore, MDR1 mRNA expression was up-regulated and sensitivity to the cytotoxic drug paclitaxel was lowered in HMVECs by tumor CM. However, these phenotypic changes were abolished when HMVECs were recultured in a tumor CM-free condition (EGM2-MV; data not shown), indicating a reversible change. In contrast, TECs showed stable MDR1 mRNA up-regulation, suggesting that an additional mechanism may be responsible for the resistance of TECs. We assumed that TECs may be affected by tumor cell factors *in vivo* and also by factors from other tumor stromal cells or hypoxia. In addition, the time course may be another factor. TECs were more influenced by the tumor microenvironment than the tumor CM-treated HMVECs because TECs were grown *in vivo* for 4 to 5 weeks. Paclitaxel sensitivity was restored by the P-gp inhibitor verapamil. However, even verapamil did not restore the apoptotic cell number to that observed

in the control, suggesting that tumor CM-induced drug resistance is, at least in part, due to MDR1/P-gp up-regulation. Moreover, other factors, such as anti-apoptotic gene up-regulation or activation of cell survival pathways, may also account for this acquired phenotype. Growth factors released from tumor and stromal cells, such as VEGF, basic fibroblast growth factor, and EGF receptor ligands, are anti-apoptotic survival factors that may affect ECs in tumor tissues.^{51,52} To determine the contribution of proteins present in tumor CM, we analyzed the MDR1 mRNA expression levels in HMVECs using heat-inactivated tumor CM. The MDR1 mRNA levels did not change when HMVECs were treated with preheated tumor CM, suggesting that proteins, such as cytokines or growth factors, from tumor cells may cause MDR1 mRNA up-regulation and drug resistance in HMVECs. On observing that the VEGF levels in tumor CM were much higher than those in HMVEC CM, we analyzed Akt phosphorylation after tumor CM treatment. Akt was activated in HMVECs by tumor CM. Because Akt is a well-known survival factor, we assumed that Akt activation is a mechanism of drug resistance. Recent studies^{53–55} have shown a correlation between Akt activation and P-gp in cancer cells. Akt activation causes YB-1 serine 102 phosphorylation, resulting in nuclear translocation.⁵⁶ We observed that the PI3K inhibitor, LY294002, blocked tumor CM-induced MDR1 up-regulation. Therefore, we assumed that Akt activation by tumor CM is a mechanism of paclitaxel resistance. Another melanoma (A375) CM also induced MDR1 mRNA up-regulation, whereas normal melanocyte CM did not. The VEGF levels in tumor CM were significantly higher than those in control or melanocyte CM (see Supplemental Figure S6 at <http://ajp.amjpathol.org>). The VEGFR kinase inhibitor, Ki8751, blocked tumor CM-induced MDR1 up-regulation, suggesting that VEGF in malignant tumor CM may cause the acquired drug resistance. In conclusion, we demonstrate, for the first time to our knowledge, that TECs are resistant to paclitaxel through MDR1 mRNA up-regulation and that NECs can acquire drug resistance through MDR1 mRNA up-regulation and nuclear translocation of YB-1 via Akt activation, caused by VEGF secreted from tumor cells.

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