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Inhibition of Multidrug Transporter in Tumor Endothelial Cells Enhances Antiangiogenic Effects of Low-Dose Metronomic Paclitaxel

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Tumor angiogenesis plays an important role in tumor progression and metastasis. Tumor endothelial cells (TECs) are a therapeutic target of antiangiogenic chemotherapy that was recently developed and is currently being investigated in the clinic with promising results. Low-dose chemotherapy, which is the long-term administration of relatively low doses of chemotherapeutic agents, has been proposed for targeting tumor angiogenesis in various types of cancers. Although the efficacy of low-dose chemotherapy has been confirmed in several clinical models, some studies show insufficient therapeutic effect for malignant cancers. As a possible mechanism of the treatment failure, it has been considered that tumor cells may acquire resistance to this therapy. However, drug resistance by TECs may also be due to another mechanism for resistance of tumor cells to low-dose chemotherapy. We reported elsewhere that TECs were resistant to the anticancer drug paclitaxel, which is a mitotic inhibitor, concomitant with P-glycoprotein up-regulation. Verapamil, a P-glycoprotein inhibitor, abrogated TEC resistance *in vitro*. Herein, we demonstrated that verapamil coadministration enhanced the effects of low-dose paclitaxel concomitant with inhibiting tumor angiogenesis in a preclinical *in vivo* mouse melanoma xenograft model. Furthermore, verapamil coadministration reduced lung metastasis. These results suggest that inhibiting P-glycoprotein in TECs may be a novel strategy for low-dose chemotherapy targeting TECs. *(Am J Pathol 2015, 185: 572–580; http://dx.doi.org/10.1016/j.ajpath.2014.10.017)*

After the role of angiogenesis in tumor progression was first recognized, antiangiogenic chemotherapy that targeted tumor blood vessels was developed.[1] Current antiangiogenic drugs, such as bevacizumab, that target vascular endothelial growth factor (VEGF) have shown a good therapeutic effect. However, these drugs produce some positive effects in patients with specific cancer types when bevacizumab is combined with conventional chemotherapy. In addition, it has been reported that drug resistance to antiangiogenic chemotherapy emerged because tumor cells can induce angiogenesis with compensatory secretion of other angiogenic growth factors, such as fibroblast growth factor-2 and angiopoietin, when VEGF is inhibited.[2]

Anticancer drugs target highly proliferative cells, such as tumor cells. Anticancer drugs have generally been administered in a short cycle with prolonged drug-free breaks at the maximum tolerated dose. Unfortunately, the high-dose anticancer drugs frequently cause substantial toxicity, resulting in adverse effects that might limit the treatment.

It was recently suggested that cytotoxic anticancer agents could target tumor vasculature because tumor endothelial cells (TECs) are more proliferative compared with normal endothelial cells (NECs).[3] Low-dose chemotherapy, which is called metronomic chemotherapy, is one of the antiangiogenic therapies. This therapy targets proliferating TECs with long-term administration of chemotherapeutic agents, such as the mitotic inhibitor paclitaxel and the DNA synthesis inhibitor 5-fluorouracil (5-FU), which show cytotoxic action on proliferating TECs at relatively low, minimally toxic doses to reduce

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adverse effects.[4–6] Several benefits of low-dose chemotherapy have been reported compared with anti-VEGF therapy and maximum tolerated dose treatment. Low-dose chemotherapy can i) reduce undesirable adverse effects caused by myelosuppression because the dose of anticancer drugs is low or by damage to NECs because it acts specifically on TECs; ii) inhibit tumor regrowth caused by the resuming of tumor angiogenesis because it does not need prolonged drug-free breaks, unlike conventional chemotherapy, such as treatment at the maximum tolerated dose; and iii) inhibit tumor angiogenesis in several aspects, such as inhibition of proliferating TECs, inducing up-regulation of the endogeneous angiogenesis inhibitor thrombospondin-1,[7] and inhibition of endothelial progenitor cell mobilization from the bone marrow.

Clinical trials with metronomic chemotherapy were undertaken for several types of solid tumors, including advanced breast cancer[8–11] and glioblastoma.[12–14] Promising results were obtained for malignancies that were poorly responsive to conventional chemotherapies.

However, low-dose chemotherapy did not improve patient survival in some cases. It is believed that tumor cells acquire drug resistance as the mechanism for the treatment failure. However, the detailed mechanism still remains unknown.

Recent studies have revealed that TECs were drug resistant compared with NECs, contrary to the traditional assumption that TECs are genetically stable and do not acquire drug resistance.[15] We also reported that TECs had cytogenetic abnormalities, such as aneuploidy in mouse tumors[16] and human renal carcinomas.[17] In addition, TECs are resistant to certain anticancer drugs, such as paclitaxel.[18,19] Recently, we found that TECs were resistant to paclitaxel concomitant with up-regulation of *MDR1* (multidrug-resistance gene)/P-glycoprotein (P-gp) in the tumor microenvironment.[18] Furthermore, the resistance to paclitaxel by cultured TECs *in vitro* was abrogated by a P-gp inhibitor, verapamil.

P-gp, a member of the ABC transporter family, is a transmembrane glycoprotein and a multidrug transporter. A variety of studies have reported that *MDR1*/P-gp played a major role in drug resistance to several anticancer drugs.[20–22] The results of a previous study suggested that even TECs could acquire resistance to antiangiogenic chemotherapy concomitant with their up-regulation of P-gp.[18] Thus, we hypothesized that inhibiting P-gp in TECs may improve the response to antiangiogenic chemotherapy.

Herein, we investigated the antiangiogenic efficacy of coadministering the P-gp inhibitor verapamil along with low-dose metronomic paclitaxel using an *in vivo* human melanoma model in mice.

Materials and Methods

Cell Line and Culture Conditions

A375SM cells (human highly metastatic melanoma cells) were a gift from Dr. Isaiah J. Fidler (MD Anderson Cancer Center, Houston, TX)[23] in 2007 and were authenticated in January 2014 by JCRB Cell Bank (Osaka, Japan) by short tandem repeat analysis. These cells were cultured in minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 and 95% air at 37°C.

Antibodies

The following antibodies were used: Alexa Fluor 647 rat anti-mouse CD31 (BioLegend, San Diego, CA), rabbit anti-mouse/human *MDR1*/P-gp (LifeSpan Biosciences Inc., Seattle, WA), rabbit anti-mouse/human cleaved caspase3 (Cell Signaling Technology Inc., Beverly, MA), Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies Inc., Gaithersburg, MD), and monoclonal anti−β-actin (AC-15; Sigma-Aldrich, St. Louis, MO).

Isolation of TECs and NECs

TECs were isolated from melanoma xenografts in nude mice, and NECs were isolated from mouse dermis as described elsewhere.[16,24] All the animal experimental procedures were approved by the local animal research authorities, and animal care was in accordance with institutional guidelines. In brief, excised tissues were minced and digested with collagenase II. After blood cells were removed by a single sucrose step-gradient centrifugation using Histopaque 1077, cell suspensions were filtered, and endothelial cells (ECs) were isolated using a magnetic cell sorting system (Miltenyi Biotec, Tokyo, Japan) with anti-mouse CD31 antibodies. CD31-positive cells were sorted and plated on fibronectin-coated culture plates and were grown in EGM-2MV medium (Lonza, Basel, Switzerland) and 15% fetal bovine serum. Diphtheria toxin (500 ng/mL; Calbiochem, San Diego, CA) was added to TEC subcultures to eliminate human tumor cells and to NEC subcultures for technical consistency. After subculture for approximately 2 weeks, isolated ECs were further purified using FITC-BS1-B4-lectin.[25] All the purified ECs were cultured in EGM-2MV and were used at passages 15 to 25.

Immunostaining

Tumor tissues were dissected out from humanely sacrificed mice. Frozen sections of excised tissues were prepared as described elsewhere.[26] To assess P-gp co-localization in tumor blood vessels, frozen sections were double stained with rat anti-mouse CD31−Alexa Fluor 647 and rabbit anti-*MDR1*/P-gp antibodies, followed by counterstaining with DAPI to stain nuclei. To detect apoptotic ECs, frozen sections were double stained with rat anti-mouse CD31−Alexa Fluor 647 and anti−cleaved caspase 3 antibodies. Stained samples were observed using a FluoView FV10i confocal microscope (Olympus America Inc., Center Valley, PA). Cleaved caspase 3−positive proportions in blood vessels were quantified