Overexpression of VEGF and alterations of tight junction expression are involved in PCB-induced dysfunction of the microvascular endothelium

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Abstract

Environmental toxicants, such as polychlorinated biphenyls (PCB) congeners, can activate vascular endothelial cells and thus increase formation of blood-borne metastases. Integrity of the vascular endothelium, regulated in part by tight junction proteins, plays a critical role in transendothelial migration of tumor cells. The present study indicates that exposure of human microvascular endothelial cells to selected ortho-chlorinated PCBs can increase transendothelial permeability and stimulate migration of tumor cells. These events are associated with overexpression of vascular endothelial growth factor (VEGF) and decreased expression of specific tight junction proteins. PCB-mediated elevation of VEGF expression was induced by phosphatidylinositol 3-kinase (PI3K) but not affected by co-treatments with antioxidants. The VEGF receptor (KDR/Flk-1) antagonist, SU1498, and the PI3K inhibitor, LY294002, inhibited PCB 104-induced hyperpermeability. In addition to upregulation of VEGF, treatment with selective PCB congeners disrupted expression of the cytosolic scaffold proteins of tight junctions, such as zonula occludens (ZO)-1, ZO-2, and AF6. In contrast, PCB exposure did not alter expression of integral membrane proteins, junctional adhesion molecule-A (JAM-A) and claudin-1. The obtained results indicate that PCB-mediated overexpression of VEGF and selective alterations of tight junction protein expression may contribute to their neurotoxic effects in the CNS.

Introduction

Vascular effects of polychlorinated biphenyls (PCBs) may influence the development of tumor metastasis. One of the key events in the metastatic formation is transendothelial migration of tumor cells. Intravascular circulating tumor cells attach to endothelium and the junctions between vascular cells are retracted (1,2). Thus, tumor cells can be captured by the vascular cells during entry and exit from the circulation and the locally activated endothelial cells can modify metastatic behavior of circulating tumor cells. Because vascular endothelial cells form a continuous monolayer which functions as a selective barrier to the passage of cancer cells from blood stream to the underlying tissues, endothelial dysfunction has a significant influence on the fate of circulating cancer cells in the blood vessel (3). For example, an increase in endothelial permeability can accelerate metastatic processes through the facilitated transendothelial migration of cancer cells (4).

Endothelial hyperpermeability can be induced either by intracellular or extracellular stimuli, such as reactive oxygen species (ROS), cytokines, and growth factors. It appears that one of the most important factors involved in the regulation of endothelial permeability is vascular endothelial growth factor (VEGF). Evidence indicates that VEGF can disrupt endothelial integrity and increase permeability across the endothelial monolayers *in vivo* or *in vitro* and promote transendothelial migration of leukocytes and cancer cells, including breast cancer cells (5).

Tight junctions are one of the main structural components which regulate the integrity of the vascular endothelium and endothelial permeability. They are composed by transmembrane proteins (such as zonula occludens [ZO]-1, ZO-2, and AF6) linked to

cytoplasmic and cytoskeletal proteins by accessory proteins (e.g., claudins, junctional adhesion molecules [JAMs], and occludin). The system of endothelial junctions is highly dynamic and reversible to allow, within minutes, passage of blood components to tissues. Alterations of tight junction expression are also involved in the metastasis formation (6).

Despite significant progress on the biology of tumor metastasis, it is still not fully understood how environmental contaminants, such as PCBs, can influence this process. Thus, the aim of the present study was to evaluate the events induced by PCBs in vascular endothelial cells which can influence and regulate the development of tumor metastasis.

Materials and Methods

Cell culture and PCBs treatment

Human microvascular endothelial cells (HMEC-1) were a generous gift from Dr. Eric Smart (University of Kentucky). HMEC-1 are an immortalized cell line obtained by transformation of human microvascular endothelial cells with the SV40 large T antigen.

Human brain microvascular endothelial cells (HBMEC) are an immortalized cell line derived from a primary cell culture through co-expression of hTERT and the SV40 large T antigen. This cell line retains most of the morphological and functional characteristics of brain endothelial cells, including expression of tight junction proteins (7).

PCBs were purchased from AccuStandard (New Haven, CT). Stock solution of PCBs was prepared in DMSO and the same amounts of DMSO as in PCBs-treated cells were added to control cultures. Levels of DMSO in experimental media were less than 0.05%.

Permeability assay

HMEC-1 were seeded on fibronectin-coated Transwell polycarbonate filters (12-mm diameter, 0.4 μm pore size) and allowed to grow to confluence. Selected PCB congeners were added for 24 h to both the lower and the upper compartment of Transwell system. After the cultures were rinsed twice with Kreb-Ringer Glucose (KRG) solution, 1.5 ml of KRG solution was added into the lower chambers of Transwell, and 0.5 ml of FITC-dextran 40 (FD-40, 1 mg/ml in KRG solution) was loaded into the upper chambers. The systems were incubated for 1 h and the assay was stopped by removing the upper chambers. Fluorescence of FD-40 was detected in aliquots (0.5 ml) from the lower chamber.

Transendothelial cell migration assay

HMEC-1 were seeded and grown to confluence on fibronectin-coated Transwell polycarbonate filters (6.5 mm diameter, 8.0 μm pore size). HMEC-1 were then washed twice with migration medium (serum-free MCDB 131 containing 1% BSA) and the calcein-labeled MDA-MB-231 cells (4.0×10^4 cells) suspended in 100 μl of the same medium were added to the monolayer of HMEC-1. After incubation for 10 h, cells were fixed with 4% formaldehyde and washed extensively with PBS. To remove non-migrating cells, cells on the upper face of the filter were gently scraped using a cotton swab and the migrating tumor cells were observed under fluorescent microscope.

Immunoblotting and Immunofluorescence

Treated endothelial cells were lysed with the standard lysis buffer. Then, protein samples were electrophoresed in SDS-PAGE and transferred onto a Hybond-ECL membrane (Amersham Biosciences, Piscataway, NJ).

Endothelial cells cultured on Type I collagen-coated chamber slide were fixed with ethanol for 30 min at 4°C and non specific binding was blocked with 3% bovine serum albumin. Samples were incubated overnight at 4°C with primary antibody and then incubated with FITC-conjugated secondary antibody. The cells were observed and photographed using

a confocal fluorescence microscope (Olympus FluoView 300; Olympus America Inc., Center Valley, PA).

Real-Time RT-PCR

Real-time RT-PCR was used to determine mRNA expression. PCR amplification was performed using commercially available pre-developed primer pairs and TaqMan probes (Applied Biosystems) according to the manufacturer's instructions.

Statistical Analysis

Data were statistically analyzed using one-way ANOVA, followed by Student's t test. Statistical probability of $p<0.05$ was considered significant.

Results and Discussion

During the formation of blood-borne metastasis, tumor cells disseminate from the primary tumor to secondary sites in various organs. Because vascular endothelial cells form a continuous monolayer which functions as a selective barrier to the passage of cancer cells from blood stream to the underlying tissues, endothelial dysfunction has a significant influence on the fate of circulating cancer cells in the blood vessel (3). Specifically, an increase in endothelial permeability can accelerate metastatic process through the facilitated transmigration of cancer cells across the microvascular endothelial monolayer (4).

Several vascular mechanisms can be responsible for facilitation of extravasation and dissemination of tumor cells. For example, upregulation of specific adhesion molecules on the endothelial surface can increase transendothelial migration of cancer cells through the increased adhesion of cancer cells to endothelial cells (1). In addition, transmigration of cancer cells can be augmented by disruption of cell junctions (7). Finally, several soluble factors, such as VEGF, can induce the disruption of endothelial integrity, which in turn may directly enhance penetration of tumor cells and facilitate establishment of cancer metastasis. Microvascular hyperpermeability in proximity to metastatic tumors also can accelerate plasma proteins extravasation to stimulate tumor growth (8).

In the present study, exposure of HMEC-1 to PCB 104 resulted in a dose-dependent increase in endothelial permeability. In addition, treatment with selected PCBs induced a dose-dependent increase in the migration of MDA-MB-231 cells across the HMEC-1 monolayers. The effective concentration of PCBs was 5 μM.

VEGF is one of the most important endothelial-derived agents regulating vascular permeability. Treatment of HMEC-1 with 10 μM PCB 104 for 6 and 12 h markedly upregulated VEGF mRNA and protein levels. To determine whether PCB 104-induces VEGF mRNA expression at the transcriptional level, confluent HMEC-1 were pretreated with actinomycin D, the inhibitor of RNA transcription, and then incubated with PCB 104 for 8 h. PCB 104-mediated VEGF mRNA levels were completely abolished by actinomycin D.

Evidence suggests that the phosphatidylinositol 3-kinase (PI3K) pathway may regulate VEGF expression via activation of Akt kinase (9). Therefore, the possibility that PCB 104 can stimulate these pathways also was examined in the present study. Treatment with PCB 104 for 15 or 30 min markedly increased phosphorylation of Akt kinase, the cellular target of PI3K. To support the regulatory role of PI3K in PCB 104-induced VEGF mRNA overexpression, pretreatment of HMEC-1 with LY294002 and wortmannin, specific inhibitors of PI3K activation, markedly and in dose-dependent manners decreased PCB 104-mediated stimulation of VEGF mRNA and protein levels.

The majority of cellular effects of VEGF, including an increase in endothelial permeability, are mediated by a high affinity tyrosine kinase receptor, known as kinase insert domain (KDR)-containing receptor or fetal liver kinase (Flk)-1 (KDR/Flk-1). To indicate

whether PCB 104-induced overexpression of VEGF can influence alterations in HMEC-1 permeability and transendothelial migration of the MDA-MB-231 cells, a series of experiments was performed in which KDR/Flk-1 was functionally blocked with the specific inhibitor SU1498. The use of this inhibitor dramatically and in a dose-dependent manner inhibited PCB 104-induced disruption of HMEC-1 barrier function.

Although the detailed mechanisms of VEGF-mediated stimulation of hyperpermeability are not known, evidence indicates that the rearrangement and altered expression of endothelial junctional proteins may be involved in this process (10). Therefore, we also studied the effects of PCBs on expression of tight junction proteins. Treatment with both coplanar and non-coplanar PCBs for 24 h markedly reduced expression of ZO-1, ZO-2 and AF6 in endothelial cells. However, tight junction transmembrane proteins, such as claudin-1 and JAM-A, were not affected by PCB treatment. In addition, mRNA levels of ZO-1, ZO-2 and AF6 were not altered as a result of PCB treatment. Thus, it appears that PCB-induced alterations of tight junction protein expression results from the translational or post-translational mechanisms rather than from transcriptional regulation.

In conclusion, the present study is the first research report indicating that exposure to highly chlorinated PCBs can induce overexpression of VEGF and diminish expression of tight junction proteins, such as ZO-1, ZO-2 and AF6 in endothelial cells. Due to their role in maintaining vascular integrity, these vascular alterations may directly contribute to the prometastatic effects of highly chlorinated PCBs.

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