Published in final edited form as:

Toxicol In Vitro. 2008 April ; 22(3): 643–651. doi:10.1016/j.tiv.2007.12.009.

Direct Antiangiogenic Actions of Cadmium on Human Vascular Endothelial Cells*

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Abstract

The vascular endothelium is a primary target of cadmium (Cd) toxicity, but little is known regarding a potential mechanism whereby Cd may inhibit angiogenesis. Recent findings showing that Cd can disrupt cadherin-mediated cell-cell adhesion suggested that Cd might inhibit angiogenesis by altering the function of VE-cadherin, a molecule that is essential for angiogenesis. To address this issue, endothelial cells (ECs) were exposed to Cd in the presence of serum and subjected to angiogenesis-related cell migration and tube formation assays. Initial examination of cytotoxicity showed that ECs are rather resistant to the acute cytotoxic effects of Cd even at concentrations up to 1mM. However, 10μM Cd decreased migration of ECs. Cd concentrations of 500nM and greater significantly reduced organization of microvascular ECs into tubes. These antiangiogenic effects were evident even when ECs were preincubated with Cd and then washed to remove free Cd, indicating that Cd acted directly on the cells rather than on the extracellular matrix. Immunolocalization studies showed that Cd caused the redistribution of VE-cadherin from cell-cell contacts. These findings indicate that Cd acts in an angiostatic manner on ECs, and that this effect may involve alterations in the localization and function of VE cadherin.

* Abbreviations

Cd = cadmium

DMSO = dimethylsulfoxide

EBM-2 = endothelial basal media-2

EC = endothelial cell

EGM-2 = endothelial growth media-2

FBS = fetal bovine serum

HMVEC = human dermal microvascular endothelial cell

HUVEC = human umbilical vein endothelial cell

PMA = phorbol 12-myristate 13-acetate

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Conflict of Interest Statement The authors have no conflicts of interest.

Introduction

Cadmium (Cd) is a widely-distributed industrial and environmental pollutant that currently ranks 8th on the Agency for Toxic Substances and Disease Registry Priority List of Hazardous Substances (ATSDR, 2005). Depending on the dose, route and duration of exposure, Cd can damage various organs including the kidney, lung, liver and testis (Elinder and Kjellstrom, 1986; Jarup et al., 1998). In addition, Cd has teratogenic and carcinogenic activities (Degraeve, 1981; IARC, 1993). Considerable evidence suggests that many of the effects of Cd in the body involve actions on the vascular endothelium (for reviews see (Nolan and Shaikh, 1986; Prozialeck et al., 2006)). For example, acute exposure to moderate doses of Cd results in edema and hemorrhaging in organs such as the testis and placenta (Parizek and Zahor, 1956; Kar and Das, 1960; Parizek, 1964; Levin et al., 1981). These effects result from the Cd-induced disruption of the adhering junctions between the endothelial cells (ECs) of the capillaries and venules in the sensitive organs. Results of more recent studies suggest the ability of Cd to disrupt endothelial cell-cell junctions may involve alterations in the function of the Ca-dependent cell adhesion molecule VE-cadherin (Prozialeck, 2000; Pearson et al., 2003; Prozialeck et al., 2006). Additional studies have shown that Cd can target vascular ECs at a variety of other levels, including cell adhesion molecules, metal ion transporters and protein kinase signaling pathways, and that these actions of Cd on ECs may contribute to the overall toxic effects of Cd in organs such as the liver, lung and kidney (for review see Prozialeck *et al.*, 2006).

Even though it is well-established that the vascular endothelium is an important target of Cd toxicity, surprisingly little has been published on the effects of Cd on the process of angiogenesis, which is the formation of new blood vessels from preexisting ones. This is an important issue because angiogenesis plays a critical role in homeostatic responses to toxicant exposure and derangements of angiogenesis may be involved in a variety of toxicant-induced pathophysiologic processes (Prozialeck et al., 2006; Straub et al., 2007).

The process of angiogenesis can be divided into several steps, each of which can be studied in vitro. These include: 1) basement membrane degradation; 2) EC migration away from the vessel in response to a chemoattractant gradient; 3) EC proliferation; and 4) morphogenesis into tube-like structures. In a series of studies in the mid-1990's, Kishimoto and coworkers reported that Cd could inhibit proliferation, migration and tube formation by human umbilical vein ECs (HUVECs) in culture (Kishimoto et al., 1994; Kishimoto et al., 1996a; Kishimoto et al., 1996b). However, the possible relevance of these studies to the actions of Cd in vivo remains somewhat unclear, because the studies were carried out in the absence of serum and utilized relatively high concentrations of Cd that also affected cell viability. These experimental conditions are quite different from the patterns of exposure that would occur in vivo. Essentially all Cd that reaches the systemic circulation is bound to proteins, cysteine or glutathione, which are present in serum (Bridges and Zalups, 2005). Moreover, the concentrations of Cd to which ECs are typically exposed in vivo are usually not acutely cytotoxic (Prozialeck et al., 2006).

In the studies described here, we have examined the cytotoxic effects of Cd on ECs in the presence of serum. Since both macrovascular and microvascular ECs are targets of Cd within the body, both cell types were studied. Next, we examined the effects of non-cytotoxic concentrations of Cd on EC migration and tube formation. Previous studies in which ECs were exposed to Cd in the absence of serum (Kishimoto et al., 1996b) suggested that Cd may act primarily on the extracellular matrix rather than on the ECs directly. Therefore, additional studies were conducted to determine whether or not Cd has direct functional effects on ECs. Finally, in an effort to begin to identify a possible mechanism through which Cd may exert its antiangiogenic effects, we examined the effects of Cd on the localization of VE-cadherin, a calcium-dependent cell adhesion molecule that is known to be an absolute requirement for

angiogenesis (Vittet et al., 1997; Liao et al., 2000), and which has been shown to be disrupted by Cd (Prozialeck, 2000; Pearson et al., 2003).

Materials and Methods

ECs

Human umbilical vein endothelial cells (HUVECs, macrovascular ECs), and human dermal microvascular ECs (HMVECs, microvascular ECs) were purchased from Cambrex (Walkersville, MD) and maintained as recommended by the manufacturer in endothelial growth media (EGM-2). For propagation, cells were removed from their flasks using trypsin supplied by the same manufacturer, and were passed at a 1:2 (HMVECs) or 1:3 ratio (HUVECs).

Cell Viability Assay

Glass coverslips coated with 0.1% gelatin (Sigma, St Louis, MO) were added to 24-well tissue culture plates and seeded with 12,000 HMVEC/well in EGM-2. After the cells were adherent, the media was replaced with a 1:5 dilution of growth media in endothelial basal media (EBM-2) for 18 hrs (similar to our chemotaxis experimental design). At the end of this period, media was replaced with EBM-2 containing 0.1% fetal bovine serum (FBS) and various concentrations of Cd (0.00000001, 0.0000001, 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, and 10mM as the chloride salt) for a 6 hr period. For studies with macrovascular ECs, cells were plated at 80,000 HUVEC/well in EGM-2 and allowed to grow to confluence. Various concentrations of Cd (0.001, 0.01, 0.1, 1, and 10mM) were added to the wells and incubated for 18 hrs in the presence of basal media with 0.1% FBS. For both cell types, viability was determined using a Live/Dead® Viability/Cytotoxicity Kit (Invitrogen/Molecular Probes, Carlsbad, CA). In these assays, ethidium homodimer and calcein AM were added to each well (0.2 μ M calcein AM and 1.0 μ M ethidium homodimer) and plates were incubated for 10 minutes. Each coverslip was then carefully washed in phosphate-buffered saline (PBS) and Milli-Q water, and the coverslips were mounted onto microscope slides using AquaPoly Mount (Polyscience Inc., Warrington, PA). Viability was determined by incorporation of ethidium homodimer into cells with “leaky” cell membranes. The nuclei of those dead or dying cells fluoresce red, whereas cytosolic esterases on the membranes of live cells convert nonfluorescent calcein AM into bright green fluorescent calcein which is visible in the cytoplasm. By viewing the samples with a broad band filter, live cells (green) and dead cells (red) can be viewed simultaneously. Images of representative samples were captured with a Nikon Eclipse E400 microscope fitted with a Spot Digital camera (Diagnostic Instruments, Sterling Heights, MI) and processed using the Image Pro Plus software package (Media Cybernetics, Silver Spring, MD).

Chemotaxis Assay

ECs at approximately 70–80% of confluence were fed the night before the assay with a 1:5 dilution of EGM-2 in EBM-2. The next morning cells were washed twice with PBS and incubated for 1 hr in 5% CO₂ at 37°C with EBM-2 + 0.1% FBS. The chemotaxis assay was performed in a 48 well chemotaxis chamber (Neuroprobe, Gaithersburg, MD). The stimulant, phorbol 12-myristate 13-acetate (PMA, 50 nM; positive control dissolved in dimethylsulfoxide (DMSO)), in the presence of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, or 100 μ M Cd, was added to the bottom chamber; PBS served as the negative control. A gelatin-coated polycarbonate membrane (8 μ m pore size) was placed on top of the wells to separate the sides. The chamber was inverted and placed in an incubator with 5% CO₂ at 37°C for 1 hour. HMVECs or HUVECs (4×10^4 cells in 40 μ l of EBM-2 + 0.1% FBS) were loaded into the top of the chamber after the chamber was righted. The top of the chamber included the identical concentrations of Cd as the bottom wells, so that no gradient of Cd should exist in any well. The chamber was then

incubated in an atmosphere of 5% CO₂ at 37°C for 4 hours to allow EC migration. Cd was present in appropriate experimental wells, while additional control wells were analyzed in the absence of Cd. At the end of the migration period, the polycarbonate membrane was removed. Non-migrated cells were carefully removed with a cotton swab, while migrated cells were fixed with methanol and stained with Diff-Quik solution II (Baxter Scientific, Deerfield, IL). Each sample was tested in quadruplicate and migrated cells were photographed using a Nikon Coolpix 5000 digital camera. Three representative high power fields were counted and the sum of the counts from those fields was compared between the various groups. Statistical analysis was performed using Analysis of Variance and post-hoc *t*-tests to determine significance of differences from control values.

Tube Formation Assays

HMVECs at approximately 70–80% confluence were provided full growth media the day before the assay. Eight-well chamber slides (Becton Dickinson, Bedford, NJ) were coated with 125 µl of growth factor reduced Matrigel (Becton Dickinson) and placed at 37°C while working with the cells. HMVECs were resuspended in Media 199 + 2% FBS and added to the Matrigel-coated chamber slides (16,000 cells/well in 0.4 ml). Cells in appropriate wells were then exposed to PMA (50 nM), DMSO (vehicle control) and/or various concentrations of Cd. After overnight incubation, the tubes that had been formed were fixed with methanol and stained with Diff Quik solution II. The sum of tube length and area was determined using AxioVision LE Version 4.2 software applied to photographs of each well taken with a 2x objective. In some studies, these photos were used to calculate the number of nodules formed, where a nodule is defined as an area in which three or more ECs can be seen making contact and branching out from one another. Tube length from four wells was averaged and values for the control and Cd-treated samples were compared using ANOVA and post-hoc *t*-tests.

In order to rule out actions of Cd on the Matrigel matrix, an alternative protocol was also used for this assay in which HUVECs or HMVECs were pretreated with various concentrations of Cd for 48 or 72 hrs, respectively. After the pre-treatment, cells were washed twice with PBS and the Matrigel assay was performed as described above, except that no Cd was present in the media.

Immunofluorescent Visualization of VE-cadherin

Gelatin coated round glass coverslips (Fisher Scientific, Pittsburg, PA) in 24 well plates were seeded with 80,000 HUVEC or HMVEC/well in EGM-2 media and grown to confluence. The plates were incubated for 18 hrs with various concentrations of Cd (HUVEC: 0.01, 0.1, 1, 10, or 100 µM; HMVECs: 0.05, 0.1, 0.5, 1, 5, 10, 50 µM) added to each well in the presence of 5% FBS. After incubation, the cells were fixed with 70% ice cold methanol for 10 min and then blocked with PBS + 10% FBS solution. The coverslips were stored in this blocking solution at 4°C until use. For immunofluorescent staining, the coverslips were removed from the wells and then incubated overnight at 4°C with mouse anti-human VE-cadherin antibody (Immunotech, Warrington, PA; 1:50 dilution). The next day, the cells were washed in PBS and Milli-Q Water and incubated for 30 minutes at room temperature with FITC-conjugated goat anti-mouse antibody (Sigma, St.Louis, MO). After incubation, the cells were washed again in PBS and water, and the coverslips were mounted on microscope slides using Aqua Poly Mount. Images were taken with a Nikon Eclipse E400 microscope fitted with a Spot Digital camera (Media Cybernetics, Sterling Heights, MI) and then processed using Image Pro Plus software (Media Cybernetics, Sterling Heights, MI).

Results

General Cytotoxic Effects of Cd on HMVECs and HUVECs

HMVECs were first exposed to various concentrations of Cd for 6 hrs in the presence of a small amount of serum (0.1% FBS). Use of a sensitive cell viability kit suggested that a concentration of 10 mM Cd killed the majority of microvascular ECs (HMVECs), while simultaneously reducing their adherence to each other and to the gelatin-coated growing surface. In contrast, 1 mM Cd and lower concentrations (listed in the Materials & Methods section) were not cytotoxic and did not appear to reduce adherence (data not shown; n=3 separate experiments).

In subsequent studies on HUVECs, shown in Fig. 1, Cd-exposure was extended to 18 hrs. When the samples are viewed with a broad-band filter, live HUVECs appear green because they take up cell-permeable calcein AM and convert it to green fluorescent calcein through their intracellular esterase activity. Cells that are dead appear red from ethidium homodimer entrance through their damaged membranes allowing red fluorescence of nucleic acids. Note that non-Cd treated HUVECs (Fig. 1A) are alive and fluoresce green. In contrast, nuclei of HUVECs intentionally killed by MeOH-treatment all fluoresce red (Fig. 1B). HUVECs treated with 10 mM Cd appear dead (Fig. 1C), and a decrease in cell density can be noted, possibly due to decreased cell-substrate adhesion. Treatment with 1 mM Cd (Fig. 1D) caused a noticeable decrease in cell adherence to the growing surface, but did not necessarily kill HUVECs. Examination of HUVECs treated with 100 μ M Cd (E) and 1 μ M Cd (F) revealed no overt evidence of cytotoxicity.

Effects of Cd on HMVEC and HUVEC migration

Migration of HMVECs and HUVECs was examined using chemotaxis chambers in the presence and absence of non-cytotoxic concentrations of Cd (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, or 100 μ M). HMVEC migration shown in Fig. 2 was induced by the chemoattractant PMA, and migration of non-Cd treated cells was used for comparison (bar shown on the left; arbitrarily set at 100%). HMVECs used in these studies were exposed to Cd for the first time during the chemotaxis assay, for a total of 4 hrs in the presence of 0.1% FBS. In the presence of PMA migration was significantly increased (i.e. 60 fold) as compared to negative control (data not shown). Fig. 2 shows results combined from 5 independent chemotaxis assays. Concentration-dependent decrease in PMA-induced HMVEC migration, as compared to the positive control, was observed at Cd concentrations of 100 μ M, 50 μ M, or 10 μ M Cd (*p<0.05). Lower concentrations of Cd also appeared to reduce HMVEC migration in a concentration-dependent manner, but did not achieve statistical significance.

HUVEC chemotaxis was also evaluated in a manner similar to that described above. Inclusion of Cd at concentrations of 50 μ M or higher during the HUVEC chemotaxis assay reduced migration in a statistically significant manner similar to that observed in the HMVECs, while lower doses did not (n=3; data not shown).

Effects of Cd on HMVEC and HUVEC tube formation

Initially, we examined HMVEC tube formation in the presence of Cd and 2% FBS for 18 hrs. Tube formation on growth factor reduced Matrigel (Fig. 3) was induced by 50 nM PMA, and tubes formed in the absence of Cd were used for comparison (bar shown on the left; arbitrarily set at 100%). Fig. 3 shows that incubation with 100 nM or 50 nM Cd had little effect on tube formation, but in contrast, concentrations of 500 nM Cd up to 100 μ M Cd significantly reduced HMVEC organization into tubules (*p<0.05).

Since it had been previously reported that Cd may act on the Matrigel rather than on ECs directly (Kishimoto et al., 1996b), we next designed a set of experiments where ECs were pre-treated with Cd, and then allowed to form tubes in the absence of the metal for 18 hrs. HUVECs were exposed to Cd for 48 hrs in their full growth media (5% serum and other growth factors), washed with PBS to remove unbound extracellular Cd, and then assayed for tube formation in the absence of Cd. With this experimental design, the Matrigel matrix would not directly be exposed to Cd. Fig. 4 shows that pre-incubation of HUVECs with Cd disrupted tube formation on Matrigel as assayed by counting the number of nodules formed (nodules are places where 3 or more tubes come together). In comparison with the positive control (PMA without Cd-treatment), pre-incubation with 50 μ M, 10 μ M, 1 μ M, and even 100 nM Cd significantly inhibited HUVEC tube formation (* p <0.05). These results are representative of 3 independent assays. Identical experiments were performed using HMVECs and yielded similar results when microvascular ECs were pre-treated with various concentrations of Cd for 72 hrs (n =3; data not shown).

Effects of Cd on the localization of VE-cadherin

Previous studies have shown that the Ca^{2+} -dependent cell adhesion molecule VE-cadherin is critical to angiogenesis (Vittet et al., 1997; Liao et al., 2000), and that Cd can disrupt VE-cadherin mediated cell-cell adhesion of HUVECs (Prozialeck, 2000), suggesting that VE-cadherin may be a target of Cd on ECs. Therefore, we examined the effects of Cd on the localization of VE-cadherin in the EC's. Fig. 5A shows a confluent monolayer of non-Cd treated HUVECs stained for VE-cadherin localization. The location of VE-cadherin immunofluorescence at junctions between cells is demonstrated by bright green fluorescence outlining the cells. Fig. 5B is a representative photo of cells treated with 100 μ M Cd for 18 hrs in the presence of 5% FBS. In comparison with immunofluorescence noted around non-Cd treated HUVECs, lower levels of VE-cadherin were noted at cell-cell junctions (shown by arrows) in addition to periodic gaps between cells. Similarly, treatment of HUVECs with Cd at concentrations of 10 μ M (C), 1 μ M (D), and 100 nM (E) for 18 hrs in the presence of serum reduced VE-cadherin localization to cell junctions in a concentration-dependent manner. In contrast, no Cd-related effect on VE-cadherin localization to cell junctions was observed in HUVEC's exposed to 10 nM Cd (F). Photos are representative of findings from 3 separate experiments. A similar set of experiments was used to examine VE-cadherin expression on HMVECs. The results were nearly identical to those described for HUVECs, where 100 nM, 500 nM, 1 μ M, 5 μ M, 10 μ M, and 50 μ M Cd appeared to alter VE-cadherin expression at cellular junctions, but 50 nM Cd did not (n =3; data not shown).

Discussion

Recent studies indicate that the vascular endothelium may be a primary target of Cd toxicity (Prozialeck et al., 2006). However, there is a relative lack of studies directly examining the effects of Cd on the process of angiogenesis under physiologically relevant conditions. Even though Kishimoto and coworkers examined the effects of Cd on HUVEC growth, migration and tube formation (Kishimoto et al., 1996a; Kishimoto et al., 1996b), those studies were performed using free uncomplexed Cd, that is, Cd in the absence of serum. When considering physiologic exposure to Cd *in vivo*, it is important to note that essentially all Cd in the blood is bound to proteins, sulfhydryl compounds, or amino acids (Bridges and Zalups, 2005; Prozialeck et al., 2006). In essence, ECs are never exposed to uncomplexed Cd under normal physiologic conditions. Therefore, we aimed to examine the extent to which Cd altered EC viability, migration, and tube formation in the presence of serum, which allows for Cd to complex with various serum components. In addition, since these Cd complexes *in vivo* are expected to interact with both microvascular and macrovascular ECs, our studies utilized both HMVECs (microvascular) and HUVECs (macrovascular). Finally, the serum concentrations

that were used for respective assays were chosen based on successful application in previous publications (e.g. chemotaxis assays performed with 0.1% serum and tube formation assays at 2% serum (Woods et al., 2003)). Similarly, Cd exposure times applied here were tailored to the individual assay. For example, 4 hrs is a typical migration period for ECs in a chemotaxis assay (Woods et al., 2003), and we aimed to determine the effect of Cd that was present during migration. Shorter migration times don't always allow for a sufficient number of cells to count, while longer time periods don't maintain a gradient and may represent chemokinesis (random migration). Tube formation assays on Matrigel typically require at least 18 hours for ECs to align and form tubes that are easily visualized upon staining, which is why an 18 hour Cd exposure was applied. The initial cytotoxicity assays were performed for 6 hours in the presence of Cd, since we anticipated the serum present in our assay to protect cells for longer than the 3 hours shown to be cytotoxic previously (Kishimoto et al., 1996a). A subsequent 18 hour exposure was analyzed to assure that tube formation assays performed for this time period would not utilize cytotoxic concentrations of Cd, since relatively high concentrations of Cd were applied. While these differing exposure times to Cd were utilized, we believe our studies may have relevance to both acute and chronic *in vivo* situations. The 4, 6, and 18 hr exposures applied in cytotoxicity, chemotaxis, and tube formation assays are perhaps most consistent with acute exposures which occur during industrial accidents or studies on experimental animals (Prozialeck *et al.*, 2006). In these situations, tissues are likely exposed to relatively high concentrations of Cd for relatively short periods of time. Alternatively, our pretreatment of ECs with low concentrations of Cd for 48–72 hrs, followed by tube formation assays, may begin to approximate conditions of chronic exposure *in vivo*, where vascular ECs would be expected to be exposed to relatively low levels of Cd for extended periods of time.

Examination of the acute cytotoxic effects of Cd on both HMVECs and HUVECs in the presence of serum indicated that these cells are remarkably resistant to Cd-induced injury. Even concentrations as high as 1 mM Cd were not cytotoxic to the majority of ECs. These data contrast with previous studies which suggested that 3 hr treatment of HUVECs with 100 μ M Cd was able to kill macrovascular ECs, while exposure to 1 mM Cd killed >90% of cells (Kishimoto et al., 1996a).

Knowing that 18 hr exposure to 1 mM Cd was not cytotoxic to the majority of ECs under these experimental conditions, we designed our chemotaxis experiments to utilize non-cytotoxic concentrations of Cd with an even shorter exposure time. Thus, our observed changes in chemotaxis could not have been attributable to acute cytotoxic injury. It should also be noted that our migration studies were performed using chemotaxis chambers, while previous cell migration studies by Kishimoto involved scraping a confluent HUVEC monolayer and watching for cell movement into the scraped area over a 24 hr period (Kishimoto et al., 1996a). While the previous paper did conclude that HUVEC migration was inhibited by Cd exposure, it was notable that the Cd concentrations at which this occurred were acutely cytotoxic.

Since EC migration is only one aspect of the angiogenic process that can be studied *in vitro*, we also examined EC tube formation. Under proper conditions, the ECs will burrow into the matrix, align themselves, and differentiate into small capillary structures over an 18 hr period. Inclusion of Cd during this process, inhibited EC tube formation. A concentration of Cd as low as 500 nM significantly reduced the number of tubes formed. As noted previously, Kishimoto et al. (1996b) reported that pre-treatment of the Matrigel matrix by Cd inhibited formation of tubes by ECs and concluded that Cd did not act on the ECs directly, but rather acted on the Matrigel. However, that study did not completely rule out the possibility that Cd from the extracellular matrix may have acted on the cells when they were applied to the matrix. In the present studies, the HUVECs were pretreated with Cd, washed to remove any excess metal, and subsequently assayed for tube formation in the absence of Cd. While this experimental

design does not completely rule out the possibility that Cd can affect Matrigel, these results strongly suggest that Cd inhibits tube formation by acting directly on the EC's themselves. This observation is consistent with recently published findings by Kolluru and coworkers showing that Cd can directly inhibit endothelial cell migration and tube formation (Kolluru et al., 2006).

VE-cadherin is a transmembrane calcium binding protein that is localized at the adherens junctions between vascular endothelial cells and plays a critical role in the process of angiogenesis (Vittet et al., 1997; Liao et al., 2000). Disruption of VE-cadherin function by monoclonal antibodies has been shown to inhibit tube formation by EC's in culture and inhibit angiogenesis in vivo (Liao et al., 2000). Other studies had shown that Cd was able to alter the localization of VE-cadherin in cultured HUVEC's (Prozialeck, 2000) and capillary EC's in mouse lung (Pearson et al., 2003). The results of the present studies indicate that under experimental conditions similar to those that inhibit tube formation by HUVEC's and HMVEC's, Cd causes a reduction in the amount of VE-cadherin that is associated with the endothelial cell-cell junctions. Taken together, these observations suggest that VE-cadherin may be a primary target through which Cd exerts its antiangiogenic effects.

In considering the implication of these findings to the actions of Cd in vivo, it is important to note that the threshold concentrations for various antiangiogenic effects of Cd described here are between 10–500 μ M, which is a range that approximates the levels of Cd to which ECs could be exposed under specific conditions in vivo. With the chronic, low-moderate levels of exposure that are typically seen in humans, blood levels of Cd are usually in the range of 10–100 nM (Nordberg et al., 1986; Jarup et al., 1998; Hotz et al., 1999). With higher levels of exposure, commonly used in animal studies, blood levels may be 5–10 times higher (Nordberg et al., 1986). However, over time, Cd can accumulate in specific tissues, most notably the liver and kidney, where tissue concentrations can reach well into the mM range (Nordberg et al., 1986) and ECs in these tissues would be exposed to Cd from both the luminal and the antiluminal compartments, at concentrations that are similar to those that inhibited angiogenesis in the present studies.

The results of the present studies could also have implications regarding the reported carcinogenic and anticarcinogenic effects of Cd. Cd exposure is recognized to result in a variety of malignancies in animals including leukemia, lung and prostate cancer and injection-site sarcomas (IARC, 1993; Waalkes, 2003). Thus, Cd has been classified as a human carcinogen by the International Agency for Research on Cancer (IARC, 1993; ATSDR, 1997). However, there are also findings which strongly suggest that under certain conditions Cd can have anticarcinogenic effects. Waalkes and coworkers have published several *in vivo* studies showing that Cd can inhibit the growth and development of several tumor types although they were not able to identify the mechanisms underlying this effect (Waalkes et al., 1991; Waalkes et al., 1993; Waalkes et al., 1996; Waalkes and Diwan, 1999). The results of the present studies suggest that one possible mechanism through which Cd might inhibit tumor formation may be through inhibiting blood vessel formation or via disrupting existing blood vessels, recognizing that without angiogenesis, tumor growth is halted at a few millimeters and metastasis is cut off (Sipkins et al., 1998).

Taken together, these data suggest that Cd acts in an angiostatic manner on ECs by disrupting their ability to migrate and form tubes. Toxicity studies suggest that in the presence of small amounts of serum, ECs are rather resistant to Cd-induced cytotoxicity. Additional evidence suggests that disruption of VE-cadherin localization on the EC surface may contribute to these angiostatic effects of Cd. Finally, results of the present studies indicate that Cd can act directly on the ECs, since pre-incubating ECs with Cd inhibits tube formation when Cd is not directly in contact with the extracellular matrix. We speculate that evidence for this angiostatic action

of Cd may already have *in vivo* support from previous studies demonstrating that under the proper conditions, Cd can suppress the growth and development of malignant tumors although additional studies are needed to resolve this issue.

Acknowledgments

Portions of this work were supported by grant R01 ES006478 from the National Institute of Environmental Health Sciences to W.C.P.. J.M.W. is supported by NIH Grant R15 AR050985 as well as an Arthritis Foundation Arthritis Investigator Award. The study sponsors had no involvement in any aspect of study design, collection, analysis, data interpretation, writing, or the decision to submit this manuscript.

References

- Angst BD, Marozzi C, Magee AI. The cadherin superfamily: diversity in form and function. *Journal of Cell Science* 2001;114:629–641. [PubMed: 11171368]
- ATSDR. Toxicologic Profile for Cadmium. Atlanta, GA: 1997.
- ATSDR. 2005. <http://www.atsdr.cdc.gov/clist.html>
- Bridges CC, Zalups RK. Molecular and ionic mimicry and the transport of toxic metals. *Toxicology and Applied Pharmacology* 2005;204:274–308. [PubMed: 15845419]
- Degraeve N. Carcinogenic, teratogenic and mutagenic effects of cadmium. *Mutation Research* 1981;86:115–135. [PubMed: 7015119]
- Elinder, CG.; Kjellstrom, T. Carcinogenic and mutagenic effects.. In: Friberg, L.; Elinder, CG.; Kjellstrom, T.; Nordberg, GF., editors. *Cadmium and Health: A Toxicological and Epidemiological Appraisal*. CRC Press; Boca Raton, FL: 1986. p. 205-229.
- Hotz P, Buchet JP, Bernard A, Lison D, Lauwerys R. Renal effects of low-level environmental cadmium exposure: 5-year follow-up of a subcohort from the Cadmibel study. *Lancet* 1999;354:1508–1513. [PubMed: 10551497]
- IARC. Cadmium and cadmium compounds. Beryllium, cadmium, mercury, and exposures in the glass manufacturing industry. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* 1993;58:119–237. [PubMed: 8022055]
- Jarup L, Berglund M, Elinder CG, Nordberg G, Vahter M. Health effects of cadmium exposure--a review of the literature and a risk estimate. *Scandinavian Journal of Work, Environment & Health* 1998;24 (Suppl 1):1–51.
- Kar AB, Das RP. Testicular changes in rats after treatment with cadmium chloride. *Acta Biologica et Medica Germanica* 1960;5:153–173. [PubMed: 13751376]
- Kishimoto T, Oguri T, Ohno M, Matsubara K, Yamamoto K, Tada M. Effect of cadmium (CdCl₂) on cell proliferation and production of EDRF (endothelium-derived relaxing factor) by cultured human umbilical arterial endothelial cells. *Archives in Toxicology* 1994;68:555–559.
- Kishimoto T, Oguri T, Yamabe S, Tada M. Effect of cadmium injury on growth and migration of cultured human vascular endothelial cells. *Human Cell* 1996a;9:43–48. [PubMed: 9183629]
- Kishimoto T, Ueda D, Isobe M, Tada M. Cadmium injures tube formation by cultured human vascular endothelial cells. *Human Cell* 1996b;9:244–250. [PubMed: 9183655]
- Kolluru GK, Tamilarasan KP, Geetha Priya S, Durgba NP, Chatterjee S. Cadmium induced endothelial dysfunction: consequence of defective migratory pattern of endothelial cells in association with poor nitric oxide availability under cadmium challenge. *Cell Biology International* 2006;30:427–438. [PubMed: 16616865]
- Levin AA, Plautz JR, Di Sant'Agnese PA, Miller RK. Cadmium: placental mechanisms of fetal toxicity. *Placenta Supplemental* 1981;3:303–318.
- Liao F, Li Y, O'Connor W, Zanetta L, Bassi R, Santiago A, Overholser J, Hooper A, Mignatti P, Dejana E, Hicklin DJ, Bohlen P. Monoclonal antibody to vascular endothelial-cadherin is a potent inhibitor of angiogenesis, tumor growth, and metastasis. *Cancer Research* 2000;60:6805–6810. [PubMed: 11156369]
- Nolan CV, Shaikh ZA. The vascular endothelium as a target tissue in acute cadmium toxicity. *Life Sciences* 1986;39:1403–1409. [PubMed: 3534498]

- Nordberg, GF.; Kjellstrom, T.; Nordberg, G. Kinetics and metabolism.. In: Friberg, L.; Elinder, CG.; Kjellstrom, T.; Nordberg, GF., editors. Cadmium and Health: A Toxicological and Epidemiological Appraisal. CRC Press; Boca Raton, FL: 1986. p. 103-178.
- Parizek J. Vascular changes at the site of oestrogen biosynthesis produced by parenteral injection of cadmium salts: the destruction of placenta by cadmium salts. *Journal of Reproductive Fertility* 1964;7:263–265.
- Parizek J, Zahor K. Effect of cadmium salts on testicular tissue. *Nature* 1956;177:1036. [PubMed: 13322022]
- Pearson CA, Lamar PC, Prozialeck WC. Effects of cadmium on E-cadherin and VE-cadherin in mouse lung. *Life Sciences* 2003;72:1303–1320. [PubMed: 12570930]
- Prozialeck WC. Evidence that E-cadherin may be a target for cadmium toxicity in epithelial cells. *Toxicology and Applied Pharmacology* 2000;164:231–249. [PubMed: 10799334]
- Prozialeck WC, Edwards JR, Woods JM. The vascular endothelium as a target of cadmium toxicity. *Life Sciences* 2006;79:1493–1506. [PubMed: 16765992]
- Sipkins DA, Cheresh DA, Kazemi MR, Nevin LM, Bednarski MD, Li KC. Detection of tumor angiogenesis in vivo by alphaVbeta3-targeted magnetic resonance imaging. *Nature Medicine* 1998;4:623–626.
- Straub AC, Stolz DB, Ross MA, Hernandez-Zavala A, Soucy NV, Klei LR, Barchowsky A. Arsenic stimulates sinusoidal endothelial cell capillarization and vessel remodeling in mouse liver. *Hepatology* 2007;45:205–212. [PubMed: 17187425]
- Vittet D, Buchou T, Schweitzer A, Dejana E, Huber P. Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proceedings of the National Academy of Sciences of the United States of America* 1997;94:6273–6278. [PubMed: 9177207]
- Waalkes MP. Cadmium carcinogenesis. *Mutation Research* 2003;533:107–120. [PubMed: 14643415]
- Waalkes MP, Diwan BA. Cadmium-induced inhibition of the growth and metastasis of human lung carcinoma xenografts: role of apoptosis. *Carcinogenesis* 1999;20:65–70. [PubMed: 9934851]
- Waalkes MP, Diwan BA, Rehm S, Ward JM, Moussa M, Cherian MG, Goyer RA. Down-regulation of metallothionein expression in human and murine hepatocellular tumors: association with the tumor-necrotizing and antineoplastic effects of cadmium in mice. *Journal of Pharmacology and Experimental Therapeutics* 1996;277:1026–1033. [PubMed: 8627513]
- Waalkes MP, Diwan BA, Weghorst CM, Bare RM, Ward JM, Rice JM. Anticarcinogenic effects of cadmium in B6C3F1 mouse liver and lung. *Toxicology and Applied Pharmacology* 1991;110:327–335. [PubMed: 1891777]
- Waalkes MP, Diwan BA, Weghorst CM, Ward JM, Rice JM, Cherian MG, Goyer RA. Further evidence of the tumor-suppressive effects of cadmium in the B6C3F1 mouse liver and lung: late stage vulnerability of tumors to cadmium and the role of metallothionein. *Journal of Pharmacology and Experimental Therapeutics* 1993;266:1656–1663. [PubMed: 8371163]
- Wolf MB, Baynes JW. Cadmium and mercury cause an oxidative stress-induced endothelial dysfunction. *Biomaterials* 2007;20:73–81. [PubMed: 16752219]
- Woods JM, Mogollon A, Amin MA, Martinez RJ, Koch AE. The role of COX-2 in angiogenesis and rheumatoid arthritis. *Experimental Molecular Pathology* 2003;74:282–290.

Fig. 1. HUVECs are resistant to Cd-induced cytotoxicity

Live (non-Cd treated; positive control) HUVECs grown to confluence introduced to calcein AM/EtBr take up cell-permeable calcein AM and convert it to green fluorescent calcein (A). In contrast, cells intentionally killed by MeOH-treatment (B) take up ethidium homodimer through their damaged membranes and exhibit nuclei which fluoresce red (negative control). Cells treated with 10 mM Cd (C) for 18 hrs in the presence of 0.1% FBS are dead and less adherent, whereas 1 mM Cd treatment (D) rendered cells less adherent, but did not necessarily kill them. Treatment of HUVECs with 100 μ M (E) or 1 μ M Cd (F) had little discernable effect on the cells. Photos are representative of 3 independent assays (Live/Dead® Viability/Cytotoxicity staining; 100x original magnification).

*p<0.05

Fig. 2. Cd inhibits PMA-induced HMVEC migration

HMVEC chemotaxis was induced in all wells using 50 nM PMA. Bars represent HMVEC migration relative to the positive control (non-Cd treated). The presence of Cd at concentrations of 10–100 μM significantly reduced migration to PMA, shown by an asterisk (*; p<0.05). HMVECs in this assay did not encounter Cd until the start of the migration period. This graph depicts combined results from 5 independent assays, where bars and error bars represent the mean $\pm$ the SEM.

*p<0.05

Fig. 3. Cd inhibits PMA-induced HMVEC tube formation

HMVEC tube formation was induced on growth factor reduced Matrigel with 50 nM PMA, where non-Cd treatment served as the positive control. Bars and error bars represent the mean tube length as percent of control values $\pm$ the SEM from 6 independent assays. The presence of Cd at concentrations of 0.5–100 μ M for 18 hrs significantly reduced tube formation, shown by an asterisk (*; $p < 0.05$). In this assay, HMVECs were not exposed to Cd until the start of tube formation.

Fig. 4. Pre-incubation with Cd inhibits HUVEC tube formation

HUVECs were incubated with or without various concentrations of Cd for 48 hours prior to assessing PMA-induced tube formation in the absence of Cd. Unbound Cd was removed with 2 PBS washes prior to incubating the cells on growth factor reduced Matrigel in 5% FBS. Bars and error bars represent the mean number of nodules $\pm$ the SEM from 3 independent assays. An asterisk (*) represents a significant reduction ($p<0.05$) in nodular contacts when compared with tubes formed by non-Cd treated HUVECs.

Figure 5. Cd disrupts VE-cadherin localization to HUVEC junctions

Confluent monolayers of HUVECs were stained by immunofluorescence for VE-cadherin expression (green), demonstrating protein localization at cell junctions in non-Cd treated cells (A). Cells treated with 100 μ M Cd (B), 10 μ M Cd (C), 1 μ M Cd (D), or 100 nM Cd (E) for 18 hrs in the presence of 5% FBS exhibited gaps between cells and reduced VE-cadherin localization to cell junctions (shown by arrows). HUVECs treated with 10 nM Cd (F) were indistinguishable from non-Cd treated cells (A). All photos are representative of typical staining observed in 3 independent assays (400x original magnification).