Presenilin 1/γ-secretase Is Associated with Cadmium-Induced E-Cadherin Cleavage and COX-2 Gene Expression in T47D Breast Cancer Cells

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Received July 1, 2008; accepted September 10, 2008

Cadmium is a heavy metal that has multiple toxic effects on human health and has been classified as a human carcinogen. E-cadherin is a major target of cadmium; however, the roles of E-cadherin and cadmium and the mechanisms of tumor progression remain to be defined. Here, we demonstrate that cadmium increases E-cadherin processing via a γ-secretase in the T47D breast cancer cell lines. This presenilin 1 (PS1)/γ-secretase-dependent cleavage of E-cadherin was accompanied by changes in reactive oxygen species or calcium. E-cadherin cleavage was blocked by a PS1 dominant-negative mutant, γ-secretase inhibitors [N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) and L-685,486], antioxidants (N-acetylcysteine and Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride), or a calcium chelating drug 1,2-bis (o-Aminophenoxo)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester. Immunofluorescence analysis confirmed the disappearance of E-cadherin staining at the cell surface. Those inhibitors attenuated cadmium-induced cytotoxicity. Additionally, cadmium treatment increased cell motility and invasion ability, which was abated by DAPT. Interestingly, cyclooxygenase-2 (COX-2) expression induced by cadmium was also inhibited by DAPT. The cadmium-induced cell motility and invasion ability were inhibited by a COX-2 inhibitor, NS398. Our data indicate a novel molecular mechanism that links cytotoxicity of cadmium and disrupted E-cadherin processing to adhesion junctions; cadmium induces COX-2 expression via γ-secretase, which increases cell motility and invasion ability. Understanding the downstream signaling cascades of cadmium that promote tumor progression might be a key to the development of novel therapeutic strategies.

Key Words: cadmium; γ-secretase; COX-2; E-cadherin; apoptosis.
cleaving β-amyloid precursor protein (APP), γ-secretase has other protein substrates, including Notch, ErbB4, CD44, and E-cadherin (Iwatsubo, 2004). Recent reports have also shown that E-cadherin cleavage during apoptosis is mediated by γ-secretase in A431 cells (Marambaud et al., 2002). Because γ-secretase inhibitors are also able to prevent Notch receptor activation, various γ-secretase inhibitors have been tested for antitumor effects (Shih le and Wang, 2007). Paris et al. (2005) showed that γ-secretase inhibitors can attenuate tumor growth and angiogenesis in tumors and in the vasculature.

The dysfunction of E-cadherin–mediated cell adhesion plays an important role in tumor progression from benign tumor to invasive, metastatic carcinoma (Hirohashi, 1998). Numerous studies have indicated that the disappearance of E-cadherin after exposure to Cd is followed by cancer migration and enhanced invasion ability. However, the mechanisms involved in the disappearance of E-cadherin and the metastatic potential of Cd remain unknown.

We investigated whether the motility and invasion activity following exposure to Cd is mediated by γ-secretase. Because Cd exposure is associated with E-cadherin disappearance, we studied the mechanisms by which Cd induces E-cadherin downregulation and how γ-secretase is involved in the effects of Cd. We show that increased production of reactive oxygen species (ROS) and changes in intracellular Ca2+ are some of the first events in T47D breast cancer cells exposed to Cd; they are followed by E-cadherin cleavage by γ-secretase, which precedes E-cadherin disappearance at the cell membrane. The γ-secretase inhibitors reduced the motility and invasiveness of T47D cells after Cd exposure. We suggest that γ-secretase activation is an important step in Cd-induced E-cadherin disappearance. And, COX-2 is a key player for the motility and invasiveness increases, along with γ-secretase.

**MATERIALS AND METHODS**

**Materials.** The γ-secretase inhibitors [N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT), L-685,486], N-(R)-[2-(hydroxymaminocarbonyl)methyl]-4-methylpentanoyl-[l]-naphthylalanyl-[l]-alanine, 2-aminoethyl amide (TAPI), GM6001, 1,2-bis(o-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM), 4′,6-diamidino-2-phenylindole (DAPI), ionomycin (A23187), MNRbII/1etraakis (1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP), and z-Ve-Ala-Asp (zVAD)-fmk were purchased from Calbiochem (La Jolla, CA). Antibody H108 against E-cadherin ectodomain was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). E-cadherin antibodies (C36) and IgG2a,k antibodies were obtained from BD Transduction Laboratories (San Jose, CA). N-acetylcysteine (NAC), CoCl2, NiCl2, PbCl2, CuCl2, and CdCl2 were purchased from Sigma (Saint Louis, MO). Alamar blue was obtained from Invitrogen (Camarillo, CA). Presenilin 1 (PS1) dominant-negative (DN) mutant (D385A) construct was a gift from Dr Kwonseop Kim (College of Pharmacy, Chonnam National University, Korea).

**Cells and culture conditions.** Human breast cancer T47D cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin, in a 5% CO2 atmosphere at 37°C. T47D cells were used because they exhibit low invasive potential. Thus, they were suitable for the examination of increased invasion.

**Cell lysates and Western blotting.** Cells in plates were washed with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation (RIPA) buffer (1% Nonidet P-40, 150mM NaCl, 50mM Tris-HCl, pH 7.5, 0.1% sodium dodecyl sulfate, 1mM phenylmethylsulfonyl fluoride, 0.1mM Na3VO4, and 100 μg/ml of leupeptin). The lysates were cleared by centrifugation (15,000 rpm, 5 min), and the protein concentrations were determined using the bicinchoninic acid method before storage at −80°C. Equivalent amounts of protein were separated on NaPAGE (4–12%; Invitrogen) gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% fat-free milk in Tris-buffered saline with 0.1% Tween 20 and incubated with the primary antibody overnight at 4°C. Immune complexes were then detected using the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

**Assays of cell viability.** The Alamar blue (Fatokun et al., 2006) was measured using fluorescence-based kits from Biosource International (Camarillo, CA). Alamar blue was measured at excitation and emission wavelengths of 555 and 600 nm, respectively. Cell viability was expressed as a percentage of the control and mean ± SE for multiple wells tested in at least three separate experiments. Statistical significance was determined using Student t-test. p < 0.05 was deemed statistically significant.

**Reverse transcription-PCR.** Total RNA from T47D cells was extracted using the TRizol (Invitrogen) reagent, according to the manufacturer’s protocol. For semiquantitative reverse transcription (RT)-PCR, cDNA was prepared from total RNA using the oligo-dT15 primer, 10mM deoxynucleoside triphosphate (dNTP), RNasin RNase inhibitor (Promega, Madison, WI), 0.1mM diithiothreitol, 5× first-strand buffer, and moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR amplification was performed using the AccuPower PCR premix (Bioneer, Daejeon, Korea), containing MgCl2 (1.5mM), KCI (30mM), Tris-HCl (pH 9.0, 10mM), dNTP (0.25mM each), 1 U of Taq DNA polymerase, stabilizer, and tracking dye, and 2 pmol of cox-2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Sense and antisense primers for cox-2 were 5′-TTCATAAGGATAGGGGAAAA-TTGCTC-3′ and 5′-AGCATCATTCTGCTGATGATCT-3′, respectively. The sense and antisense primers for GAPDH were 5′-CCATGTTCGCTATGGG-TGTGAACCA-3′ and 5′-GCCAGTAGAGCCAGGATGATGTC-3′, respectively. The predicted sizes of the PCR products were 305 bp for cox-2 and 251 bp for GAPDH. The PCR products were quantified using the Image Gauger densitometry software (V3.12; Fuji film, Tokyo, Japan). Each arbitrary unit (AU) of cox-2 mRNA expression was normalized by the AU of GAPDH mRNA expression.

**Real-time quantitative RT-PCR.** Real-time quantitative RT-PCR analysis was performed using SYBR Green PCR core reagent, in two-step RT-PCR protocol according to manufacturer’s protocol (Applied Biosystems, Warrington, UK). Initial denaturation at 95°C for 10 min was followed by 40 amplification cycles of 95°C for 15 s and 58°C for 1 min. Primers for cox-2 were sense 5′-GAGAAAATCTTACAACCGGA-3′ and antisense 5′-CACAAGTTTCCA-AATCCCCTTGTG-3′ and for β-actin: sense 5′-AGAAAAATCGGACACCA-CC-3′ and antisense 5′-GGGGTGTTGAAGGTCTCAAA (Maret et al., 2007). The relative quantification was normalized to the β-actin gene expression levels. PCR reactions were performed using ABIPrism 7900 SDS (Applied Biosystems). The mean threshold cycle (Ct, the first cycle at which an exponential growth of PCR product is detected) value of stimulated sample was compared to that of unstimulated control sample using the Ct value of β-actin as an internal control. ΔCt is the difference in Ct values derived from cox-2 gene (in each sample assayed) and β-actin gene, while ΔΔCt represented the difference between paired samples.

**Immunocytochemistry and antibodies.** The cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized in the blocking buffer (1% goat serum, 0.1% bovine serum albumin in PBS) containing 0.2% Triton X-100 at room temperature for 10 min, and blocked in blocking buffer for 1 h at room
temperature. They were then incubated with anti–E-cadherin antibody (C36) (1:200) (BD Transduction laboratories) in blocking buffer at 4°C overnight. For the negative control, the cells were incubated with mouse IgG2a,k (1:200) (BD pharmaning, San Diego, CA). The cells were incubated with Alexa 488–conjugated goat anti-mouse antibodies (1:1000) (Molecular Probes, Eugene, Oregon) for 1 h, washed in PBS, and then photographed using a fluorescence microscope. Images were assembled using PhotoShop (Adobe, San Jose, CA).

**Cell migration and invasion assays.** For migration and invasion assays, the QCM 24-well colorimetric cell migration assay (ECM508; Chemicon, Temecula, CA) and the Cell Invasion Assay Kit (ECM550; Chemicon) were used. Assay steps were performed according to the manufacturer’s protocol, except that some steps were optimized. Briefly, for the migration assay, cells that had been stabilized by passage two to three times were harvested using trypsin-ethylenediaminetetraacetic acid solution. Harvested cells were grown to 70% confluence in six-well plates, washed twice with PBS, and incubated for another 18–24 h in serum-free medium for cell starvation. The cells were then incubated in various conditions and were detached, counted, and dissolved in serum-free medium. Cells (6 × 10^5) were brought into the upper chamber, which was then inserted into the lower chamber with 500 μl of medium with 10% serum. Each chamber was incubated for 24 h at 37°C in 5% CO2. After removing medium from the top side by gentle pipetting, the upper chamber was placed into a clean well containing 400 μl of cell-staining solution and incubated for 20 min at room temperature to stain migrated cells. The upper chamber was rinsed with water to remove nonmigrated cells and air-dried according to the manufacturer’s instructions. The stained upper chambers were then transfected to clean wells containing 200 μl of extraction buffer and incubated for 15 min at room temperature. The optical density at 560 nm was then measured by transferring the extraction buffer (100 μl) to a 96-well microtiter plate. For DAPI staining, the membranes, along with the cells that migrated across the membrane, were fixed with 2% paraformaldehyde in PBS and stained with DAPI. Stained cells in three representative fields were photographed using a digital camera and counted. These assays were carried out in triplicates.

The invasion assay was similar to the migration assay, except that the upper chamber was preincubated with warm, serum-free medium for 1–2 h to rehydrate the extracellular matrix layer. The stained upper chambers were then transferred to clean wells containing 200 μl of extraction buffer (10% acetic acid).

**Statistical analysis.** Data were expressed as mean ± SD. Data were either initially analyzed by ANOVA followed by Duncan post hoc test. The level of significance was set at p < 0.05.

**RESULTS**

**Cd-Induced E-cadherin Cleavage in Breast Cancer Cells**

To determine the time and concentration dependence of the effect of Cd on intracellular levels of E-cadherin, we treated T47D cells with various concentrations (1–100 μM) of CdCl2. After incubation for 1–48 h, E-cadherin C-terminal fragment (E-cad/CTF) levels in total lysates were analyzed by Western blotting. Cd treatment of T47D cells resulted in the time-dependent production of E-cad/CTFs in A431 cells. To study the involvement of γ-secretase in E-cadherin processing by Cd in breast cancer cells, we first examined the effect of γ-secretase inhibitors on Cd proteolysis of E-cadherin in the breast carcinoma cell line T47D. The γ-secretase inhibitors, DAPT and L685,486, completely blocked the production of E-cad/CTF2 by 25 μM Cd in T47D cells (Fig. 2A). T47D cells were also transfected with PS1, which is a component of the γ-secretase complex, or a PS1 DN mutant to block γ-secretase activity. Transfection of wild-type PS1 increased E-cad/CTF2 production without Cd exposure (Fig. 2B). Transfection of the PS1 DN mutant increased C-terminal fragments of APP by blocking γ-secretase activity. As expected, transfection of the PS1 DN mutant blocked E-cad/CTF2 production by low cytotoxic dose of Cd (5 μM) (Fig. 2B). These observations suggest that the E-cad/CTF2 production by Cd was a result of PS1/γ-secretase activity. Since MMPs are the obvious candidate to cleave E-cadherin, we also investigated the effect of MMP inhibitors on Cd-induced E-cadherin processing. Figure 2A showed that MMP inhibitors, such as TAPI or GM6001, did not affect E-cadherin processing by 25 μM Cd. We also rule out the possibility that E-cad/CTF1 band represent MMP-mediated E-cadherin products because MMP inhibitors treatment could not attenuate E-cad/CTF1. Cd induces oxidative stress and increases intracellular Ca2+ levels (Biagioli et al., 2008; Yokouchi et al., 2008). Thus, we examined the effects of ROS and calcium on the production of E-cad/CTF2 by Cd. To block oxidative stress or Ca2+, T47D cells were treated with NAC or BAPTA-AM, respectively. Both drugs (10 mM NAC and 10 μM BAPTA-AM)
blocked the production of E-cad/CTF2 by 100 μM Cd (Fig. 2C). In addition, H2O2-induced oxidative stress or the calcium ionophore A23187 induced E-cad/CTF2 production. Since NAC has been shown to be a chelator of divalent cations, NAC was removed from the media before adding Cd to rule out Cd-NAC complex formation. The results showed that NAC also blocked E-cadherin cleavage by Cd (Fig. 2D). To further confirm that Cd-induced E-cad/CTF2 production is mediated by ROS, we also tested MnTmPyP, which is a cell-permeable superoxide dismutase and catalase mimetic (Day et al., 1997). In total, 50 μM MnTmPyP also blocked Cd-induced E-cad/CTF2 production (Fig. 2E). These results suggest that Cd induces γ-secretase–dependent E-cad/CTF2 production via oxidative stress and increased Ca2+ in cells.

**Cd Decreased E-Cadherin Levels at the Cell Surface**

Cd disrupts the E-cadherin distribution on the cell surface (Pearson and Prozialeck, 2001; Prozialeck, 2000). The Cd-mediated cleavage of E-cadherin cytoplasmic domains proximal to the transmembrane region would be expected to decrease the E-cadherin level on the cell surface. To assess this, we used immunofluorescent microscopy to examine alterations in the E-cadherin distribution on the cell surface as a result of Cd treatment. E-cadherin was located primarily at cell-cell contacts between T47D cells. Staining for E-cadherin at the plasma membrane of T47D cells with the anti–E-cadherin antibody (C36) was reduced after treatment with Cd. Consistent with the cleavage of E-cadherin by Cd (Fig. 1), Cd treatment disrupted cell-cell adhesion and decreased plasma membrane staining of cytoplasmic E-cadherin in T47D cells (Fig. 3). There was no signal in negative controls using IgG2a,k as primary antibody (data not shown). Preincubation with an antioxidant (NAC) or an intracellular calcium chelating drug (BAPTA-AM) significantly delayed the loss of E-cadherin staining at the cell surface after Cd exposure. Further, DAPT treatment partially preserved E-cadherin at cell-cell contacts. However, preincubation with a caspase inhibitor (zVAD) or an MMP inhibitor (GM6001) did not delay the loss of E-cadherin staining at the cell membrane after Cd exposure. These results suggest that inhibitors of caspase (zVAD) or MMP (GM6001) did not block the effect of Cd on cell junctions and cadherin cleavage. Treatment of GM6001 did not block E-cad/CTF2 production by Cd (Fig. 2A). It should be noted that GM6001 did not block γ-secretase–mediated E-cad/CTF by apoptosis either (Marambaud et al., 2002). Our results suggest that E-cadherin cleavage and disassembly of the adherens junction by Cd are caused by γ-secretase activation via ROS or Ca2+ signaling.
Cd Induced the Release of the E-Cadherin Ectodomain in Breast Cancer Cells via γ-Secretase

E-cadherin cleavage via γ-secretase after Cd exposure may induce the release of the E-cadherin ectodomain from the cell plasma membrane. To confirm this, we measured E-cadherin levels in the medium after Cd treatment. Cd increased E-cadherin levels in the medium, and DAPT partially attenuated the Cd-induced release of E-cadherin (Fig. 4). However, MMP inhibitor, GM6001, did not block Cd-induced release of E-cadherin.

Cd Induces Cytotoxicity via ROS/Calcium/γ-Secretase

To investigate the effect of Cd on the viability of T47D cells, the Alamar blue assay was performed after the exposure to various concentrations of Cd for 2, 4, 8, and 24 h.

Exposure to 25–100μM Cd significantly decreased cell viability in a dose-dependent manner (Fig. 5). We also examined the mechanisms involved in the Cd-induced cytotoxicity by investigating the effects of NAC, BAPTA-AM, and DAPT. Consistent with the inhibitory effect of DAPT on cleavage of E-cadherin by Cd (Figs. 3 and 4), DAPT partially blocked Cd-induced cytotoxicity (Fig. 5). Furthermore, BAPTA-AM and NAC could block cytotoxicity induced by exposure to 100μM Cd for 8 h. In our study, caspase inhibition by zVAD treatment did not attenuate Cd-induced cytotoxicity either (data not shown). Our results suggest that Cd-induced cytotoxicity is mediated by ROS/calcium/γ-secretase.

E-Cadherin Cleavage by γ-Secretase Is Specific to Cd Exposure

We investigated whether other heavy metals could increase the cleavage of E-cadherin by γ-secretase. To confirm the Cd specificity of E-cad/CTF2 production, T47D cells were exposed to cobalt (Co), nickel (Ni), lead (Pb), copper (Cu), and cadmium (Cd) ions. After 8 h of exposure, Western blots were performed to detect E-cadherin cleavage. Only Cd
increased E-cad/CTF2 production (Fig. 6). Although other metals can induce apoptosis, E-cad/CTF2 was not produced by them. These results suggest that E-cadherin cleavage by \( \gamma \)-secretase is specific to Cd exposure.

**Cd Increased Cell Motility and Migration via \( \gamma \)-Secretase**

We evaluated the effect of Cd on the biological properties of cells that are important for metastasis: cell motility and invasion into the basement membrane. Cd treatment for 2 h increased T47D cell migration by approximately 1.6 times (Fig. 7A). The inhibition of \( \gamma \)-secretase by DAPT decreased Cd-induced T47D cell migration. Treatment with 5\( \mu \)M Cd for 24 h also increased T47D cell motility (Fig. 7B). The matrix invading ability of breast cancer cells is associated with metastatic potential. To investigate changes in the metastatic potential of T47D cells induced by Cd, we assessed their invasion ability. The invasion ability was approximately 2–3.5 times higher in Cd-treated T47D cells than in control cells; this effect was abated by DAPT (Fig. 7C). We also performed a modified migration assay using DAPI staining to confirm cell migration. The results indicated that Cd increased cell migration and DAPT blocked the effect (Fig. 7D).

**Cd Increases Cell Motility and Migration via COX-2**

Because a previous study described COX-2 induction following Cd exposure (Figueiredo-Pereira et al., 2002), we analyzed the expression of COX-2 in T47D cells and the effect of a COX-2 inhibitor (NS398) on the motility and invasion ability of T47D cells after Cd exposure. Since COX-2 has been linked with metastatic potential, it is worth examining the role of COX-2 on Cd-induced cell motility and invasion ability. To establish whether Cd affects cox-2 gene expression, we carried out semiquantitative RT-PCR or real-time RT-PCR using gene-specific primers. Cd (100\( \mu \)M) induced cox-2 mRNA in a time-dependent manner (Figs. 8A and 8D). After exposure to Cd, the levels of cox-2 mRNA reached a maximum at 6 h. Exposure to Cd concentrations between 1 and 10\( \mu \)M for 24 h caused considerable induction of cox-2 mRNA (Fig. 8B). The GAPDH mRNA levels did not change significantly in these conditions. However, a \( \gamma \)-secretase inhibitor (DAPT) inhibited the increase of cox-2 mRNA expression by Cd exposure (Fig. 8C). Real-time quantitative experiments indicated a \( \gamma \)-secretase inhibitor (DAPT)–inhibited cox-2 mRNA expression following Cd exposure (Fig. 8E). These findings suggest that Cd upregulates cox-2 gene expression via \( \gamma \)-secretase in cultured T47D cells.

To test the possibility that the increased cell motility and invasion ability by Cd may be mediated through COX-2 expression, we assessed whether COX-2 inhibition could block the increased motility and invasion ability after Cd exposure. The inhibition of COX-2 by NS-398 decreased the Cd-increased T47D cell motility and invasive ability (\( p < 0.05 \); Figs. 8F and 8G). Our observations indicate that Cd upregulates cox-2 gene expression via \( \gamma \)-secretase, resulting in increased cell motility and invasion ability.

**DISCUSSION**

Previous studies have shown that Cd has specific damaging effects on cadherin-dependent junctions. Exposure to Cd
causes cells in culture to separate from each other and to change morphologies; they become rounded. Based on this, it has been suggested that a loss of cadherin-associated adhesion at the cell surface may contribute to metastasis. Given that E-cadherin is a tumor suppressor, it is reasonable to hypothesize that E-cadherin cleavage is an important step in tumorigenesis caused by Cd exposure.

Despite evidence suggesting a connection between Cd and E-cadherin, there has been little evidence illustrating the mechanisms connecting Cd-induced signaling and E-cadherin downregulation at the cell surface. In this study, we used two concentrations of Cd, high cytotoxic dose (100 \mu M) and low cytotoxic dose (5 \mu M). Although 100 \mu M concentration is higher than physiological range of cadmium, they are within the range of concentration seen at localized regions such as lung, in acute, high-dose exposure (Pearson and Prozialeck, 2001). Our observations not only show the direct activation of \( \gamma \)-secretase by Cd but also provide a possible molecular mechanism for how Cd induces E-cadherin cleavage: via \( \gamma \)-secretase at cell-cell contacts. Our results show that exposure to Cd causes alterations in the pattern of E-cadherin localization, via \( \gamma \)-secretase. Figure 1A shows a band at \(-75\) kDa whose steady-stage level declines after cadmium treatment. However, it may be the result of nonspecific staining by immunoblotting because the \(-75\)-kDa band in Figure 2A does not correlate with E-cadherin/full-length. We also ruled out the possibility that the \(-75\)-kDa band represents the precursor of E-cad/CTF2 because DAPT treatment fails to produce \(-75\)-kDa band (Fig. 2A). Additionally, we showed that oxidative stress also induced E-cadherin cleavage via \( \gamma \)-secretase. Until now, NAC has been widely used as a scavenger of ROS on cadmium-induced cytotoxicity. Tandon et al. (2003) reported that NAC or mannitol reversed the Cd-induced alterations of blood and liver glutathione, blood catalase, SOD, and other antioxidants without lowering blood cadmium contents. These results show that the NAC effect is not likely via chelating Cd\(^{2+}\) ions. There is a recent report that NAC upregulates catalase and blocks ROS generation by Cd (Oh and Lim, 2006). Our findings are significant in light of the important role of \( \gamma \)-secretase in regulating epithelial barrier function and metastatic capability. The disruption of adherens junctions at cell borders is associated with progression toward tumor malignancy and promotes invasion and metastasis. It is important to examine the relationship between the Cd-induced changes in cadherin localization and other effects of Cd. From the observed effects of Cd on T47D cells (Fig. 1), we speculated that \( \gamma \)-secretase activation by Cd disrupted adherens junctions and in turn promoted cellular migration and invasion, which are key steps in metastasis. Our demonstration that a \( \gamma \)-secretase inhibitor blocked Cd-induced cell motility and invasion ability may indicate that \( \gamma \)-secretase is a key mediator of Cd-mediated cancer development. Similarly, Notch signaling...
FIG. 8. Cd increases the migration/motility and invasion of T47D cells via COX-2. (A, D) Time-dependent cox-2 gene expression after exposure to 100μM Cd. (B) Dose dependency of cox-2 gene expression after Cd treatment for 24 h. (C, E) Cells were preincubated with 2.5μM DAPT and then exposed to 100μM Cd for 2 h. (A–C) Semiquantitative RT-PCR detection of cox-2 and GAPDH gene expression was conducted as described in the Materials and Methods. The results are representative of at least two independent experiments. (D, E) Real-time RT-PCR detection of cox-2 and β-actin gene expression was conducted as described in the Materials and Methods. Values are the mean ± SE. *P < 0.05 versus untreated control; #P < 0.05 versus Cd-treated group.

activation, which also occurs via γ-secretase, has been reported in the development of breast cancer (Shih Ie and Wang, 2007).

Previous report showed that E-Cad/CTF2 promotes the lysosomal/endosomal degradation of the transmembrane APP derivatives, C99 and C83, and inhibits production of the β-amylloid precursor protein intracellular domain (Agiostrati-dou et al., 2006). To date, the cellular functions of E-cad/CTF are still unknown. Our observation implicates E-Cad/CTF2 in gene expression or cancer development by Cd. Further studies will be required to fully understand the role of E-cad/CTF2 on Cd-mediated cellular events.

There is growing evidence that COX-2 promotes not only proliferation but also invasion ability (Singh et al. 2005; 2007; Tsuji et al., 1997). COX-2 can promote tumorigenesis (Chang et al., 2006). The cox-2 gene, a target of Cd (Figureiredo-Pereira et al., 2002; Seok et al., 2006), is overexpressed in several epithelial malignancies, including breast cancer. Given that a γ-secretase inhibitor blocked Cd-induced cox-2 expression (Fig. 8), the apparent increase in cox-2 expression by Cd was likely due to the γ-secretase activity. Our observations suggest that γ-secretase activation is a key event in the Cd-induced cascade.

Prozialeck et al. proposed that Cd interacts with a putative Ca+2-binding domain of E-cadherin and thereby disrupts the E-cadherin interaction with catenin (Prozialeck and Lamar, 1999). The increased Ca+2 levels in cytosol might be due to the released Ca+2 from E-cadherin by Cd2+. In our study, γ-secretase activation by Cd is mediated by Ca2+ (Fig. 2C). Given that intracellular calcium chelating drug (BAPTA) or antioxidant (NAC) blocked the loss of E-cadherin at surface by Cd (Fig. 3), intracellular events caused by Cd are also necessary to disrupt cell-cell junction. It suggests that E-cadherin cleavage is one of key mechanisms for disappearance of E-cadherin from the surface.

Previous reports that Cd induces nuclear translocation of β-catenin (Thevenod et al., 2007), and β-catenin increases cox-2 mRNA (Araki et al., 2003; Lee and Jeong, 2006), also provides supporting evidences for a role of γ-secretase in the Cd-induced signaling pathways. Thus, we examined the function of COX-2 in the Cd-regulated motility and invasion ability that may involve γ-secretase. Evidence indicates that COX-2 promotes transformation and invasion abilities (Brown and DuBois, 2005). For example, the inhibition of COX-2 reduces the motility of HCA-7 colon carcinoma cells (Chang et al., 2006). In cerebrovascular endothelial cells, COX-2 is also associated with Cd-induced ICAM-1 expression (Seok et al., 2006), which was correlated with the metastatic potential of each of the cell lines. The most metastatic MDA-MB-435 breast cancer cells showed the highest level of COX-2 expression (Schmid et al., 2002). Our observations, in which COX-2 inhibition blocked Cd-mediated motility and invasion, provide support for COX-2-mediated metastasis.

Because it is a major component of cigarette smoke, Cd is thought to be involved in smoking-associated vascular malfunctions (Kolluru et al., 2006). Although Cd does not directly produce ROS, antioxidants attenuate Cd toxicity, suggesting that this metal induces an increase in ROS production (Stohs and Bagchi, 1995). Our observation that γ-secretase can mediate cytotoxicity, COX-2 expression, cell migration, and invasion following Cd exposure raises the possibility that γ-secretase may be involved not only in Cd-induced gene expression but also in other biological properties such as metastasis.

FUNDING

ACKNOWLEDGMENTS

We thank Dr Kwonseop Kim for PS1 DN mutant construct. We also thank Dr K. S. Ahn for her valuable comments and Mrs. S. Y. Hur for her assistance in preparing the manuscript.

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