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REVIEW

Cadherins and NCAM as Potential Targets in Metal Toxicity

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Cell adhesion molecules are cell surface proteins that play critical roles in cell recognition and cell adhesion. These adhesion molecules, which include the cadherins, integrins, occludins, and a variety of immunoglobulin-like molecules, are essential for a wide variety of physiologic processes such as epithelial barrier function, tissue development, learning and memory, and immune responses. In light of the evidence that toxic metals can affect many of these processes, investigators have begun to examine the possibility that cell adhesion molecules may be targets for metal toxicity. This review summarizes the results of recent studies showing that certain cell adhesion molecules, particularly the cadherins family of Ca^{2+}-dependent cell adhesion molecules and the immunoglobulin family of Ca^{2+}-independent cell adhesion molecules, may be important early targets on which toxic metals such as a Cd, Hg, and Pb act to produce their toxic effects. These metals, and in some cases their organic compounds, can target cell adhesion molecules at multiple levels, including protein-protein interactions, post-translational modification, and transcriptional regulation. Moreover, by interfering with the normal function of the cadherin family of cell adhesion molecules, some of these metals may activate the β-catenin nuclear signaling pathway. These studies have provided important new insights into the molecular mechanisms of metal toxicity and have opened several exciting avenues of research.

Key Words: **cadherins; β-catenin; cadmium; lead; mercury; methylmercury; NCAM; trimethyltin.**

Toxic metals such as lead, mercury, and cadmium pose serious risks to human health (for reviews, see Jarup *et al.*, 1998; ATSDR, 1997, 1999, 2000). As a result of the extensive use of these metals in industry and in consumer products, these agents are widely disseminated in the environment. Since these metals are nonbiodegradable, they can persist in the environment and produce a variety of adverse effects in living organisms. In humans, exposure can result in damage to a variety of organ systems including the lung, liver, kidney, bone, immune system, and nervous system. In some cases, these metals also have the potential to be mutagenic, teratogenic, and carcinogenic. The risks of exposure to lead, mercury, and cadmium are so serious that the Agency for Toxic Substances and Disease Registry currently ranks all three metals among the top 10 on its priority list of hazardous substances (ATSDR website: http://atsdr.cdc.gov/).

One of the most active areas of biomedical research over the past 15 years has focused on the molecular mechanisms by which cells interact with each other. Studies in this area have led to the discovery of a variety of specialized cell surface proteins that play critical roles in cell recognition and cell adhesion. These cell adhesion molecules, which include the cadherins, integrins, occludins, and a variety of immunoglobulin-like molecules, are cell surface proteins that serve as mechanical components of the structures that maintain cell-cell and cell-substrate attachments. In many cases, they are also linked to intracellular signaling pathways and serve important roles as regulators of cell function and gene expression. In these contexts, cell adhesion molecules are essential for a wide variety of physiologic processes such as epithelial barrier function, tissue development, learning and memory, and immune responses (for reviews, see Edelman, 1985, 1986; Edelman and Crossin 1991; Grunwald, 1996a, 1996b, 1998; Knudsen *et al.*, 1998; Takeichi, 1990; Walsh and Doherty, 1997).

In light of the evidence that toxic metals can affect many of these processes, investigators have begun to examine the possibility that cell adhesion molecules may be targets for metal toxicity. Results of these pioneering studies suggest that certain cell adhesion molecules, particularly the cadherins family of Ca^{2+}-dependent cell adhesion molecules and the immunoglob-

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ulin family of Ca^{2+}-independent cell adhesion molecules, may be important early targets on which some metals act to produce their toxic effects (for reviews, see Prozialeck, 2000; Reuhl and Dey, 1996; Reuhl and Grunwald, 1997). These studies have provided important new insights into the molecular mechanisms of metal toxicity and have opened several exciting avenues of research. In order to highlight some of the most recent work on the role of cell adhesion molecules in metal toxicity, a symposium "Cell Adhesion Molecules as Potential Targets in Metal Toxicity" was held at the 2001 Meeting of the Society of Toxicology in San Francisco. The purpose of this report is to summarize the work presented in that symposium.

Multiple Roles for Divalent Cations in Regulation of Cadherin Cell Adhesion Molecules Reveal Potential Targets for Heavy Metal Toxicants (Gerald B. Grunwald)

The cadherins represent one of the largest and most diverse classes of calcium-dependent cell adhesion molecules. Thus far, over 40 cadherins have been described with the best characterized being the classical cadherins such a P-, N-, and E-cadherin (for reviews, see Angst *et al.,* 2001; Gallin, 1998; Nollet *et al.,* 2000; Redies, 2000). These classical cadherins have similar molecular weights and amino acid compositions, but differ in their tissue distribution patterns, immunological characteristics, and cell-binding specificities. The cadherins are especially important in the formation and maintenance of adhesions among epithelial and neural cells.

Cadherin function is associated with important events at all stages of the life cycle. For example, the earliest discernible morphogenetic event in mammalian development, compaction of the 8-cell morula, is mediated by E-cadherin (Vestweber and Kemler, 1984). The subsequent complex cellular rearrangements that occur during gastrulation and neurulation are controlled in part by differential E- and N-cadherin expression. Later events in the histogenesis of differentiated tissues also involve cadherin function. This is especially well-documented in nervous tissues, where studies of N-cadherin expression and perturbation have demonstrated a major role for N-cadherin in the organization of nervous tissues and in processes such as axon growth and synaptogenesis. In both early and late stages of neural development, antibody-mediated or molecular genetic perturbation studies have clearly demonstrated that disruption of cadherin expression and function leads to abnormal development (Bronner-Fraser *et al.,* 1992; Chazal *et al.,* 2000; Covault, 1989; Detrick *et al.,* 1990; Grunwald, 1998; Lagunowich *et al.,* 1994; Tepass *et al.,* 2000).

The cadherins are transmembrane proteins that include an extracellular domain, a transmembrane region, and an intracellular domain (Alattia *et al.,* 1999; Grunwald, 1996a; Norvell and Green, 1998; Ringwald *et al.,* 1987). The extracellular domain includes the Ca^{2+}-binding sites, as well as the adhesive regions of the molecule. The binding of Ca^{2+} causes the molecule to assume a rigid conformation and orients the adhesive regions so that the cadherin from one cell can interact, in a homologous manner, with the same cadherin from an adjacent cell (Nagar *et al.,* 1996; Pokuta *et al.,* 1994; Shapiro, 1995). In most cases, the intracellular domain of the cadherin is linked to the actin cytoskeleton via a family of proteins known as catenins (Wheelock *et al.,* 1996). The catenin family is made up of three major isoforms: α-catenin, β-catenin, and γ-catenin. Both β-catenin and γ-catenin can directly bind to the cadherin C-terminal domain, while α-catenin is indirectly attached to the cadherin through the β-catenin/γ-catenin complex. Disruption of cadherin linkages to the catenins renders the cadherin incapable of adhesion, leading directly to impaired function (Ozawa *et al.,* 1990; Pavalko and Otey, 1994). It should be noted that in addition to serving as a structural protein, β-catenin may also function as an intracellular signaling molecule and regulator of gene expression (Behrens, 1999; Ben-Ze'ev, 2000; Cadigan and Nusse, 1997; Gumbiner, 1995). Under certain conditions, β-catenin can be released from the junctional complexes into the cytosol, where it may either be targeted for proteasomal degradation by the adenomatous polyposis coli (APC) gene product, or it can be translocated to the nucleus, where it can bind to transcription factors such as TCF/LEF1 and stimulate the expression of various genes that regulate apoptosis and cell-cycle control (Barker *et al.,* 2000; Behrens, 1999; Ben-Ze'ev, 2000; Dale, 1998; Shtutman *et al.,* 1999). The degradation of cytosolic β-catenin is regulated by the wingless/Wnt signaling pathway. Activation of this pathway by Wnt ligands leads to an inhibition of the degradation of β-catenin, an increase in the distribution of the β-catenin to the nucleus, and subsequent alterations in gene expression. In this system, β-catenin serves a critical role in transducing the Wnt signal to the nucleus. Activation of the β-catenin signaling pathway has been implicated as a key regulator of a variety of physiologic processes including cell growth and differentiation, the induction of apoptosis and carcinogenesis (Behrens, 1999, 2000; Ben-Ze'ev, 2000; Cadigan and Nusse, 1997; Dale, 1998).

In considering the possible mechanisms by which metals might affect cadherin function, it is important to note that the cadherins are calcium-binding metalloproteins with specific binding sites for calcium in the extracellular domain. It is this critical interaction with calcium that provides one target for potential perturbation through calcium mimicry by metals (Grunwald, 1998; Reuhl and Grunwald, 1997). Depletion of calcium from the extracellular environment prevents cadherin-mediated cell adhesion due to a resulting conformational change in the cadherins. This shape change is detectable both by altered binding of conformation-sensitive antibodies as well as by the resulting enhanced trypsin sensitivity. A number of recent studies suggest that cadmium, and possibly other toxic metals, may disrupt cadherin function by displacing Ca^{2+} from its binding sites on the cadherin molecules (Duizer *et al.,* 1999; Lacaz-Vieira, 1997; Leussink *et al.,* 2000; Prozialeck, 2000; Zimmerhackl *et al.,* 1998).

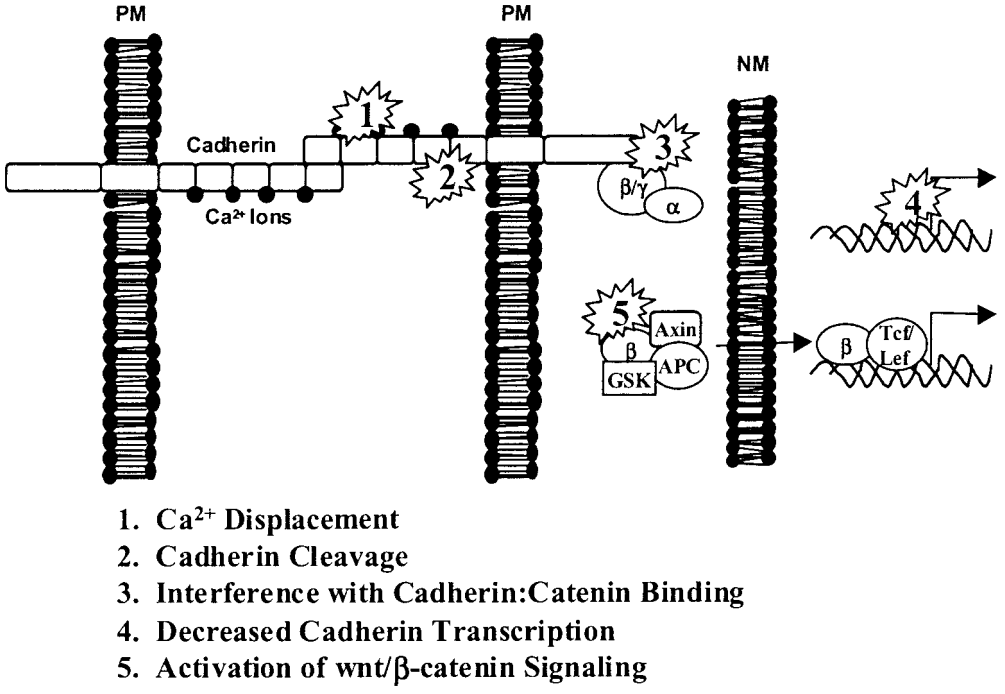

FIG 1. Structural organization of the cadherin/catenin complex showing potential targets of metal toxicity.

In addition, many other studies of metal toxicity have pointed to perturbations of cellular calcium and other essential divalent cations such as zinc as likely mechanisms of action. Indeed, it has been recognized for some time that a principal general mechanism by which lead and other toxic metals interfere with normal biological functions may be through either mimicking or inhibiting the physiologic actions of essential metals (Markovac and Goldstein, 1998; Mazzei *et al.*, 1994; Mills and Johnson, 1985; Pounds and Rosen, 1988). Thus, additional targets for metal perturbation of cadherin function could be through one or more of the many metal-dependent signaling pathways that regulate cadherin function. Indeed, one such indirect role for calcium in cadherin function has been revealed by the demonstration that some cadherin-mediated functions likely occur through the activation of calcium channels in the plasma membrane (Bixby *et al.*, 1994; Dougherty *et al.*, 1991). Metals such as lead have been shown to block Ca^{2+} channels (Alkoudon *et al.*, 1990; Ma *et al.*, 1998). Furthermore, the effects of heavy metals on calpain activity are of special interest since it has been previously suggested that N-cadherin is a substrate for calpain (Covault *et al.*, 1991) and metals such as lead can inhibit calpain activity (Audesirk *et al.*, 1998). Recent studies discussed in this symposium suggest that metals such as mercury can increase the generation of a 110-kDa calpain cleavage product of N-cadherin, with a concomitant loss of N-cadherin function.

A model summarizing how the cadherin/catenin complex may be targeted by toxic metals is illustrated in Fig. 1. This model is consistent with the data discussed above and in the hypothesis-driven experiments described in this symposium. As depicted in the model, there are several points at which cadherin perturbation by lead, cadmium, mercury, and other metals can be proposed based on knowledge of the biochemistry of cadherins and heavy metal interactions with proteins. Toxic metals may perturb cadherin function directly by competing for calcium-binding sites on the cadherins. Since calcium is known to affect the structure and adhesive-binding function of these adhesion molecules, such competition would be expected to modulate cadherin function. This could result in direct inhibition of adhesive function through alteration of the extracellular domain. Altered cadherin conformation could also perturb the association with the catenins and the underlying cytoskeleton, which would in itself result in suboptimal cadherin function. Furthermore, altered cadherin conformation resulting from calcium displacement could result in positive or negative modulation of the cadherins' susceptibility to proteolysis, via the metalloproteases that cleave cadherins to produce fragments such as the soluble 90-kDa ectodomain fragment of N-cadherin (NCAD-90) or the membrane-associated 110-kDa fragment (NCAD-110) (Covalt *et al.*, 1991; Paradies and Grunwald, 1993; Roark *et al.*, 1992). Finally, since cadherins and catenins also serve as signaling molecules, in addition to their adhesive function, perturbation could result in aberrant intracellular signaling responses. Indeed, the experiments described in the following presentations suggest that toxic metals may act in part through the direct or indirect perturbation of cell adhesion molecule expression and function, leading either to gross dysmorphogenesis or more subtle

derangements that ultimately become manifest as functional deficits.

The E-Cadherin/β-Catenin Complex as a Target of Cadmium Toxicity (Walter C. Prozialeck)

Cadmium (Cd^{2+}) is an important industrial and environmental pollutant that poses a serious health risk to humans and animals (reviewed in ATSDR, 1997; Jarup *et al.*, 1998). Depending on the dose, route, and duration of exposure, Cd^{2+} can cause damage to various organs including the lung, liver, kidney, bone, and placenta. In addition, Cd^{2+} has been shown to be carcinogenic (for review, see IARC, 1993; Waalkes, 2000). While the importance of Cd^{2+} as an environmental health problem has long been recognized, relatively little is known about the specific mechanisms by which Cd^{2+} produces its toxic effects. This presentation will summarize the results of recent studies suggesting that the E-cadherin/β-catenin complex may be a primary target of Cd^{2+} toxicity in various types of epithelial cells, and that this mechanism may help to explain some of the toxic effects of Cd^{2+} in specific target organs.

The finding that E-cadherin might be a target of Cd^{2+} toxicity stemmed from an observation that my colleagues and I made about 11 years ago while we were studying the effects of Cd^{2+} on cells of the established porcine renal epithelial cell line, LLC-PK_1. We found that exposure to 10–20 μM Cd^{2+} for 1–4 h caused the cells to separate from each other and change morphologically from epithelioid to rounded, an effect that coincided with the loss of E-cadherin from the cell-cell contacts and a reorganization of the actin cytoskeleton (Prozialeck and Niewenhuis, 1991a,b). These effects resembled those that occurred when the cells were incubated in the presence of the Ca^{2+} chelator EGTA (Prozialeck, 2000). Moreover, they occurred at Cd^{2+} concentrations and times of exposure that did not cause the loss of cell membrane integrity or alter cellular levels of ATP or glutathione, indicating that they represented relatively specific toxic actions of Cd^{2+} on the E-cadherin-dependent junctions between the cells (Prozialeck, 2000; Prozialeck and Lamar, 1995). The disruption of cadherin-dependent cell junctions by Cd^{2+} is not cell specific, as we have also found similar effects on N-cadherin junctions in rat osteosarcoma cells (Prozialeck, 2000) and on VE-cadherin junctions in human umbilical vein epithelial cells (Prozialeck, 2000; Lamar, 2000). Additionally, by using a model of Cd^{2+}-induced lung injury in mice, we found that Cd^{2+}-treated animals showed a decrease in the amount of E-cadherin in the epithelial cells of the alveoli and small bronchioles (Prozialeck, 2000) and of VE-cadherin in vascular endothelial cells (Pearson *et al.*, 2000). These data indicate that cadmium can disrupt cadherin-dependent cell-cell junctions in many types of epithelial cells *in vitro*, and that under certain circumstances can disrupt cadherin-dependent cell junctions *in vivo*.

The hypothesis that we developed to account for these observations was that Cd^{2+} might displace Ca^{2+} from its extracellular binding sites on E-cadherin and interfere with the normal adhesive function of the molecule (Prozialeck, 2000; Prozialeck, and Lamar, 1995; Prozialeck and Niewenhuis, 1991b). To further explore this possibility, we studied the interaction of Cd^{2+} with recombinant and synthetic polypeptide analogs of the extracellular Ca^{2+}-binding motifs of E-cadherin (Prozialeck, 2000; Prozialeck and Lamar, 1996, 1999). The results of these studies showed that Cd^{2+} could, in fact, displace Ca^{2+} from its binding sites on the polypeptides. In addition, results of circular dichroism (CD) spectroscopy studies with one of the polypeptides, termed Peptide B (sequence KVSATDADDDVLL) showed that Cd^{2+} and Ca^{2+} had significantly different effects on the secondary structure of the polypeptide (Prozialeck and Lamar, 1999). Both Cd^{2+} and Ca^{2+} caused a shift in the CD spectrum of the polypeptide that was characterized by a decrease in the molar ellipticity at 220 nm. However, the shift produced by Cd^{2+} was about 3 times the magnitude of that produced by Ca^{2+}, indicating that Cd^{2+} caused a distortion of the peptide B molecule. This is significant because if Cd^{2+} and Ca^{2+} can differentially affect the secondary structure of a simple peptide, such as peptide B, it would seem reasonable to expect that Cd^{2+} could cause even more pronounced distortions of complex polypeptides such as the E-cadherin molecule. It seems likely that such a distortion of secondary and tertiary structure could profoundly affect the spatial orientation of the adhesive regions of E-cadherin.

The finding that Cd^{2+} can interfere with the adhesive function of E-cadherin raises an intriguing question as to whether or not the disruption of E-cadherin-mediated cell-cell adhesion results in the breakdown of the E-cadherin/β-catenin complex. Such an effect could be especially significant in light of β-catenin's role as a signaling molecule and regulator of gene expression (Ben-Ze'ev *et al.*, 2000 and Behrens, 1999). In order to begin to explore this issue we examined the effects of Cd^{2+} on the cellular localization of E-cadherin and β-catenin in relation to the levels of expression of the β-catenin-regulated protooncogenes c-jun and c-myc in LLC-PK_1 cells. As seen in Fig. 2, E-cadherin and β-catenin were colocalized at the junctional contacts between the cells, with little or no β-catenin immunofluorescence present in the cell nuclei. Exposure to 10 μM Cd^{2+} for 4 h caused a pronounced loss of E-cadherin from the cell borders and the appearance of gaps between the cells. These effects coincided with a decrease in the amount of β-catenin labeling at the cell-cell junctions and a pronounced increase in the β-catenin labeling in the nuclei and perinuclear regions. Additional studies indicated that β-catenin began to accumulate in the nucleus after as little as 30 min to 1 h of Cd^{2+} exposure (not shown). To determine if the translocation of β-catenin to the nucleus might lead to an increase in β-catenin-stimulated gene transcription, we examined the effects of Cd^{2+} on the levels of mRNA encoding the *c-jun* and *c-myc* protooncogenes, genes which had previously been shown to be at least partially regulated by the β-catenin nuclear signaling system (Behrens, 2000; He *et al.*, 1998; Mann *et al.*, 1999). Interest-

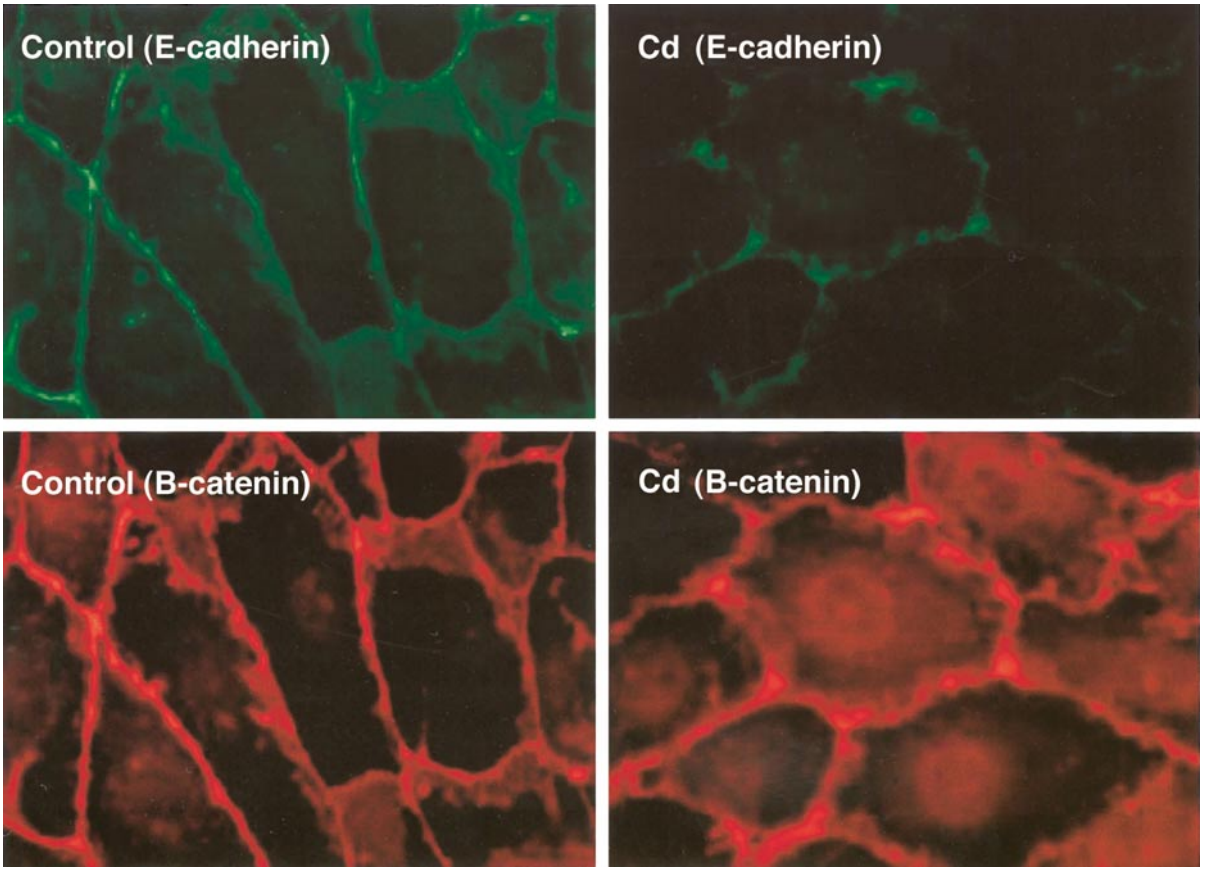

FIG 2. Effects of Cd^{2+} on the cellular localization of E-cadherin and β-catenin in LLC-PK_1 cells. LLC-PK_1 cells on glass coverslips were treated with 10 μM Cd^{2+} for 4 h. E-cadherin and β-catenin were visualized by indirect immunofluorescence using a dual-labeling procedure. The samples were fixed and permeabilized in −20°C methanol for 10 min, and then blocked with 10% goat serum in phosphate-buffered saline (PBS) for 10 min. The samples were incubated for 45 min at 37°C in PBS containing the primary antibodies, a mouse anti-human E-cadherin (Transduction Laboratories, Lexington, KY) at 1/100 dilution, and a polyclonal rabbit anti-β-catenin (Zymed Laboratories, South San Francisco, CA) at a 1/100 dilution, rinsed quickly in PBS, and then incubated for 30 min at 37°C in PBS containing the secondary antibodies, an FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co, St. Louis, MO) at a dilution of 1/50, and a TRITC-conjugated goat anti-rabbit IgG (Sigma) at a dilution of 1/100. The samples were rinsed, mounted on glass slides in Aqua Polymount (Polysciences, Inc, Warrington, PA), and viewed immediately using a Nikon Eclipse 400 fluorescence microscope and 100× objective. Digital images of a single field were captured using a Spot Jr. digital camera (Diagnostics Instruments, Sterling Heights, MI) and processed by using the Image-Pro Plus software package (Media Cybernetics, Silver Spring, MD).

ingly, *c-jun* mRNA was increased by 1 h, continued to increase at 2 h, and remained elevated until at least 4 h, while *c-myc* was increased by 2 h and began to decline by 4 h (not shown). The increases in the levels of *c-jun* and *c-myc* expression were similar to those previously reported by other investigators (Matsuoka and Call, 1995) and suggest that the Cd^{2+}-induced translocation of β-catenin to the cell nucleus might lead to an increase in β-catenin-mediated gene transcription. This finding could have important implications regarding the mechanisms of Cd^{2+} carcinogenesis in that it suggests a possible epigenetic mechanism by which Cd^{2+} could trigger the expression of protooncogenes such as *c-jun* and *c-myc* (Pearson and Prozialeck, 2001).

In summary, the results of these studies indicate that the cadherin/catenin complex may be a primary target of Cd^{2+} toxicity in many types of epithelial cells. This ability of Cd^{2+} to disrupt the cadherin/catenin complex may help to explain the mechanisms of Cd^{2+} toxicity in specific target organs, such as the lung, and could have important implications regarding the mechanisms of Cd^{2+} carcinogenesis.

Disturbances of the N-Cadherin Adhesion Complex in Neural Cells Following Mercury Exposure (P. Markus Dey)

The neurotoxicant mercury and its organic congener methylmercury represent major health threats to the developing fetus (ATSDR, 2000). Both inorganic mercury and methylmercury cause a spectrum of neurological disturbances depending on the dose, duration, and developmental age at exposure. Infants prenatally exposed to high levels of methylmercury exhibit abnormal cerebellar and cerebral cytoarchitecture, per-

sistence of fetal cortical structure, large numbers of heterotopic neurons, and aberrant neuritic orientation of neurons (Choi, 1986, 1989; Choi *et al.*, 1978; Takeuchi, 1977). Recent prospective human studies indicate that low, environmentally relevant exposure to methylmercury during fetal development results in persistent cognitive and psychomotor deficits (Grandjean *et al.*, 1998). Studies in developing animals confirm that low-level methylmercury can perturb dendritic spine formation, arborization, and neuritogenesis, with consequent behavioral impairment (Burbacher *et al.*, 1989; Stoltenburg-Didinger and Markwort, 1990).

N-cadherin is one of the major cell adhesion molecules that mediate neural morphogenesis (Takeichi, 1988). It is highly expressed on neural aggregates, migrating neural cells, on neurites during axon elongation, and tract formation, and diminishes once neurites have synapsed with their targets (Tanaka *et al.*, 2000). The ability of N-cadherin to support neural development is determined both by the expression of the molecule and by the competency of its associations with key cytoskeletal elements. N-cadherin levels are regulated at the transcriptional level, and specific promoter regions for N-cadherin have been identified (Li *et al.*, 1997). Posttranslational regulation of N-cadherin expression occurs, in part, via developmentally regulated proteolytic loss of the molecule at cell surfaces (Geiger *et al.*, 1990; Paradies and Grunwald, 1993). N-cadherin proteolysis at neural cell surfaces results in the generation of two major forms retaining the N-cadherin extracellular domain, a 110-kDa membrane-associated (NCAD110) isoform and a 90-kDa (NCAD90) soluble N-cadherin fragment (Covault *et al.*, 1991, Paradies and Grunwald 1993; Roark *et al.*, 1992). Perturbation in transcriptional and/or posttranslational control of N-cadherin during development is associated with adverse effects on both brain morphology and neurobehavior (Bennett *et al.*, 1997; Dey *et al.*, 2000). Interactions among the intracellular domain of N-cadherin, the catenins, and the neuronal cytoskeleton stabilize N-cadherin-mediated adhesion and cell signaling during neural development (Knudsen *et al.*, 1998).

Disturbed expression of N-cadherin alters neural development. Administration of an N-cadherin blocking antibody during embryonic chick brain development disrupts neuroepithelial structure and results in marked disorganization of diencephalic gray matter *in ovo* (Gaenzler-Odenthal and Redies, 1998). *In vitro* studies indicate that perturbation in N-cadherin expression can inhibit neurite outgrowth and pathfinding in neural cell culture models (Bixby and Zhang, 1990), and impair long-term potentiation in hippocampal slices (Tang *et al.*, 1998). Importantly, many of these N-cadherin-mediated defects phenocopy those observed in animal models of metal exposure (as reviewed in Reuhl and Dey, 1996; Reuhl *et al.*, 1994).

The fact that N-cadherin functions as a morphogenetic regulator of brain development raises the possibility that exposure to mercurials might impair neurodevelopment by disturbance of N-cadherin expression and function. To address this question, *in vitro* experiments were undertaken to investigate the ability of inorganic mercury or methylmercury to perturb N-cadherin expression and function. Studies using Day 11 chick cortical cells indicate changes in N-cadherin-catenin adhesion complex expression following exposure to mercury chloride. Twenty-four hours of exposure of cortical cell cultures to either 1 or 10 μM mercury chloride resulted in loss of N-cadherin-β-catenin association, as demonstrated by Western blot analysis. Analysis of β-catenin expression in N-cadherin immunoprecipitates from detergent-extracted cell supernatant from control and mercury-treated cultures revealed a dose-dependent decrease in the amount of β-catenin bound to N-cadherin after mercury exposure (data not shown). This occurred in the absence of changes in total cellular β-catenin or N-cadherin protein expression (data not shown). As seen in Fig. 3, these changes in the N-cadherin-catenin assocation were accompanied by an increase in the generation of the truncated N-cadherin isoform NCAD110. Immununoprecipitation of cell culture supernatants of control and mercury-treated cultures with anti N-cadherin antibody also indicates the increased production of the soluble N-cadherin proteolytic fragment NCAD90 following mercury exposure (Fig. 3). Western blotting for total β-catenin in Triton-soluble and insoluble fractions from these cell lysates indicated that mercury treatment also altered β-catenin cellular distribution, with increased amounts of β-catenin associated with the Triton-insoluble fraction (data not shown).

Cerebellar cell culture studies indicate that mercurials can also affect N-cadherin mRNA expression. Semiquantitative PCR analysis of N-cadherin expression revealed that exposing cerebellar cells to methylmercury resulted in a significant decrease in N-cadherin mRNA expression. Fourty-eight hours of exposure to either 0.2 or 2 μM methylmercury resulted in a dose-independent 25% reduction in cerebellar N-cadherin mRNA levels when compared to control cultures.

The genetic and epigenetic mechanisms by which methylmercury disturbs N-cadherin expression and/or function may provide insight into the importance of N-cadherin dysregulation in a variety of human developmental brain disorders, such as autism and other idiopathic retardation syndromes. Development of toxicological models of cell adhesion molecule-mediated neural dysgenesis will have broad applications as tools in the investigations into mechanisms of birth defects and neuropathologies of unknown etiology.

Calcium-Independent Adhesion Molecules in Heavy Metal Neurotoxicity (Kenneth R. Reuhl)

The calcium-independent adhesion molecules constitute a large family of morphoregulatory molecules of the immunoglobulin superfamily. Each member possesses one or more immunoglobulin-like domains, consisting of approximately 100 amino acids bridged by a disulfide linkage. Many, but not

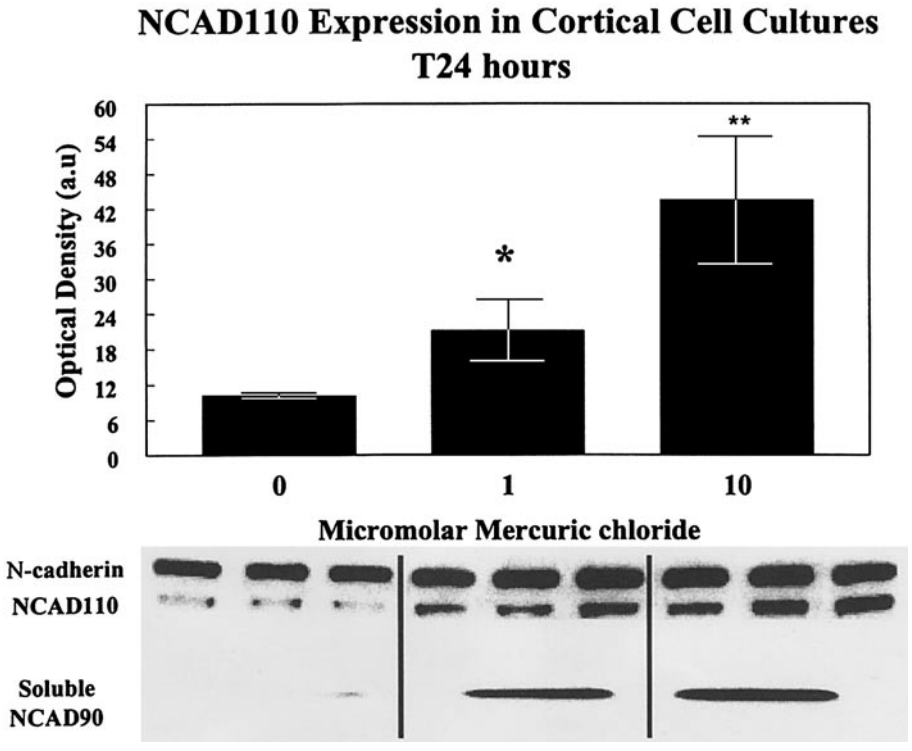

FIG 3. Production of truncated (NCAD110 and NCAD90) N-cadherin isoforms following exposure to $HgCl_2$. Embryonic day 11 chicken cortical cell cultures were exposed for 24 h to 0, 1, or 10 μM $HgCl_2$. The cell pellet and cell culture media was obtained, resolved on SDS-PAGE gels, and immunoblotted for N-cadherin. Mercury treatment resulted in a dose-dependent increase in the NCAD110 membrane fragment isoform and increased generation of the soluble NCAD90 fragment ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$).

all, also have a single membrane-spanning region and a variably sized intracellular domain (Figure 4). These cell adhesion molecules show complex spatial and temporal patterns of expression within the CNS reflecting their protean roles during development and adulthood.

The neural cell adhesion molecule (NCAM) is the best characterized member of the calcium-independent adhesion molecules and serves as representative of this family. NCAM is a surface glycoprotein that plays a key role in a variety of developmental processes in the CNS (Chozal *et al.,* 2000; Doherty *et al.,* 1992, 1995; Ronn, 1998). As may be seen in Fig. 4, the NCAM molecule is characterized by five Ig loops and two fibronectin Type III repeats in the highly conserved extracellular region. At least three major NCAM isoforms (180, 140, and 120 kDa) are present in the adult brain, all generated by alternative splicing of transcripts encoded by a single gene located on chromosome 9 of mouse, 8 of rat, and 11 in humans (Ronn *et al.,* 1998). The three NCAM forms have highly conserved amino terminal domains and differ primarily in the length of the intracellular domain. NCAM180 has the longest intracellular domain; this region of the molecule interacts with the spectrin/fodrin component of the cytoskeleton (Pollerberg *et al.,* 1987). NCAM140 has a shorter cytoplasmic domain and lacks direct cytoskeletal linkages, while NCAM 120 lacks a membrane-spanning element entirely and is linked to the plasma membrane by a glycosylphosphatidyl inositol (GPI) anchor.

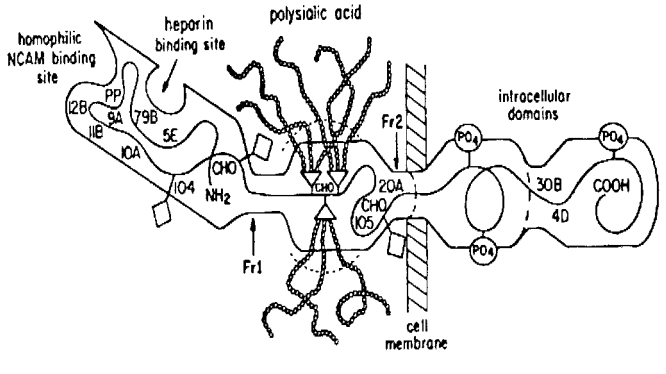

FIG 4. Structure of NCAM.

In addition to the molecular variability provided by alternative splicing, NCAM is subjected to a number of posttranslational modifications, the most important of which is the developmentally regulated addition of variable amounts of polysialic acid (PSA) residues to the 5th immunoglobulin loop of the extracellular domain. Long chains of PSA residues, connected by an a-2,8 linkage, are added to the core NCAM molecule by several sialyltransferase enzymes resident in the Golgi apparatus (Regan, 1991). The sialyltransferases are themselves un-

der complex developmental regulation, and their appropriate activity is critical for the proper expression of PSA-NCAM. Appropriate temporal and spatial expression of PSA-NCAM is necessary for morphogenetic events requiring positional rearrangement of neural elements, such as neuronal migration, neurite outgrowth and branching, and development of synaptic fields. During brain development, neurons are heavily decorated with PSA-NCAM, which may constitute up to 30% of the molecule's weight. The PSA residues provide a large, negatively charged volume which inhibits close intercellular proximity and adhesion, thereby preventing the initiation of contact-mediated events previously described.

To date, there have been relatively few detailed studies of metal effects on calcium-independent adhesion molecules, and most of these have focused on NCAM. The large extracellular domain, essential for specific binding, possesses several motifs potentially vulnerable to direct metal binding. The transmembrane and intracellular domains, which participate in second messenger cascades and also physically stabilize the molecule within the plasma membrane, are vulnerable to proteolysis. NCAM-cytoskeleton linkages are likely targets of metals, as many metals have demonstrated ability to disrupt cytoskeletal integrity (Chou, 1989; Mills and Fern, 1989; Wang and Templeton, 1996). Finally, the biosynthetic machinery of the cell (particularly the rough endoplasmic reticulum and Golgi apparatus) represents documented targets of toxic metals and their impairment would affect NCAM function. As such, alterations in NCAM have been demonstrated following exposure to lead (Breen and Regan, 1988a,b; Cookman *et al.*, 1987; Doyle *et al.,* 1992a,b; Regan, 1989, 1993; Dey *et al.,* 2000), trimethyltin (Dey *et al.*, 1997), and methylmercury (Dey *et al.*, 1999; Reuhl and Dey, 1996).

Methylmercury has been shown to affect NCAM in both developing and adult animals. Unlike lead, which appears to exert its effect primarily on NCAM-PSA biosynthesis by sialyltransferases, methylmercury is able to alter both the regulation of posttranslational polysialation and the intact NCAM molecule at the plasma membrane. Direct effects of methylmercury on the NCAM molecule have been described (Reuhl *et al.,* 1994). Cultured neurons showed a rapid loss of NCAM immunostaining on the plasma membrane following exposure to methylmercury, initially involving the most distal portion of the neurite and proceeding centripetally to involve the perikaryon. This response preceded disassembly of neuritic microtubules or neurite retraction. Following removal of methylmercury from the medium, NCAM reappeared on the neurons over a period of several hours. This delay reflects the insertion of newly synthesized NCAM, as its reexpression could be blocked by protein synthesis inhibitors. The mechanism by which methylmercury blocks NCAM immunoreactivity at the plasma membrane is not clear but may involve removal of NCAM by an extracellular protease or masking of the antigenic-binding site. Alternatively, methylmercury is a well-recognized cytoskeletal toxicant and may amputate the attachment of NCAM to the cytoskeleton. There is evidence in support of this mechanism, as methylmercury can activate intracellular calpain, which can cleave the intracellular NCAM linkage with the cytoskeleton.

The effects of methylmercury on NCAM have also been demonstrated in brain of developing animals. Dey *et al.* (1999) examined the effects of methylmercury on rat cerebellar PSA-NCAM and found a reduction of PSA-NCAM in brain homogenates, synaptosomes, and isolated growth cones on Postnatal Day 15 and in whole brain homogenates on Postnatal Day 30. Golgi sialyltransferase activity was reduced on Day 15 but had returned to control levels by day 30. Methylmercury treatment also resulted in transient reexpression of PSA-NCAM in cerebellum. Acute treatment resulted initially in reduction of PSA-NCAM within the hippocampus and other limbic structures. However, if methylmercury treatment ceases, PSA-NCAM levels may rebound and exceed control levels. This enhancement likely represents reactive/reparative sprouting of synaptic networks and possibly reparative axonal branching.

Given the key role of NCAM and other calcium-independent adhesion molecules in brain development and function, it is not surprising that their disruption has significant consequences for neurological integrity. The most intriguing evidence implicating NCAM in heavy metal neurotoxicity has emerged from studies of learning and memory. It is now clear NCAM plays an important role in the synaptic remodeling which occurs during the acquisition and consolidation of new information (Doyle *et al.,* 1992a,b; Muller *et al.,* 1996; Schuster *et al.*, 1998; Stork *et al.,* 2000). During the consolidation phase of learning which occurs 6–12 h after training, PSA-NCAM expression is transiently upregulated at the synapse to facilitate the release, repositioning, and/or expansion of the synaptic complex. Immunohistochemical studies have confirmed the reexpression of PSA-NCAM in the limbic system (particularly dentate granule cells) following training with a variety of behavioral paradigms. The anatomical location of labeled cells varies according to the particular behavioral test employed, suggesting that PSA-NCAM expression might be useful in mapping neural pathways involved in the learning of specific behaviors. Dysregulation of PSA-NCAM reexpression during the consolidation phase of learning causes significant impairment of long-term memory. Similar learning deficits are seen in mutant mice unable to express PSA-NCAM180 (the isoform preferentially localized at the synapse) (Cremer *et al.,* 1994; Stork *et al.,* 2000). Regulation of NCAM expression and function may represent an important mechanism underlying behavioral abnormalities in metal neurotoxicology.

Conclusions

This symposium reviewed an emerging area in metal toxicology: the impact of metals on expression and function of cell adhesion molecules. The targeting of these critical cell proteins by metals may explain, in part, both the cytotoxic and the

teratogenic/carcinogenic properties of several metals, including cadmium, lead, trimethyltin, and methylmercury. The data presented in this symposium demonstrate that metals target both calcium-dependent (cadherins) and calcium-independent (NCAM) molecules at multiple levels, including protein-protein interactions, posttranslational modification and transcriptional regulation. The finding that metals may also stimulate the β-catenin signaling pathway opens a new and exciting avenue of research concerning the molecular mechanisms of metal-induced carcinogenesis. Clearly the impact of metals on cell adhesion molecules and related signaling pathways is an important area of research, and it is hoped that this symposium will stimulate further investigation into this emerging area.

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