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Changes of Tight Junction and Epithelial Cells after Treatment of Cadmium

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Cadmium (Cd) affects cell proliferation, differentiation, apoptosis and other cellular activities and can cause numerous molecular lesions that would be relevant to carcinogenesis. The mechanism of adverse effects of Cd has been poorly understood and, especially on the tight junction. Since there is rare information about the effect of Cd on tight junction protein, we here investigated whether Cd can alter the localization of the proteins. This study examined Cd effects on of tight junction (occludin, ZO-1, and ZO-2) using MDCK cell culture.

The change of MDCK cell and tight junction was investigated after treatment of cadmium with phase contrast microscopy, TEER, cell viability, Transmission electron microscopy and confocal laser microscopy.

After treatment of cadmium, transendothelial electrical resistance decreased with time and concentration dependent manner. AlamarBlue assay revealed that decreased cell viability also decreased with time and concentration dependent manner. The tight junction moved down between intercellular spaces with decreased density and the cellular thickness around cell junctions decreased with increasing concentration and exposure time of $CdCl_2$. The MDCK cells eventually showed cell death with. Confocal laser microscopy revealed that immunofluorescent reaction of occludin, ZO-1 and ZO-2 decreased. Occludin, ZO-1 and ZO-2 were disrupted at tight junction.

These data suggest that after treatment of Cd, increased permeability of MDCK cell monolayer increased. This might be accompanied with disruption of occludin, ZO-1 and ZO-2.

Key words : MDCK cell, $CdCl_2$, Tight junction, Occludin, ZO-1, ZO-2

Introduction

Cadmium (Cd) is a highly toxic metal that can be found in food and water in contaminated areas. Cd is absorbed by the gastrointestinal tract and is distributed quickly to the kidney and liver. Because Cd is nonessential, it most likely utilizes other metal transporters to gain entry into cells (Olivi et al. 2001). Cd is a component of paint, batteries, and other electronic devices and a contaminant of metal ores, pesticides, and fertilizers. Acute, high-level exposure to Cd causes damage to many organs, including lung, brain, kidney, and liver (Bonham et al. 2003). Cd is an environmental and occupational toxin with no known physiological function. The wide environmental distribution of Cd has led to an increased interest in its toxicity and biological effects (Thevenod 2003). Food crops grown on Cd-containing soils or on soils naturally rich in this

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metal constitute a major source of nonworkplace exposure to Cd other than exposure from cigarette smoking (Satarug et al. 2000, 2003).

Cd exposure, even at lower levels may contribute to chronic renal failure (Hellstrom et al. 2001). Prolonged urinary Ca loss caused by Cd is sufficient to promote skeletal demineralization, which may lead to increases in bone fragility and risk of fractures (Wu et al. 2001). Maternal Cd exposure may result in adverse reproductive outcomes (Nishijo et al. 2002). Exposure to Cd in early life resulted in rapid weight gain and early onset of puberty (Johnson et al. 2003). Cd is thought to be responsible for cancer in lung (Waalkes 2003), kidney (Hu et al. 2002), breast (Band et al. 2002), and prostate and colorectum (Ekman 1999). Cd causes apoptosis in renal tubular cells in vitro (Alvarez-Barrientos et al. 2001). It is known that cadmium interferes with calcium homeostasis, and this could play an important role in cadmium toxicity (Faurskov and Bjerregaard 1997, Zimmerhackl et al. 1998). Cd is one of the most toxic testicular toxicants known, because Cd is known to alter the expression of a variety of proteins, from protooncogenes (Hechtenberg et al. 1996) to testicular tight-junction proteins (Chung and Cheng 2001).

The tight junction is well developed and functions as barriers between epithelial cells in skin, renal tubular cells, endothelial cells in brain cortical capillaries (blood-brain barrier), and testicular Sertoli cells (blood-Sertoli cell barrier). The occlusive property of the barrier can be attributed, in part, to the presence of a continuous ring of tight junction between neighboring cells (Bolton et al. 1998), and delivery of ions and solutes from blood to brain is limited by the selectivity of blood-brain barrier (Rubin and Staddon 1999).

Tight junction is complexes of plasma membrane proteins connected to the cytoarchitecture via membrane associated accessory proteins. Claudin, copolymerized occludin, and junctional adhesion molecule are integral membrane proteins interacting with those of neighboring plasma membrane and form TJ barrier (Martin-Padura et al. 1998, Furuse et al. 1999). Cytoplasmic tight junction accessory proteins [Zonular occludens (ZO)-1, ZO-2, cingulin] connect tight junction to the actin cytoskeleton.

The mechanism of adverse effects of Cd on the epithelial cell, especially on the tight junction has been poorly understood. MDCK is extensively studied culture models of renal epithelial cells derived from canine distal convoluted tubule epithelial cell. In the present study, we found that Cd disrupted the morphology and tight junction in MDCK. Since there is rare information about the effect of Cd on tight junction protein, we here investigated whether Cd can alter the localization of the proteins.

Materials and Methods

1. Materials

Transwell polycarbonate membrane inserts (0.4-μm pore) and cell culture plates were purchased from Corning Costar (Acton, MA, USA). Cell culture plates were purchased from Corning Costar (Acton, MA, USA). Chamber slides were purchased from Nunk (Roskilde, Denmark). Dulbecco's minimum Eagle medium (DMEM) was purchased from JBI (Seoul, Korea). Epithelial volt-ohm meter (EVOM) and electrode (STX-2) were purchased from World Precision Instruments (Sarasota, FL, USA). All other nutrients, salts, antibiotics and etc. used in culture media were purchased from Sigma (St. Louis, MO, USA). Antibodies (mouse anti-occludin, rabbit anti-ZO-1, anti-ZO-2, goat anti-rabbit-IgG-FITC, and goat anti-mouse-IgG-FITC) were purchased from Zymed Laboratories (San Francisco, CA, USA). MDCK cell was purchased from ATCC (Manassas, VA, USA).

2. Subculture of MDCK cells

MDCK (Madin Darby Carnine Kidney tubular) cell

was cultured in 15 mL Dulbecco's minimum Eagle medium (DMEM) supplemented with 10% fetal bovine serum 100 μg/mL penicillin/streptomycin, 45 μg/mL polymyxin B in T-75 flask. After confluence, the cells were trypsinizied and counted using a hemocytometer. Total MDCK cells in T-75 flask were suspended with 15 mL DMEM containing 10% fetal calf serum, 100 μg/mL penicillin/streptomycin, 45 μg/mL polymyxin B, DMSO (5%), aliquoted into 2 mL bottles and stored in liquid nitrogen for later use. A 100-mM stock solution of cadmium chloride ($CdCl_2$) was prepared and added to cells cultured in plates.

3. Culture of MDCK cells

One bottle (2 mL) MDCK cells from liquid nitrogen was rapidly thawed and added to 45 mL of DMEM for centrifugation (2000 × g, 10 min). The pellet was dissolved with 15 mL DMEM and counted with hemacytometer. Cell viability was determined by Trypan-Blue exclusion test. MDCK cells were plated at a density of 5×10^4 cells/cm^2 in 96- or 24-well tissue culture plates, chamber slides, and 3×10^5 cells/cm^2 on Transwell insert. The culture surfaces of Transwell inserts were treated with rat-tail collagen (50 μg/mL). MDCK cells were cultured DMEM containing 10% fetal calf serum, 100 μg/mL penicillin/ streptomycin, 45 μg/mL polymyxin B. The cells were grown in a 37 ˚C incubator in 5% CO_2 and 95% room air. The culture medium was changed every other day with changing media (DMEM containing 10% fetal calf serum, 100 μg/mL penicillin/streptomycin, and 45 μg/mL polymyxin B). The growing cells were observed with phase-contrast microscope (CK2, Olympus, Tokyo, Japan).

4. Cadmium treatment

Cadmium chloride was dissolved in culture media at various concentration from 0.1 μM to 100 μM and was treated at culture plate with time interval (3, 6, 9, 12, 24 hours).

5. Assessment of transepithelial electrical resistance (TEER)

The MDCK cells were plated on 12-well Transwell inserts. The resistances of monolayers were monitored every other day until they reached a steady state. Once stable resistances were obtained ($> 200 \Omega \cdot cm^2$), the cells were treated with $CdCl_2$. TEER across MDCK cell monolayer was measured using EVOM. The TEER ($\Omega \cdot cm^2$) was obtained from the displayed electrical resistance on the layout screen by subtraction of electrical resistance of a collagen-coated filter without cells and a correction for filter surface area. TEER was measured just before adding $CdCl_2$ and after media refresh at time schedules. Measurements were taken in triplicate (mean ± SD). Controls were treated with media without $CdCl_2$. TEERs were normalized relative to controls and presented as mean ± SD. The results are reported as a percent of control.

6. AlamarBlue assay

Viability of MDCKs after treatment of $CdCl_2$ was assessed using AlamarBlue assay (Serotec, Kidlington, Oxford, UK). Confluent MDCK cells were exposed to various concentration of $CdCl_2$. AlamarBlue (final concentration 10%) were added to the culture plates. Controls were treated with media without $CdCl_2$. The culture plates were returned to incubator for 2 hr. The absorbance was measured at wavelength of 570 nm using Spectra MAX 340 (Molecular Devices, Sunnyvale, CA, USA). Background absorbance measured at 600 nm was subtracted from the 570 nm absorbance (n=5). Viability was expressed as a percentage of control.

7. Transmission electrron microscopy

$CdCl_2$ was added to Transwell insert 48 hr after the cells were plated at a density of 3×10^5 cells/cm^2.

Cells were exposed to this toxic compound upto 24 hr at 37°C. Control plates were treated with media without $CdCl_2$. After exposure, Transwell inserts were briefly washed with PBS and fixed in 2.5% glutaraldehyde. Insert membranes were removed and post-fixed in 1% (v/v) osmium tetroxide. After dehydration with a graded series of alcohol concentrations, the samples were impregnated with epoxy resins. The ultrathin sections were contrasted with uranyl acetate and lead citrate for electron microscopy study. Electron micrographs were taken with a JEM-200CX transmission electron microscope (Jeol, Tokyo, Japan).

8. Confocal laser microscopy of MDCK cells

MDCK cells grown on chamber slide (Nunc, Roskilde, Denmark) were exposed to $CdCl_2$ as previously described. Controls were treated with media without $CdCl_2$. After culture medium being removed, monolayer was washed with prewarmed phosphate buffered saline (PBS, 0.01 M). Cells were fixed with 3% paraformaldehyde (in PBS) for 20 min at RT, and permeabilized with 0.1% Triton-X 100 (in PBS) for 10 min at RT. After fixing and permeabilization, monolayer was blocked with 1% bovine serum albumin (BSA)/PBS for 60 min at RT. Confluent monolayers from each treatment group were incubated with anti-occludin (5 μg/mL), anti-ZO-1 (5 μg/mL), or anti-ZO-2 (5 μg/mL) primary antibody for 1 hr at RT. The cells were rinsed with 1% BSA/PBS, followed by incubation with FITC conjugated with secondary antibodies (5 μg/mL) for 1 hr at RT in the dark. The fluorescent-stained cells were rinsed three times with PBS before being mounted with coverglass with 50% glycerol-PBS and sealed. Photographs were taken with a confocal laser microscope (LSM-510 meta, Zeiss, Berlin, Germany).

9. Statistical methods

Data are expressed as mean±SD. Differences between mean values were tested for the significance using Student's *t*-test ($P < 0.05$).

Results

1. Phase contrast microscopy

Incubating MDCK cells in the presence of $CdCl_2$ caused observable cellular changes (Fig. 1). The adverse effects of $CdCl_2$ were dose and time dependent. The initial and principal change was distinct demarcation of MDCK cells from neighboring cells. The incubation of MDCK cell with $CdCl_2$ caused oval shaped cells and eventually detached the cells from the plating surfaces.

2. Assessment of transendothelial electrical resistance (TEER)

The effects of $CdCl_2$ on TEER were measured in MDCK monolayers over 24 hr (Figs. 2 and 3). Blank collagen-coated Transwell inserts were used as an indicator of background effects on TEER and showed consistency at $105 \pm 6\Omega \cdot cm^2$. The results were expressed as a percentage of control. TEER decreased significantly and reached the bottom level after 9 hours (1 μM). TEER decreased significantly after 3 hours (5 to 100 μM) (Fig. 2A). The fresh media change without $CdCl_2$ after 10 minutes showed increased TEER (Fig. 2BCD) This suggests that alterations of MDCK cell permeability are dependent on the length and severity of $CdCl_2$.

3. Cell viability analysis of $CdCl_2$-treated MDCK cells with AlamaBlue assay

To test the possibility that changes in TEER resulted from the death of cells or the subsequent formation of holes in monolayer, the cell viability was measured using AlamarBlue assay. The cell viability using AlamarBlue Assay was measured at 2 hours later after adding the dye when the reaction was stabilized (Fig.

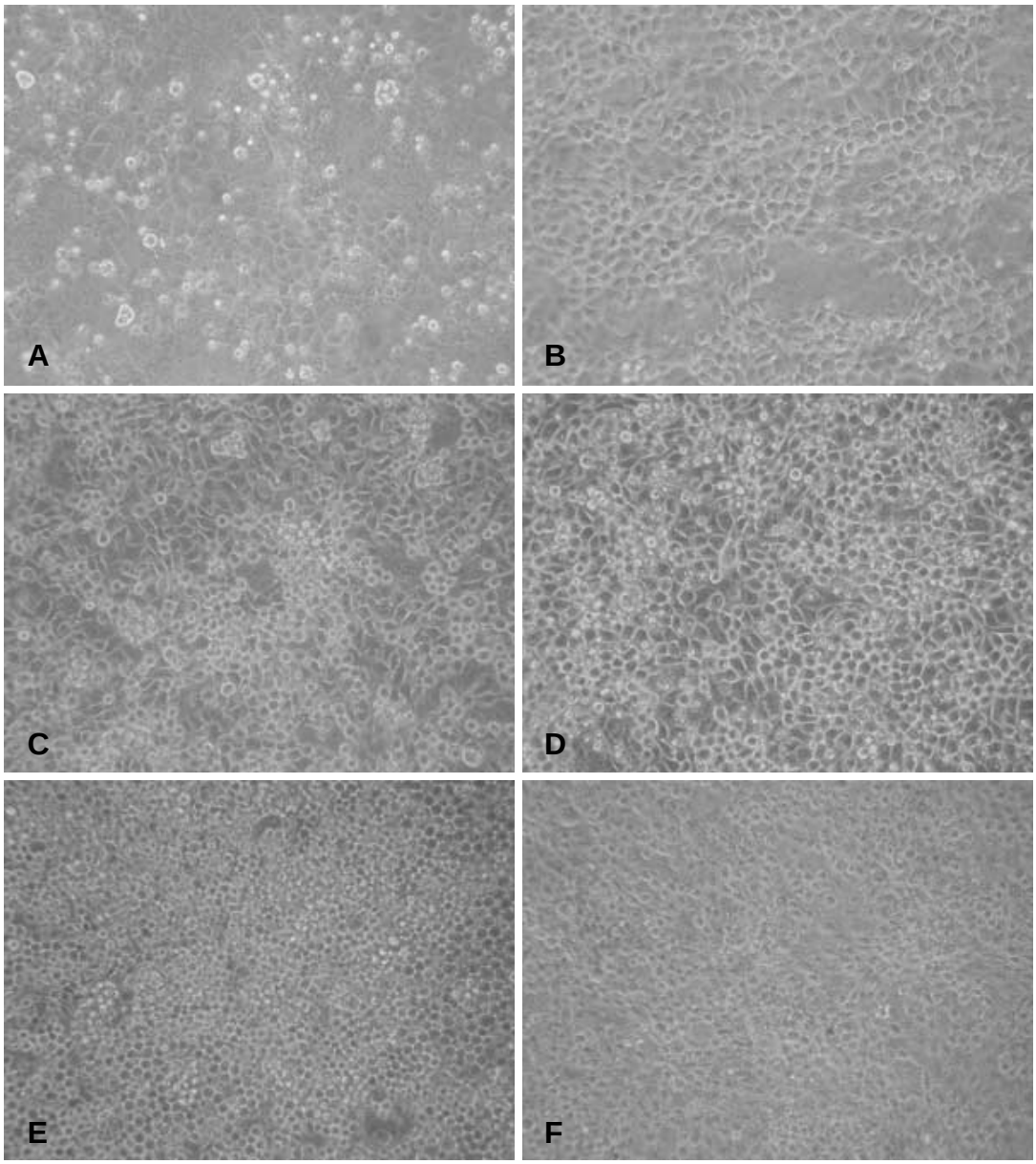

Fig. 1. Phase contrast Micrographs of MDCK cells. In control (1A), cell boundaries are not distinct. With increasing concentration and exposure time after $CdCl_2$ treatment, the cell boundaries became distinct (1B: 5 μM 3 hr, 1C: 5 μM 9 hr, 1D: 10 μM 9hr, 1E: 25 μM 9 hr, 1F: 25 μM 12 hr). 100×.

3A). $CdCl_2$ concentration of 0.1 μM (24 hr) and 1 μM (9 and 24 hr) decrease cell viability as compared to the control. $CdCl_2$ concentration of 5 μM and more greatly decreased the cell viability (Fig. 3B).

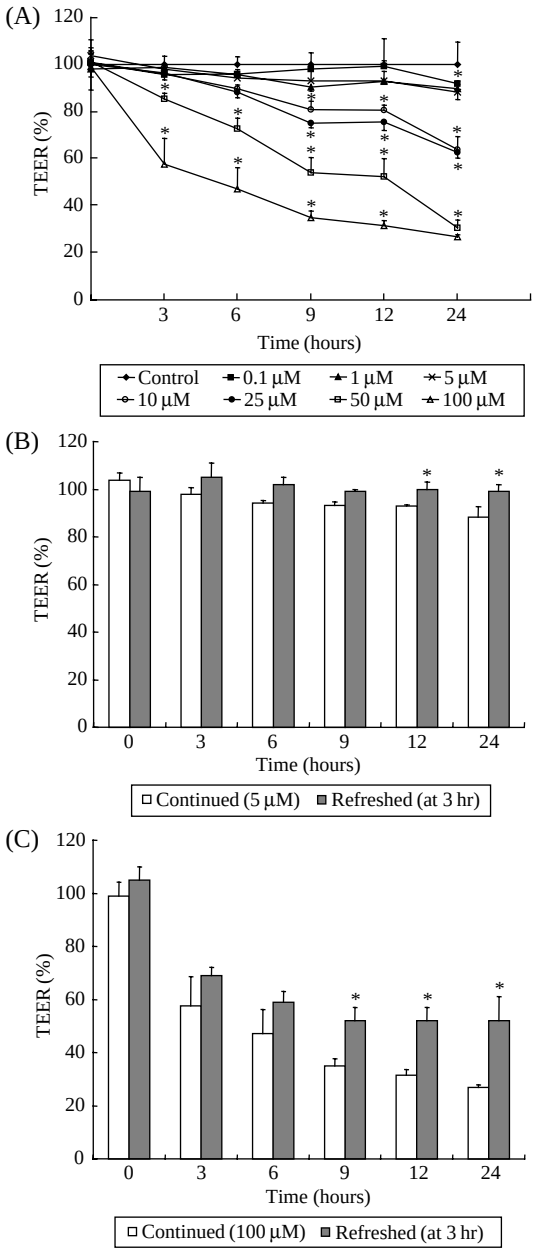

Fig. 2. $CdCl_2$-induced transendothelial electrical resistance (TEER, Ω · cm^2) in MDCK celll monolayer measured with EVOM. 2A: Significant decrease in TEER at 1 μM (9 hr) and 5～100 μM (3～24 hr) with dose- and time-dependent pattern. 2BC: Fresh media replaced after 10 min increased TEER. Data are shown as mean±SD with $*P < 0.05$ (n=5).

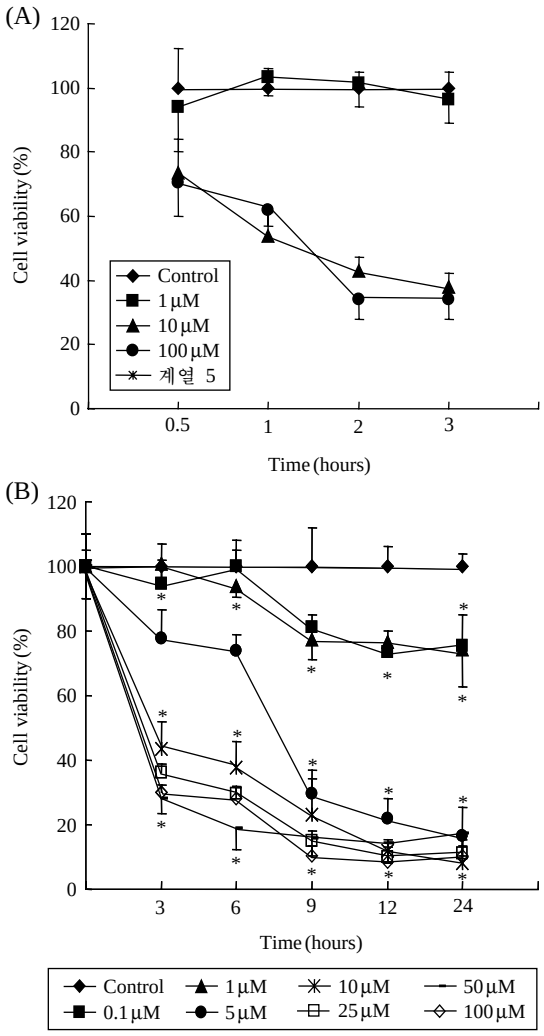

Fig. 3. Percentage of cell viability of MDCK cells after $CdCl_2$ treatment using AlamarBlue assay. 3A: The cell viability was stabilized and measured at 2 hr after adding dye. 3B: Treatment of $CdCl_2$ shows significant decrease in viability at 0.1 μM (24 hr), 1 μM (9 and 24 hr), 5～100 μM (3～24 hr) when compared with control level. Data are shown as mean±SD with $*P < 0.05$ (n=5).

4. Transmission electron microscopy

The ultrastructure of control MDCK cell represented uniformly thick cells between which distinct tight junction at apical cytoplasm (Fig. 4AB). The tight

junction moved down between intercellular spaces with decreased density (Fig. 4CE) and the cellular thickness decreased (Fig. 4DE) with increasing concentration and exposure time of $CdCl_2$. The MDCK cells eventually showed cell death with increased electron density of cytoplasm (Fig. 4F).

5. Confocal laser microscopy for tight junction proteins

The localization of occludin, ZO-1, and ZO-2 was examined after $CdCl_2$ treatment (Fig. 5). At tight junction, immunofluorescent staining showed localized immunoreaction of occludin (Fig. 5A), ZO-1 (Fig. 5E), and ZO-2 (Fig. 5I) in control MDCK cells. The proteins were distributed continuously around at the periphery of MDCK cells in contact with neighboring cells. After exposure to $CdCl_2$ with increasing concentration and times, MDCK cells showed gradual decrease in immunofluorescent reaction of occludin (Fig. 5BCD), ZO-1 (Fig. 5FGH), and ZO-2 (Fig. 5JKL) in tight when compared to the control.

Discussion

Cadmium (Cd), a metal toxin of continuing worldwide concern, is accumulated in the environment due to its extremely long half-life. Its compounds are classified as human carcinogens. Daily intake of Cd, albeit in small quantities, is associated with a number of adverse health effects which are attributable to distinct pathological changes in a variety of tissues and organs. Cd affects cell proliferation, differentiation, apoptosis and other cellular activities and can cause numerous molecular lesions that would be relevant to carcinogenesis. Besides, Cd is suggested as a strong mutagen which induces predominantly multilocus deletions (Filipic et al. 2006). Recent reports have shown that occludin plays an important role in maintaining endothelial or epithelial solute barriers (Hirase et al. 1997, Tsukita and Furuse 1999). In addition, zonular occludens proteins (ZO-1, 2, 3) are important molecules regulating barrier functions (Denker and Nigam 1998, Itoh et al. 1999). This study focuses on its adverse effect on MDCK tight junction in vitro.

We studied the effects of Cd on the TEER across cultured monolayers of MDCK cells. TEER is a useful physiological marker of cell barrier integrity. It is well known that the establishment of tight junction correlates with development of TEER (Gonzales-Marisca et al. 1985). The resistance between $100 \sim 300 \Omega \cdot cm^2$ in our MDCK model is enough to show barrier function as in other model using MDCK cells grown as monolayers on polycarbonate filters (Zimmerhackl et al. 1998, Raub et al. 1992). The MDCK permeability, measured as TEER, decreased in a dose- and time-dependent pattern when monolayers were treated with $CdCl_2$ ($0.1 \sim 100$ μM). These data indicate that $CdCl_2$ increases permeability with a similar pattern as seen in airway epithelial cells (Chapman et al. 2002) and intestinal epithelial cells (Rao et al. 1997).

$CdCl_2$ concentration of 0.1 μM (24 hours) and 1 μM (9, 24 hours) decreased cell viability as demonstrated by AlamarBlue assay. 5 μM or more of $CdCl_2$ significantly decreased the cell viability in all experimental period. These data suggest that Cd decrease cell viability with time and dose dependent pattern. In other study using MDCK cell, $CdCl_2$ changed the distribution of E-cadherin and α-catenin report (Zimmerhackl et al. 1998). But there is no report found that suggests Cd effect on tight junctional protein. In this report, $CdCl_2$ effect on alterations of tight junction studied.

The ultrastructure of MDCK cell and tight junction represented that $CdCl_2$ caused flattening of cytoplasm around intercellular junction and migration of tight junction to basal compartment with decreasing electron density. It is suggested that these ultrastructural changes may decrease the permeability of MDCK cell barrier. Under normal condition, tight junctional proteins are located as a continuous network pattern

around intercellular space. The fluorescent immunostaining showed that $CdCl_2$ caused gradual loss of occludin, ZO-1, and ZO-2 junctional localization.

It is known that tight junctional proteins response is variable by different stress and cell types. The junctional localization of these proteins is no longer observed at the region where endothelial cells are not in contact with one another (Madara et al. 1993, Kevil et al. 2000). These disruptions in tight junctional proteins at cell-to-cell contact sites correlate with increased paracellular permeability (Kevil et al. 2000, Mark and Davis 2002).

Occludin with a molecular mass of approximately 65 kDa has NH_2 and COOH termini in cytoplasm with two extracellular loops projecting into paracellular space (Denker and Nigam 1998). Three membrane-associated guanylate kinase-like homologues (MAGUKs, ZO-1, ZO-2, and ZO-3) have been identified as component of TJs (Mitic and Anderson 1998). Members of this MAGUKs family are often found at the site of cell-to-cell contact and may function to couple extracellular signaling pathways with the cytoskeleton-like actin (Denker and Nigam 1998). It has been known that the COOH terminal of occludin binds ZO-1, ZO-2, and ZO-3, indicating that occludin is a membrane partner for ZO-1, ZO-2, and ZO-3 (Haskins et al. 1998), but recent finding suggested that the three MAGUKs were recruited to claudin (Itoh et al. 1999). Many lines of evidence suggest that paracellular permeability is influenced by the state of perijunctional actin (Rajasekaran and Rajasekaran 2003, Wittchen et al. 2003).

Some of the second messenger and signaling pathways important for the assembly of tight junction have been identified. Multiple signaling transduction pathways have been implicated in tight junction biogenesis including src kinase (Kevil et al. 2001), Ca^{2+} (Stuart et al. 1996), G proteins (Denker et al. 1996), and c-AMP (Ishizaki et al. 2003). The mechanism of action leading to that damage cannot be further elucidated with the techniques used. In particular, it cannot be clarified whether the protein is degraded or whether $CdCl_2$ directly effects on tight junction.

In conclusion, Cd induces decrease of TEER and cell viability with dose and time dependent pattern, loss of occludin, flattening of cytoplasm around tight junction, and loss of ZO-1, and ZO-2 at tight junction. This study suggests that $CdCl_2$ induces increased paracellular permeability of MDCK cell barrier that is accompanied with loss of occludin, ZO-1 and ZO-2. Because tight junctional proteins appear at times to be cell-specific and stimulant-specific, much remains to be studied concerning the direct role, signaling pathways and genes that are affected by $CdCl_2$. Further studies are needed to elucidate how $CdCl_2$ produced by various diseases affect the role of tight junction. Therapeutic strategies aimed at controlling the function of tight junction by intervening $CdCl_2$-mediated changes have wide applicability in health science.

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Legends for figures

Fig. 4. Transmission electron micrographs of MDCK cells cultured on 0.4 μm pore Transwell inserts. The tight junctions (Arrows) are found apical side of intercellular junction between thick MDCK cells (Fig. 4AB). The tight junction moved down between intercellular spaces with decreased density (Fig. 4CE) and the cellular thickness decreased (Fig. 4D) with increasing concentration and exposure time of $CdCl_2$. The MDCK cells eventually showed cell death with increased electron density of cytoplasm (Fig. 4F). (4C: 5 μM 3 hr, 4D: 5 μM 3 hr, 4E: 10 μM 6 hr, 4F: 100 μM 12 hr). Bars equal 1 μm.

Fig. 5. Immunofluorescent staining showing occludin (A-D), ZO-1 (E-H), and ZO-2 (I-L) protein localization in MDCK cells after treatment of $CdCl_2$. Immunofluorescent staining showed localized immunoreaction at tight junction of controls (AEID). After exposure to $CdCl_2$ with increasing concentration and times, MDCK cells showed gradual decrease in immunoflurescent in tight junction (5BFJ: 5 μM 6 hr, 5CGK: 10 μM 6 hr, 5DHL: 25 μM 12 hr). 400 ×.

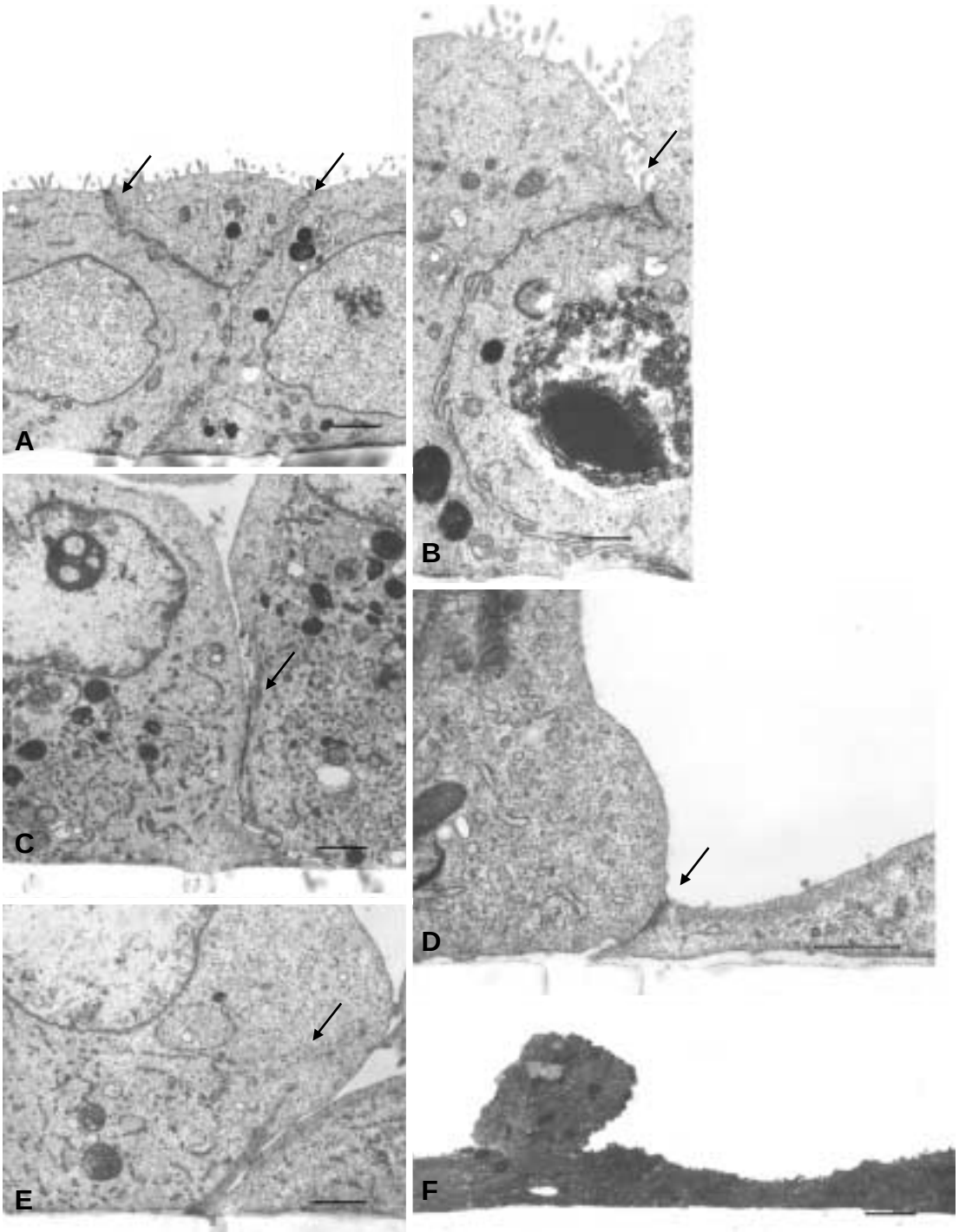

Fig. 4

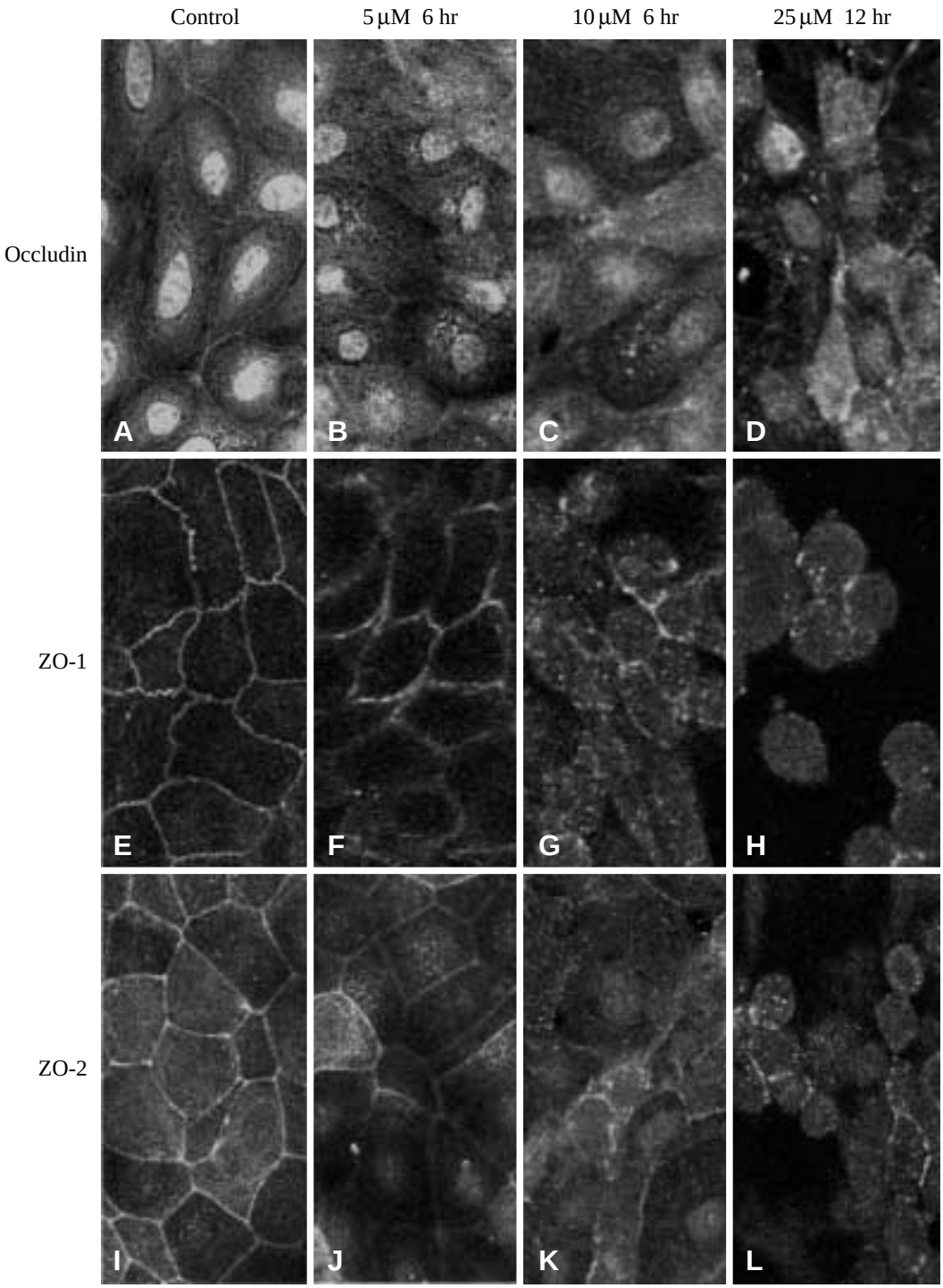

Fig. 5

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카드뮴투여 후 나타나는 치밀이음부와 상피세포의 변화

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간추림 : 카드뮴은 세포의 증식, 분화, 세포자멸 등에 영향을 주며 암을 유발시킨다. 카드뮴의 작용기전은 잘 알려져 있지 않으며, 특히 치밀이음부에 대해서는 더욱 알려져 있지 않다. 그러므로 이 연구에서는 카드뮴의 상피세포 치밀이음부에 대한 연구에 대한 영향을 연구하고자 하였다.

MDCK 세포배양에서 카드뮴을 투여하고 위상차현미경, TEER, 세포생존률, 공초점레이져현미경, 투과전자현미경을 이용하여 배양세포와 치밀이음부의 변화를 관찰하였다.

카드뮴을 투여하면 TEER 가 감소되었으며, AlamarBlue Assay 결과, 역시 세포의 생존률이 감소되었다. 치밀이음부는 세포사이연접에서 아래로 이동하면서 전자밀도가 낮아졌으며, 다른 세포와 인접한 부위에 있는 세포질의 두께가 감소하였다. 최종적으로 세포의 사멸이 나타내기도 하였다. 공초점레이져현미경을 통해 관찰한 결과, occludin, ZO-1, ZO-2의 면역형광반응은 감소하고 불규칙해지는 경향을 나타내고 있었다. 이러한 변화는 시간과 농도에 비례하여 나타났다.

이러한 결과로 보면, 카드뮴을 투여하면 MDCK 세포장벽에서 투과성이 증가되고, 이것은 치밀이음부를 구성하는 occludin, ZO-1, ZO-2 의 변화에서 오는 것으로 보인다.

찾아보기 낱말 : MDCK 세포, 카드뮴, 치밀이음부, Occludin, ZO-1, ZO-2

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