Regulation of blood-testis barrier dynamics: an in vivo study

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Summary

An in vivo model was used to investigate the regulation of tight junction (TJ) dynamics in the testis when adult rats were treated with $CdCl_2$. It was shown that the $CdCl_2$-induced disruption of the blood-testis barrier (BTB) associated with a transient induction in testicular TGF-β2 and TGF-β3 (but not TGF-β1) and the phosphorylated p38 mitogen activated protein (MAP) kinase, concomitant with a loss of occludin and zonula occludens-1 (ZO-1) from the BTB site in the seminiferous epithelium. These results suggest that BTB dynamics in vivo are regulated by TGF-β2/-β3 via the p38 MAP kinase pathway. Indeed, SB202190, a specific p38 MAP kinase inhibitor, blocked the $CdCl_2$-induced occludin and ZO-1 loss from the BTB. This result clearly illustrates that $CdCl_2$ mediates its BTB disruptive effects via the TGF-β3/p38 MAP kinase signaling pathway. Besides, this $CdCl_2$-induced occludin and ZO-1 loss from the BTB also associated with a significant loss of the cadherin/catenin and the nectin/afadin protein complexes at the site of cell-cell actin-based adherens junctions (AJs). An induction of α_2-macroglobulin (a non-specific protease inhibitor) was also observed during BTB damage and when the seminiferous epithelium was being depleted of germ cells. These data illustrate that a primary disruption of the BTB can lead to a secondary loss of cell adhesion function at the site of AJs, concomitant with an induction in protease inhibitor, which apparently is used to protect the epithelium from unwanted proteolysis. α_2-Macroglobulin was also shown to associate physically with TGF-β3, afadin and nectin 3, but not occludin, E-cadherin or N-cadherin, indicating its possible role in junction restructuring in vivo. Additionally, the use of SB202190 to block the TGF-β3/p-38 MAP kinase pathway also prevented the $CdCl_2$-induced loss of cadherin/catenin and nectin/afadin protein complexes from the AJ sites, yet it had no apparent effect on α_2-macroglobulin. These results demonstrate for the first time that the TGF-β3/p38 MAP kinase signaling pathway is being used to regulate both TJ and AJ dynamics in the testis, mediated by the effects of TGF-β3 on TJ- and AJ-integral membrane proteins and adaptors, but not protease inhibitors.

Key words: Blood-testis barrier, Tight junctions, Adherens junctions, TGF-β3, α_2-Macroglobulin, p38 MAP kinase

Introduction

A single type A1 spermatogonium (diploid, 2n) in rodents, such as rats and mice, gives rise to 256 mature spermatids (haploid, n) in the seminiferous epithelium during spermatogenesis (for reviews, see de Kretser and Kerr, 1988; Cheng and Mruk, 2002; Lui et al., 2003b). For this event to happen, preleptotene and leptotene spermatocytes must traverse the blood-testis barrier (BTB, also known as the seminiferous epithelium barrier) at late stage VIII and early IX of the epithelial cycle (Russell, 1977). The BTB is constituted largely by inter-Sertoli cell tight junctions (TJ). However, cell-cell actin-based adherens junctions (AJ, e.g. ectoplasmic specialization, a testis-specific AJ type) and possibly cell-cell intermediate filament-based desmosome-like junctions (for reviews, see de Kretser and Kerr, 1988; Pelletier, 2001; Cheng and Mruk, 2002), are also found at the BTB site. As such, these junctions must undergo extensive restructuring during this process of germ cell migration. Yet the mechanism(s) that regulates BTB dynamics remains largely unknown (for a review, see Cheng and Mruk, 2002).

Recently completed in vitro studies have shown that Sertoli cell TJ dynamics are regulated, at least in part, by transforming growth factor-β3 (TGF-β3) (Lui et al., 2001; Lui et al., 2003a) and tumor necrosis factor-α (TNF-α) (Siu et al., 2003a) via the TGF-β3/MEKKs/p38 mitogen activated protein (MAP) kinase and the TNF-α/integrin-linked kinase (ILK)/p130 Crk-associated substrate (Cas)/MAP kinase signaling pathways, respectively. More importantly, a preliminary in vivo study has shown that the event of BTB disruption induced by cadmium chloride ($CdCl_2$) indeed was mediated via the TGF-β3/MEKKs/p38 MAP kinase pathway (Lui et al., 2003d). Yet it remains to be determined whether proteases, protease inhibitors, AJ integral membrane proteins and their associated peripheral adaptors, and signaling molecules are also involved in TJ restructuring and, in particular, the subsequent germ cell loss from the epithelium as a result of BTB damage. In brief, in this study we sought to investigate whether a primary loss of the BTB function can lead to a secondary disruption of the AJs. We also investigated the roles of proteases and protease inhibitors in this event, since recent in vitro studies have shown that AJ dynamics in the testis are regulated by the intricate interactions between proteases and protease inhibitors under

the influence of cytokines (Mruk et al., 1997; Mruk et al., 2003). Furthermore, we have examined whether TGF-β3 can also utilize the p38 MAP kinase pathway to regulate AJ dynamics. These results are physiologically and clinically important in understanding the regulation and functional relationships of TJ and AJ dynamics.

Materials and Methods

Animals

Male Sprague-Dawley rats (outbred) weighing 250-340 g were purchased from Charles River Laboratories (Kingston, NY). The use of animals in this study was approved by the Rockefeller University Animal Care and Use Committee (Protocol Numbers 00111 and 03017).

Disruption of the BTB by $CdCl_2$

$CdCl_2$ was prepared as a 0.1% solution (wt/vol in Milli Q water), and was administered to groups of rats (*n*=3-5 rats per time point) by intraperitoneal injection (i.p.) at 3 mg/kg body weight (b.w.) to induce BTB damage as previously described (Hew et al., 1993b; Setchell and Waites, 1970; Grima and Cheng, 2000). Thereafter, rats were killed by CO_2 asphyxiation at 1, 3, 5, 7, 12, 16, 20, 24, 48, and 96 hours after $CdCl_2$ treatment, testes were removed from the scrotum, and the weight of each testis was recorded (Table 1). Testes were immediately frozen in liquid nitrogen and stored at –80°C until used. Preliminary experiments were also conducted using 1 mg/kg b.w. of $CdCl_2$ with *n*=4 rats per time point. In most of the animals, however, the BTB damage was not obvious at the above time points when examined by immunohistochemistry and/or immunofluorescence microscopy using occludin and ZO-1 as markers. Only 1 of 4 rats in the 48-hour group and 2 of 4 rats in the 72-hour group had visible signs of BTB damage. Consequently, a higher dose (3 mg/kg b.w.) of $CdCl_2$ was used instead in three subsequent experiments since all animals (*n*=3-5 rats per time point) responded uniformly at this dosing when the seminiferous epithelial barrier was examined by electron microscopy, immunohistochemistry, and immunofluorescence microscopy. However, it is of important to note that the changes detected in selected target proteins, such as α_2-macroglobulin and occludin, in rats receiving the lower concentration (1 mg/kg b.w.) of $CdCl_2$ were similar to those receiving 3 mg/kg b.w. $CdCl_2$ (see Results and Fig. 2). More important, such changes in protein expressions were detected in all animals receiving 1 mg/kg b.w. $CdCl_2$, similar to the 3 mg/kg b.w. treatment group. This suggests that changes in these target proteins indeed occurred long before any physical damage to the BTB was visible. Using 3 mg/kg b.w. of $CdCl_2$ vascular damage in the testis was visible by ~24 hours, since testes recovered from rats at this time point and thereafter were reddish in color, apparently loaded with red blood cells. Routine histological analysis indeed identified red blood cells in the interstitium, a clear indication of vascular barrier damage which permitted the leakage of blood cells via the opened (i.e. disrupted) endothelial tight junctions. However, it is important to note that physical damage to the BTB was detected as early as ~7-12 hours by immunohistochemistry and immunofluorescence microscopy (see Figs 3 and 4), prior to the disruption of the vascular barrier. This is consistent with an earlier report (Setchell and Waites, 1970) that the BTB was compromised by $CdCl_2$ prior to vascular damage. These preliminary results are also consistent with another report (Hew et al., 1993b) that when $CdCl_2$ was applied at doses of 1.5 mg/kg b.w. and above, the testes of most rats displayed damaged vascular and seminiferous epithelial barriers.

Table 1. Number of animals used and their average testicular weights (per testis) following treatment of adult rats (~280-290 g b.w.) with 3 mg/kg b.w. of $CdCl_2$

Time after $CdCl_2$ treatment (hours)	No. of animals used	Testicular weight (g) (mean ± s.d.)
0 (Ctrl)	4	1.49±0.09
1	3	1.55±0.09^{ns}
3	3	1.52±0.09^{ns}
5	3	1.55±0.09^{ns}
7	3	1.61±0.10^{ns}
12	4	1.59±0.18^{ns}
16	5	1.65±0.18^{ns}
20	4	1.66±0.19^{ns}
24	5	1.65±0.14^{ns}
48	3	1.44±0.04^{ns}
96	3	1.00±0.05**

*Only data of a representative experiment is shown. Another two sets of experiments with *n*=3-5 rats per time point also yielded similar results, but, these data were not pooled because the animal body weight ranges in these other two experiments were ~250-270 g and 320-340 g, respectively and different from this set.

ns, not significantly different from control rats at time 0 by Student's *t*-test; **Significantly different from control rats at time 0, $P<0.05$.

Treatment of rats with MAP kinase inhibitor

To examine the effect of SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, Calbiochem, San Diego, CA], a specific p38 MAP kinase inhibitor (Ravanti et al., 1999), on the $CdCl_2$-induced TJ and AJ damages, one of the testes from each rat (*n*=3-4 rats) was injected intratesticularly with SB202190 (5 μM, assuming the testicular volume of ~1.6 ml/testis, ~8 nmol SB202190 was used per testis) prior to $CdCl_2$ treatment (3 mg/kg b.w. via i.p.) as described (Lui et al., 2003d). SB202190 was prepared in DMSO (~1 mg/ml) and diluted in saline as described (Lui et al., 2003d). The other testis of the same rat received ~200 μl of 1% DMSO in saline (vol/vol) (vehicle control).

Electron microscopy

Electron microscopy was used to examine the effects of $CdCl_2$ on the integrity of the BTB in the testis following $CdCl_2$ treatment (3 mg/kg b.w., i.p.). In brief, rats were treated with $CdCl_2$ (3 mg/kg b.w., i.p.) and killed as described above. Rats without $CdCl_2$ treatment were used as controls. Capsules of testes were incised to release the seminiferous tubules. Tubules were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate (pH 7.4, 22°C) for 2-4 hours at room temperature. Thereafter, tubules were post-fixed in 1% OsO_4 solution for 1 hour on ice, and stained in 1% uranyl acetate for 1 hour at room temperature. Tubules were then dehydrated in ascending concentrations of ethanol as follows: 70% ethanol for 30 minutes, 95% for 10 minutes and 100% for 1 hour. After dehydration, tubules were incubated in propylene oxide for 45 minutes, and infiltrated with EMbed (Electron Microscopy Sciences, Fort Washington, PA). After overnight infiltration, used EMbed was discarded and replaced with fresh EMbed, and the whole specimen was then incubated at 60°C for 2 days. Ultra-thin (~80 nm) sections were prepared on a Reichert-Jung Ultracut E microtome and post-stained with uranyl acetate/lead. Sections were examined and photographed on a JEOL 100CXII electron microscope at 80 kV. Electron microscopy was performed at the Rockefeller University Bio-Imaging Resource Center.

Immunoblot analysis

For immunoblotting, testis lysates were prepared as follows. Briefly, 0.1 g of testis from a rat at each specified time point was suspended in 1 ml lysis buffer A [0.125 M Tris, pH 6.8, at 22°C containing 1% SDS (wt/vol), 2 mM EDTA, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonylfluoride (PMSF), 1.6% 2-mercaptoethanol

Table 2. Sources of primary antibodies used for immunoblotting experiments

Antibody	Vendor	Catalog no.	Lot no.	Dilution used
Rabbit anti-TGF-β3	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-82	A222	1:500
Rabbit anti-TGF-β2	Santa Cruz Biotechnology	sc-90	B0603	1:500
Rabbit anti-TGF-β1	Santa Cruz Biotechnology	sc-146	D0203	1:500
Rabbit anti-p38-MAPK	Cell Signaling Technology (Beverly, MA)	9212	3	1:1000
Rabbit anti-p-p38-MAPK	Cell Signaling Technology	9211S	3	1:1000
Rabbit anti-ERK 1/2	Cell Signaling Technology	9102	3	1:1000
Rabbit anti-occludin	Zymed Laboratories (San Francisco, CA)	71-1500	11067632	1:250
Rabbit anti-ZO-1	Zymed Laboratories	61-7300	30175033	1:250
Mouse anti-E-cadherin	BD Transduction Laboratories (San Jose, CA)	610182	9	1:2500
Rabbit anti-N-cadherin	Santa Cruz Biotechnology	sc-7939	F061	1:500
Goat anti-nectin-3	Santa Cruz Biotechnology	sc-14806	K261	1:500
Rabbit anti-β-catenin	Santa Cruz Biotechnology	sc-7199	D171	1:500
Rabbit anti-γ-catenin	Santa Cruz Biotechnology	sc-7900	J139	1:500
Rabbit anti-l-afadin	Sigma	A0349	012K4875	1:2000
Rabbit anti-p120^{ctn}	Santa Cruz Biotechnology	sc-1101	K080	1:500
Mouse anti-c-Src	Santa Cruz Biotechnology	sc-8056	C051	1:500
Goat anti-actin	Santa Cruz Biotechnology	sc-1616	E0503	1:200
Mouse anti-vimentin	Santa Cruz Biotechnology	sc-6260	B252	1:500
Mouse anti-α-tubulin	Santa Cruz Biotechnology	sc-8035	G182	1:500
Rabbit anti-MMP2	Chemicon International (Temecula, CA)	AB809	21082339	1:1000
Rabbit anti-MMP3	Chemicon International	AB811	21100782	1:1000
Rabbit anti-uPA	American Diagnostica (Greenwich, CT)	1190	010906	1:100
Rabbit anti-cathepsin L	(Lee et al., 1986)			1:500
Rabbit anti-cystatin C	Upstate Biotechnology (Lake Placid, NY)	06-458	21767	1:500
Rabbit anti-α_2MG	(Cheng et al., 1990)			1:750

(vol/vol), 1 mM sodium orthovanadate, and 0.1 μM sodium okadate] in siliconized microfuge tubes. All samples within a treatment group were processed simultaneously to eliminate inter-experimental variations. Samples were sonicated twice, 10 seconds each with a 30-second interval, while being incubated on ice. The clear supernatant was collected (15,000 ***g***, 4°C for 10 minutes) and used as testis lysate. Protein concentration was determined by Coomassie Blue dye-binding assay using BSA as a standard (Bradford, 1976). Equal amounts of proteins from testis lysates (~50 μg per lane) of an experiment were resolved by SDS-PAGE under reducing conditions. Proteins were electroblotted onto nitrocellulose membranes and immunostained with commercially obtained primary antibodies [except for rabbit anti-rat cathepsin L (Lee et al., 1986) and rabbit anti-rat α_2-macroglobulin (Cheng et al., 1990), which were prepared against purified protein isolated from Sertoli cell-enriched culture media, and previously characterized in our laboratory]. All primary antibodies were known to cross-react with the corresponding target proteins in rats according to the manufacturers (Table 2). Depending on the origin of the primary antibody, one of the following secondary antibodies conjugated to horseradish peroxide (HRP) was used: bovine anti-rabbit IgG (Cat sc-2370), bovine anti-goat IgG (Cat sc-2350) and goat anti-mouse IgG (Cat sc-2005) were purchased from Santa Cruz Biotechnology. Target proteins on nitrocellulose membranes were visualized by an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) as described (Lui et al., 2003d). The same blot was incubated in a stripping buffer [2% (wt/vol) SDS and 100 mM 2-mercaptoethanol in 62.5 mM Tris, pH 6.7, 22°C] at 50°C to remove the initial primary and secondary antibodies so that at least three target proteins could be visualized in the same blot without noticeable loss of a target protein as described (Siu et al., 2003a). By this method, target proteins within the same category, such as different proteases, can be compared. Each immunoblotting experiment was repeated at least three times using different sets of sample from different testes.

Immunohistochemistry

Streptavidin-biotin peroxidase immunostaining was carried out as described previously (Siu et al., 2003a; Siu et al., 2003b) using Histostain-SP^{TM} kits (Zymed Laboratories). Cryosections of testes embedded in OCT compound (Miles Scientific, Elkhart, IN) (~8 μm thick) were obtained using a microtome in a cryostat (Hacker, Fairfield, NJ) at –20°C. Frozen sections were mounted on poly-L-lysine (molecular mass >150 kDa)-coated slides and air-dried at room temperature, fixed in modified Bouin's solution [4% (vol/vol) formaldehyde in picric acid] for 5 minutes, followed by treatment with 3% (vol/vol) H_2O_2 in methanol for 20 minutes to block the endogenous peroxidase activity. Thereafter, sections were incubated with 10% non-immune goat serum to block the nonspecific binding sites, and were subsequently incubated with primary antibodies in a moist chamber at 4°C overnight. Primary antibodies were used at the following dilutions: rabbit anti-ZO-1 (1:150), rabbit anti-α_2-macroglobulin (α_2-MG; 1:350), rabbit anti-occludin (1:100) and rabbit anti-N-cadherin (1:150). Afterwards, sections were incubated with biotinylated goat anti-rabbit antibody for 30 minutes followed by streptavidin-peroxidase for 10 minutes. Aminoethylcarbazole (AEC) mixture was applied to sections as substrate for 5-10 minutes to permit color development, which appeared as reddish-brown precipitates. Sections were washed, counterstained with Hematoxylin, mounted in GVA mount (Zymed Laboratories), and examined and photographed using an Olympus BX-40 microscope (Olympus Corp., Melville, NY) equipped with an Olympus QImaging Micropublisher cooled digital camera (Olympus Corp.). All images were acquired and analyzed with Adobe Photoshop (Version 7.0). Controls include sections incubated with: (i) normal rabbit serum or preimmune serum instead of the primary antibodies, (ii) PBS in place of the primary antibodies, and (iii) normal rabbit serum in place of biotinylated secondary antibody.

Immunofluorescence microscopy

Co-localization of occludin and ZO-1 to the same site in the BTB of the rat seminiferous epithelium was performed by immunofluorescence microscopy as earlier described (Lee et al., 2003; Mruk et al., 2003). In brief, frozen sections of testes (~8 μm) from normal rats and those treated with $CdCl_2$ (3 mg/kg b.w., i.p.) 7, 12 or 24 hours earlier were mounted on poly-L-lysine-coated slides, air dried at room temperature, and fixed in modified Bouin's fixative. After blocking the non-specific binding sites in testis sections using

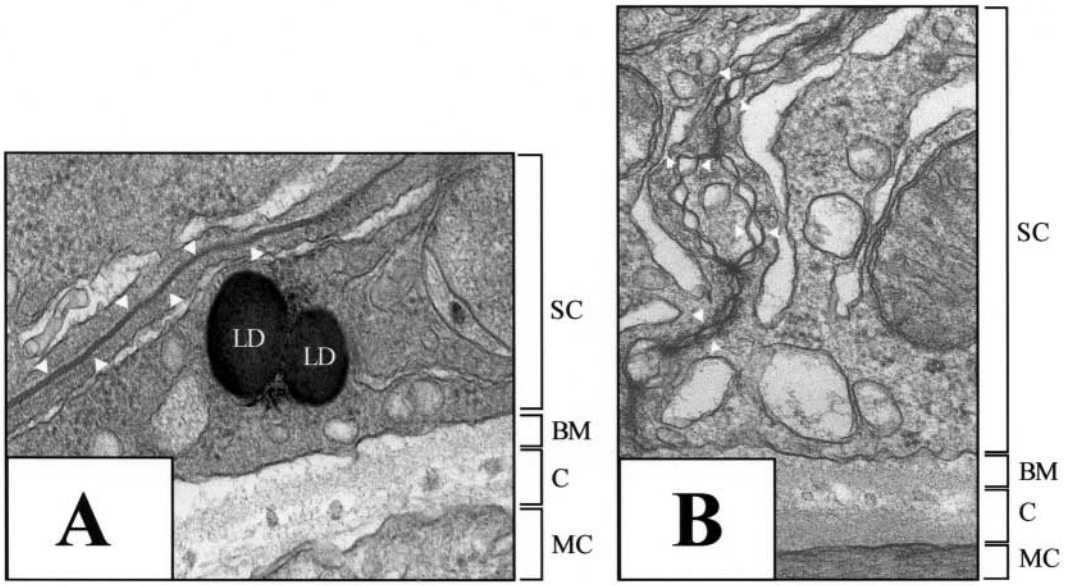


Fig. 1. Ultrastructural analysis of the integrity of the blood-testis barrier (BTB) following treatment of rats with $CdCl_2$ (3 mg/kg b.w. via i.p.). (A,B) Electron micrographs of (A) the BTB between two adjacent Sertoli cells at the seminiferous epithelium of a control rat, as an example of a tight junction (TJ), which appears as electron dense material (arrowhead). Magnification, ×67,500; (B) a rat testis 24 hours after $CdCl_2$ treatment illustrating that the TJ were disrupted (arrowheads). Magnification, ×45,000. LD, lipid droplet; SC, Sertoli cell; BM, basement membrane; C, collagen network; MC, myoid cell layer.

10% normal goat serum, sections were incubated with a rabbit polyclonal anti-occludin antibody (35°C overnight at 1:100 dilution), to be followed by a goat-anti rabbit IgG-Cy-3 and mouse monoclonal anti-ZO-1 IgG-FITC (Cat. 33-9100, Lot 20269234, Zymed) (35°C overnight at 1:100 dilution). Sections were then washed, mounted in Vectashield, and fluorescence microscopy was performed using an Olympus BX40 microscope equipped with Olympus UplanF1 fluorescence optics. All images were digitally acquired using an Olympus Q-Color 3 cooled digital camera and analyzed with Adobe Photoshop (Version 7.0) in a Macintosh G4 workstation.

Immunoprecipitation

Testis lysates for immunoprecipitation were prepared as described above but in lysis buffer B [10 mM Tris, pH 7.4 at 22°C, containing 1% (vol/vol) NP-40, 0.15 M NaCl, 2 mM PMSF, 2 mM EDTA, 2 mM N-ethylmaleimide, 1 mM sodium orthovanadate, 0.1 μM sodium okadate, and 10% (vol/vol) glycerol]. Equal amounts of proteins (~400 μg) from testis lysates were pre-incubated with 1% (vol/vol) normal rabbit serum for 3 hours, to be followed by another 3-hour incubation with 10 μl protein A/G PLUS-agarose (Santa Cruz Biotechnology), and samples were then centrifuged at 1000 ***g*** for 5 minutes. This step is important to eliminate non-specific interactions of serum proteins in the following step. Thereafter, supernatant was collected and incubated with 0.5 μl anti-α_2MG antibody or 2 μg of anti-TGF-β3 at 4°C overnight, with agitation using a rotator (Glas-Col, Terre Maute, IN) at ~24 cycles/minute. 20 μl of a protein A/G PLUS-agarose suspension was then added to precipitate the immunocomplexes. Immunoprecipitates were washed four times with lysis buffer B by resuspension and centrifugation (1000 ***g***, for 5 minutes). 50 μl of SDS sample buffer [0.125 M Tris, pH 6.8 at 22°C, containing 1% SDS (wt/vol), 1.6% 2-mercaptoethanol (vol/vol) and 20% glycerol (vol/vol)] was then added, followed by heating at 100°C, for 10 minutes to extract and denature protein from the immunocomplexes. Protein A/G PLUS-agarose was removed by centrifugation (1000 ***g***, for 5 minutes), and supernatant was resolved by SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes and blots were probed with different antibodies. All antibodies used for immunoblotting described herein are the same as described above, except that anti-TGF-β3 is a monoclonal antibody (Cat MAB643, Lot BHT04) from R&D Systems (Minneapolis, MN). Testis lysates obtained from rats at 20 hours after $CdCl_2$ treatment were used as positive controls to verify if a primary antibody indeed reacted with its target protein. Testes lysates without any incubation with antibodies or incubating only with normal rabbit serum or rabbit IgG during the immunoprecipitation step served as negative controls.

Statistical analysis

Statistical analyses were performed by analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) tests or Student's *t*-tests using the GB-STAT Statistical Analysis Software Package (version 7.0; Dynamic Microsystems, Silver Spring, MD).

Results

Ultrastructural change in the BTB after $CdCl_2$ treatment

In normal rat testes (controls, Fig. 1A), TJs that constitute the BTB (arrowheads) between two apposing Sertoli cells were readily identified. Yet TJs at the site of the BTB between two Sertoli cells became damaged and opened after treatment of rats with 3 mg/kg b.w. $CdCl_2$ 24 hours earlier (Fig. 1B). This pattern is somewhat similar to TJs found in rat testes at ~14 days of age, before the BTB was formed (Byers et al., 1993). This result also illustrates that a disruption of the BTB in the seminiferous epithelium is one of the major phenotypic changes in the testis following $CdCl_2$ treatment in vivo.

Changes in the endogenous levels of different target proteins

Changes in TGF-βs, the downstream signaling molecules and TJ-associated proteins

Previous studies have reported that cytokines, such as TGF-β3 and TNF-α, play a crucial role in regulating Sertoli cell-TJ dynamics both in vitro (Lui et al., 2001; Lui et al., 2003a; Siu et al., 2003a) and in vivo (Lui et al., 2003d). Consistent with results of these earlier studies, the $CdCl_2$-induced BTB damage also led to an approximate fourfold induction of TGF-β3 and -β2 between 16 and 24 hours (Fig. 2A,C), but not TGF-β1, suggesting that both TGF-β2 and -β3 are involved in BTB dynamics. While the level of p38 MAP kinase remained relatively stable, an induction in p-p38 MAP kinase (the

activated phosphorylated form of p38) (Fig. 2A,D), but not ERK 1/2 (Fig. 2A,E) or JNK 1/2 (data not shown), was detected. In contrast, the endogenous levels of occludin (a TJ-integral membrane protein) and ZO-1 (a TJ peripheral adaptor protein) remained steady in the testes until they began to decline by about 16 hours post-treatment and were virtually undetectable by 24 hours and thereafter (Fig. 2A,F). Such reciprocal relationship of TGF-β2/-β3 and occludin in response to BTB damage is consistent with earlier reports that a transient reduction in TGF-β3 was associated with an induction of occludin and ZO-1 during Sertoli cell TJ-barrier assembly in vitro (Lui et al., 2001; Lui et al., 2003a).

Changes in AJ-associated proteins

When the levels of the two principal ES structural protein complexes, namely cadherin/catenin and nectin/afadin in the testes were examined during $CdCl_2$-induced BTB damage, the pattern of their expressions was similar to the two TJ proteins (Fig. 2A,G-I). The endogenous levels of all the cadherins and catenins examined were reduced during the $CdCl_2$-induced BTB disruption, although N-cadherin and β-catenin levels began to drop earlier (~16 hours and 7 hours, respectively) than did E-cadherin (~20 hours) and γ-catenin (~48 hours) (Fig. 2A,G,H). Nectin-3 and l-afadin showed a similar pattern of reduction as the cadherins and catenins, which started to tumble by 20 hours and 16 hours post-treatment, respectively (Fig. 2A,I). Two other AJ signaling molecules, $p120^{ctn}$ and its upstream activator c-Src, were transiently induced in contrast to the AJ structural protein complexes. A surge of $p120^{ctn}$ protein (~3-fold) in the testis was observed between 12 and 24 hours post-treatment, and c-Src production was also significantly induced (~2-fold) between 7 and 16 hours (Fig. 2A,J).

Changes in cytoskeletal proteins

Besides the TJ- and AJ-associated proteins, the cytoskeletal structural proteins were also affected during the $CdCl_2$-induced BTB damage (Fig. 2A,K). The expression of actin remained stable until 24 hours after $CdCl_2$ administration, but was reduced by as much as 50% of the control value by 48-96 hours (Fig. 2A,K). This result is consistent with an earlier report illustrating that $CdCl_2$ exposure can induce fragmentation of actin microfilament bundles in rat seminiferous epithelium (Hew et al., 1993b). The protein levels of vimentin, a structural component of the intermediate filament network, and α-tubulin, a structural protein of the microtubule network, were also found to drop significantly, beginning at 16 hours and 48 hours, respectively (Fig. 2A,K).

Changes in proteases and protease inhibitors

Since there are several studies indicating the crucial roles of proteases and protease inhibitors in TJ and AJ dynamics (Mruk et al., 1997; Mruk et al., 2003; Okanlawon and Dym, 1996; Siu et al., 2003a; Siu and Cheng, 2004), we sought to investigate their protein levels in the testis during $CdCl_2$-induced TJ and AJ restructuring (Fig. 2A,L-N). For MMP-2, a member of the metalloproteinase family and a known product of Sertoli cells in the testis (Longin et al., 2001), the expression of its pro-form was apparently induced mildly by 5 hours post-treatment and persisted until 12-16 hours before returning to its basal level (Fig. 2A,L). The levels of MMP-3, another MMP member, and uPA, a serine protease, both of which are also products of Sertoli cells (Longin and Le Magueresse-Battistoni, 2002; Vihko et al., 1988) were reduced by 20 hours post-treatment (Fig. 2A,L,M). In contrast, cathepsin L, a Sertoli cell cysteine protease (Chung et al., 1998; Erickson-Lawrence et al., 1991), was significantly induced by ~3-fold beginning at 16 hours (Fig. 2A,M). One of its specific inhibitors, cystatin C, also displayed a similar trend of increase, except that its expression dropped significantly at 96 hours post-treatment (Fig. 2A,N) while the protein level of cathepsin L remained high in the testis (Fig. 2A,M). The level of another Sertoli cell secreted non-specific protease inhibitor, α_2-MG (Cheng et al., 1990), was also induced by as much as 4-fold starting at 12 hours, and remained high until 96 hours (Fig. 2A and N). It is of interest to note that some of these patterns of changes in target proteins in testes were also detected when a lower dose of $CdCl_2$ (1 mg/kg b.w.) was used. An induction of α_2-MG was detected by 24 hours post $CdCl_2$ treatment, which persisted until 96 hours (Fig. 2B). This pattern of changes, while somewhat delayed, is consistent with results using the higher dose of $CdCl_2$, 3 mg/kg b.w., shown in Fig. 2A. Also, the testicular protein level of occludin was also significantly reduced at 24 hours post $CdCl_2$-treatment (results not shown), consistent with results shown in Fig. 2A. However, we opted to use 3 mg/kg b.w. $CdCl_2$ instead of 1 mg/kg b.w. because visible damage to the seminiferous epithelial barrier was detected by immunohistochemistry and immunofluorescence microscopy in all animals in all three experiments (n=3-5 rats per time point) beginning at ~7 hours (see following and Figs. 3 and 4). Moreover, changes in testicular weight were not detected using 3 mg/kg b.w. of $CdCl_2$ until 96 hours post-treatment (Table 1).

Immunohistochemical localization of ZO-1 in the seminiferous epithelium after $CdCl_2$ treatment

The localization of ZO-1, a TJ peripheral adaptor found at the site of the BTB (Byers et al., 1991), in the seminiferous epithelium was examined to assess its changes during the $CdCl_2$-induced BTB damage. Immunoreactive ZO-1 appeared as reddish-brown precipitates at the basal compartment of normal rat seminiferous tubules consistent with its localization at the site of the BTB (Fig. 3A,C; see arrowheads in Fig. 3A). Non-specific staining was only detected in the blood vessels in the interstitium of the control sections (Fig. 3B), illustrating that the staining shown in Fig. 3A,C-H is specific to ZO-1. Control sections using PBS in place of the primary antibodies, and normal rabbit serum instead of biotinylated secondary antibody, also yielded no detectable staining (data not shown). Immunoreactive ZO-1 precipitates formed an almost continuous belt at the base of the each tubule in virtually every stages of the epithelial cycle (see white arrowheads, Fig. 3A). Weak staining was also found around early elongating spermatids, consistent with an earlier report (Byers et al., 1991). This immunostaining pattern of ZO-1 in the seminiferous epithelium persisted until 7 hours after the $CdCl_2$ treatment (Fig. 3D). Thereafter, the intensity of ZO-1 staining declined and became a discontinuous ring by 12 hours post-

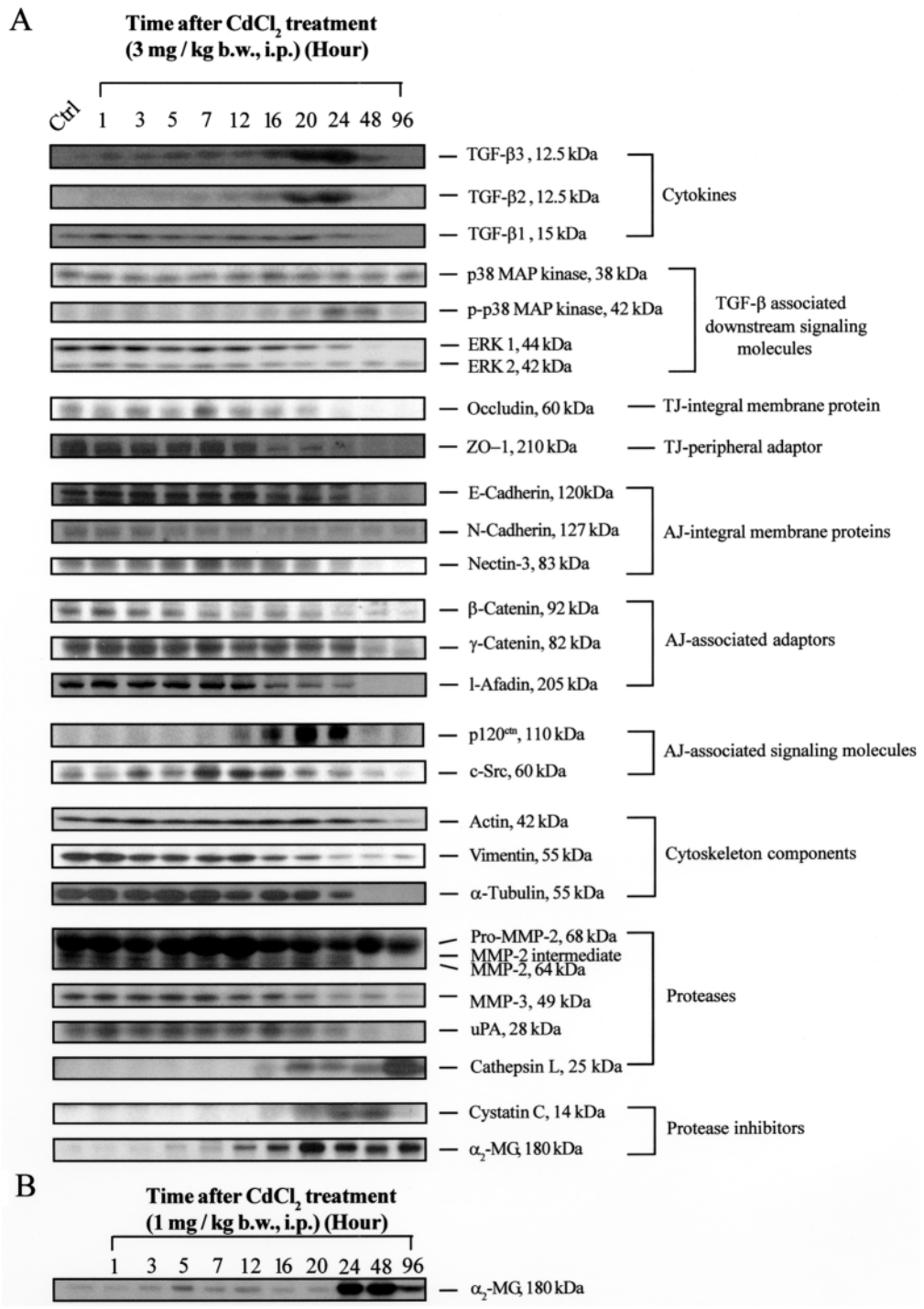
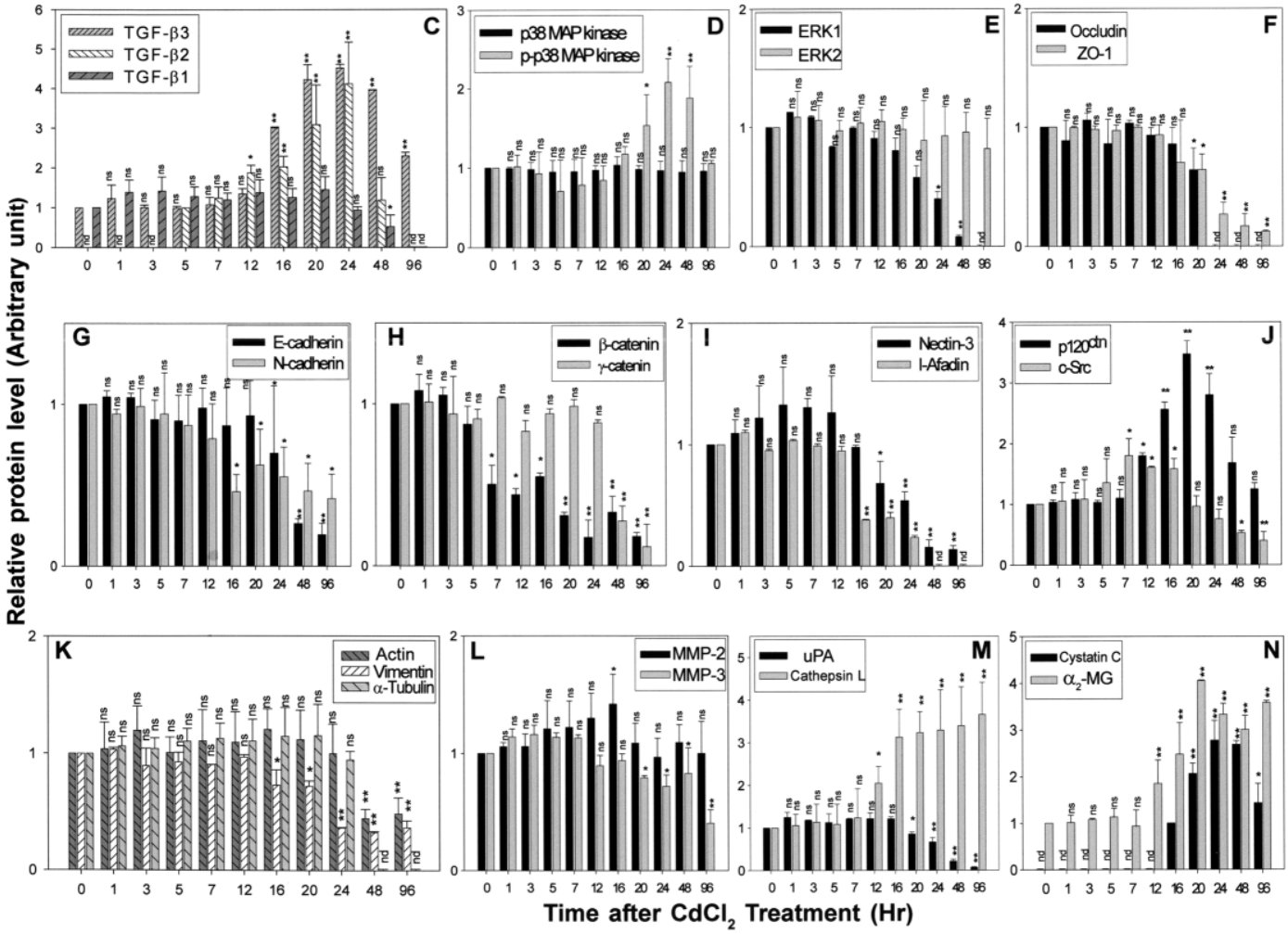


Fig. 2. Changes in cytokine levels, different TJ- and AJ-associated molecules, cytoskeleton components, proteases and protease inhibitors in rat testes during $CdCl_2$-induced BTB damage. (A) Rats that had been treated with $CdCl_2$ [3 mg/kg body weight by intra-peritoneal injection (b.w., i.p.)] were sacrificed and testis lysates were prepared as described in Materials and Methods. Proteins (~100 μg per lane) from these lysates were resolved by SDS-PAGE and subjected to immunoblot analysis using the corresponding primary antibody as indicated. (B) Immunoblot analysis to quantify the levels of α_2-MG of testis lysates from rats receiving 1 mg/kg b.w. $CdCl_2$. (C-N) Densitometry scans of immunoblots, such as those shown in A. The protein level of a corresponding target protein at time 0 was arbitrarily set at 1. Each bar is mean±s.d. of three to five determinations from three to five rats of each experiment. Two other sets of experiments yielded virtually identical results. nd, non-detectable; ns, not significantly different by ANOVA where a given sample was compared to all other samples within an experimental group; *significantly different, $P<0.05$; **significantly different, $P<0.01$.

treatment (Fig. 3E). By 20 hours, immunoreactive ZO-1 at the base of the tubules became very weak (Fig. 3F), and the staining was virtually undetectable at 24 hours and 96 hours (Fig. 3G,H). The population of germ cells in the seminiferous tubules also declined significantly by 24 hours, suggesting a loss of cell adhesion function at the site of Sertoli-germ cell interface. By 96 hours, virtually all tubules were devoid of germ cells, confirming that a primary loss of BTB function can lead to a secondary loss of Sertoli-germ cell adhesion function.

Co-localization of occludin and ZO-1 in the seminiferous epithelium during $CdCl_2$-induced BTB damage to assess BTB integrity by immunofluorescence microscopy

Occludin and ZO-1 are two important interacting proteins that co-exist in TJ fibrils in a stoichiometric ratio of 1:1 (Furuse et al., 1994). Herein, we demonstrate that the losses of occludin and ZO-1 during $CdCl_2$-induced BTB damage are indeed in concert with each other, as revealed by immunofluorescence microscopy. Merged images showed that occludin and ZO-1 co-localized at the basal compartment of the seminiferous tubules in both normal



(Fig. 4C compare with 4A,B) and $CdCl_2$-treated (Fig. 4F,I,L,O compare with D,E; G,H; J,K and M,N, respectively) rat testes. In normal testis, occludin and ZO-1 formed continuous belts of fluorescent staining near the basement membrane at the basal compartment of the seminiferous epithelium, consistent with their localization at the BTB. As the time after $CdCl_2$ treatment lengthened, the immunofluorescent staining of both proteins and their merged signals gradually weakened. By 24 hours, although some patches of weak staining were still visible in some tubules (Fig. 4J-L), staining of both proteins became hardly detectable in almost 90% of the tubules examined (Fig. 4M-O). These results also illustrate the use of immunofluorescence microscopy as a novel technique to monitor BTB integrity.

Immunohistochemical localization of α_2-MG in the seminiferous epithelium after $CdCl_2$ treatment

The pattern of localization of immunoreactive α_2-MG in the seminiferous epithelium of normal rat testes, as shown in Fig. 5A, is consistent with an earlier report (Zhu et al., 1994). The middle and right panels of Fig. 5A are the magnified views of the left panel that show the cellular association of α_2-MG in the seminiferous epithelium. α_2-MG was confined largely to the perinuclear region of Sertoli cells and around the heads of elongating and elongated spermatids at the site of apical ES in stages XI-VII, except at spermiation, as well as in the interstitium associated with blood vessels and possibly Leydig cells (Fig. 5A). Very intensive α_2-MG staining was observed at the basal compartment as well as around the head of spermatids at the site of ES during $CdCl_2$-induced BTB damage (Fig. 5B, see also micrographs of higher magnifications in the middle and right panels), which persisted until 96 hours (Fig. 5C,D), although became weaken after 20 hours (Fig. 5C,D compare with Fig. 5B). The specificity of the α_2-MG staining shown in Fig. 5A-D was confirmed when control sections were incubated with pre-immune rabbit serum, since the reddish-brown precipitates were barely detectable in control sections (Fig. 5E).

α_2-MG structurally associates with the actin-based nectin/afadin ES structural protein complex, but not cadherins, catenin, $p120^{ctn}$ and occludin

In order to further investigate the role of α_2-MG in the TJ/AJ dynamics in the testis, an immunoprecipitation study was performed using an anti-α_2-MG antibody and lysates of testes from rats with or without $CdCl_2$ treatment. α_2-MG was found to structurally link to nectin-3 and l-afadin in normal testis and in the testis 24 hours post $CdCl_2$ treatment but not to the cadherin/catenin/$p120^{ctn}$ complex (Fig. 6). It was also structurally linked

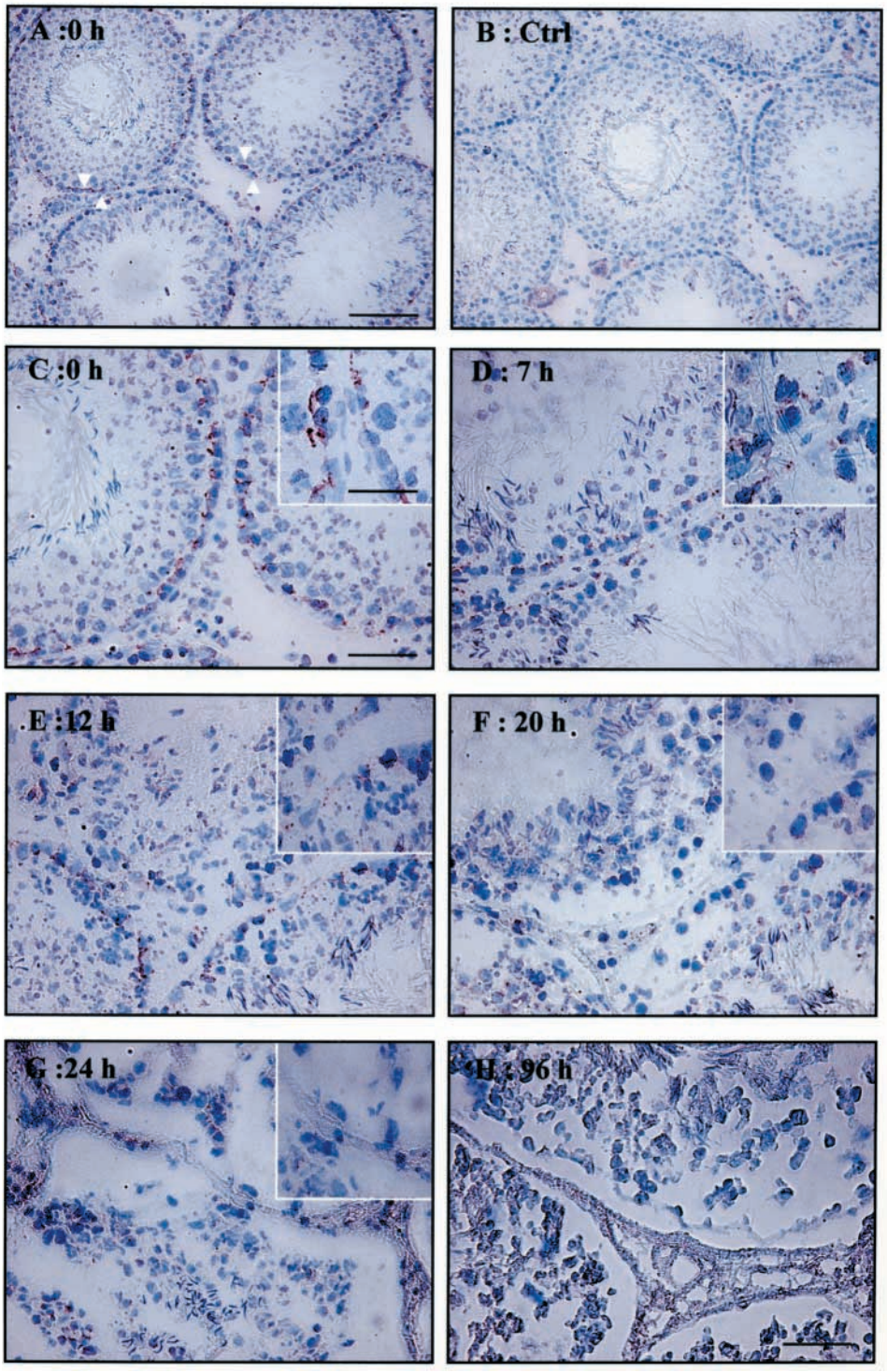


Fig. 3. Immunohistochemical localization of ZO-1 in the seminiferous epithelium of adult rat testes during $CdCl_2$-induced BTB damage. (A) Low magnification of a cross section of a rat testis at 0 hours immunostained with an anti-ZO-1 antibody, and (B) a control section where normal rabbit serum was substituted for the primary antibody. (C-H) Micrographs at high magnifications of rat testes after $CdCl_2$ treatment (3 mg/kg b.w.i.p.). Immunoreactive ZO-1 appears as reddish-brown precipitates localized at the basal compartment consistent with the site of the BTB (see arrowheads in A). These micrographs are typical of the results from at least 120 cross sections from three testes of different rats. Scale bar: (A,B) 200 μm; (C-H) 80 μm; (C inset, applies to insets in D-G) 20 μm.

to two major cytoskeletal proteins actin and vimentin (Fig. 6). However, α_2-MG did not associate with occludin. There was no structural association between α_2-MG and cathepsin L either, possibly because the specific inhibitor of the cysteine protease cathepsin L in the testis is cystatin C (Fritz et al.) (Fig. 6). Since immunoprecipitation of testis lysates with α_2-MG, and the resultant blot subsequently stained with an anti-TGF-β3 antibody, revealed a minor protein band close to the 12.5 kDa TGF-β3 band, it was difficult to unequivocally demonstrate that α_2-MG structurally associated with TGF-β3. Therefore, an anti-TGF-β3 antibody was used instead for immunoprecipitation using testis lysates as shown in Fig. 6. α_2-MG indeed was shown to associate with this cytokine in normal testis, which was also induced in testis by 24 hours post-$CdCl_2$ treatment (Fig. 6), consistent with data shown in Fig. 2. Controls using testis lysates incubated with normal rabbit serum or without incubation with any antibodies during immunoprecipitation produced no detectable band (data not shown).

Changes in the protein levels and immunostaining pattern of TJ- and AJ-associated molecules, proteases, protease inhibitors and cytokines in $CdCl_2$-treated rat testes after SB202190 administration

A recent in vitro study demonstrated that TGF-β3 might regulate Sertoli cell TJ dynamics via the p38 MAP kinase pathway (Lui et al., 2003a). Furthermore, the intratesticular injection of SB202190, a specific p38 MAP kinase inhibitor, delayed the $CdCl_2$-induced occludin loss at the site of BTB in vivo (Lui et al., 2003d). In this study, we sought to expand these earlier studies to investigate if cytokines can also regulate AJ dynamics via the p38 MAP kinase pathway. When SB202190 was administrated to the rat testis prior to $CdCl_2$ treatment and testes were collected 16 hours after $CdCl_2$ administration, it was found that SB202190 not only blocked the $CdCl_2$-induced loss of occludin and ZO-1, but also blocked the loss of N-cadherin, E-cadherin, β-catenin, nectin-3 and l-afadin from the seminiferous epithelium (Fig. 7). It also suppressed the

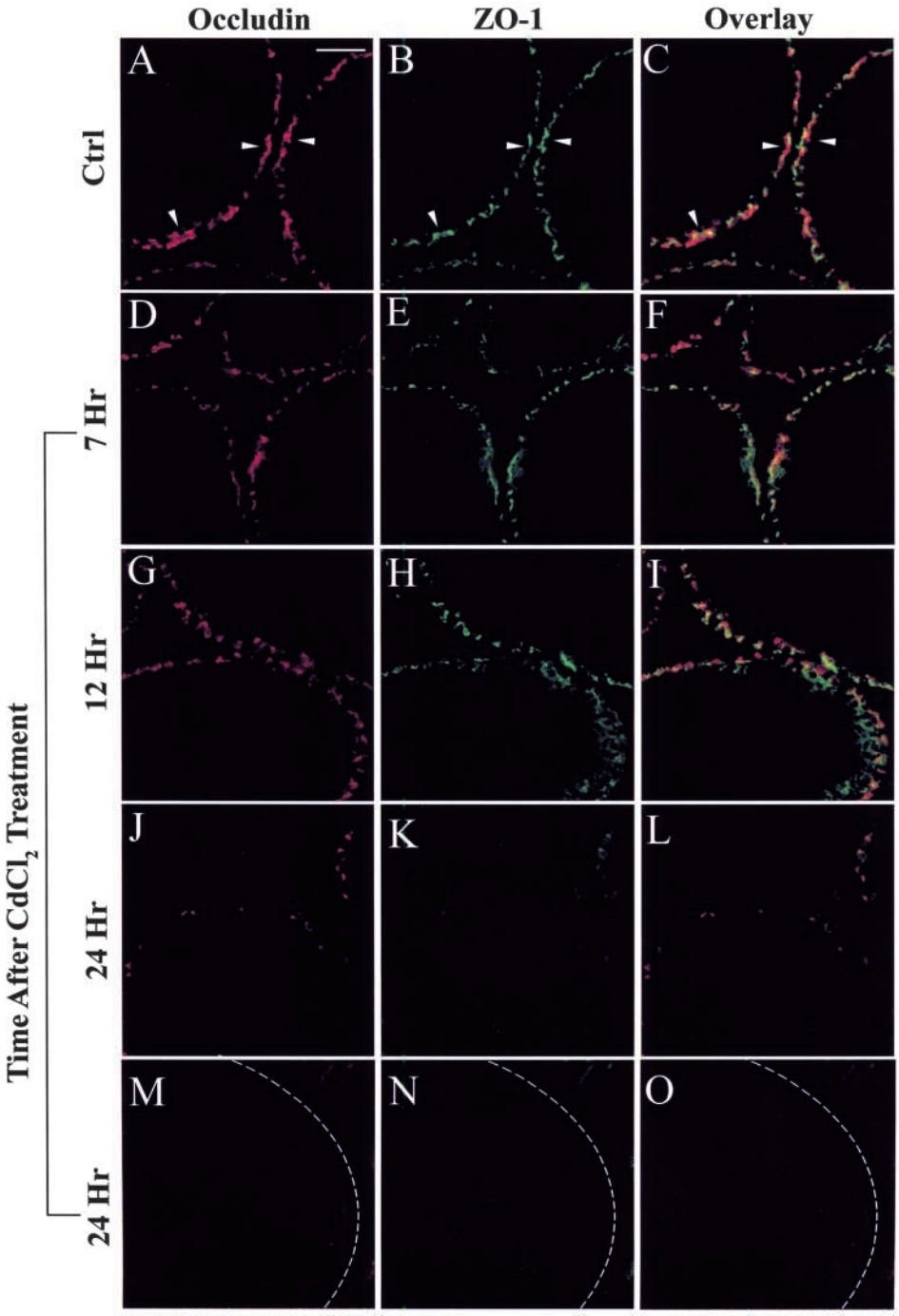


Fig. 4. Immunofluorescence microscopy to identify occludin and ZO-1 in the seminiferous epithelium of rat testes at the BTB site during $CdCl_2$-induced BTB damage. (A-O) Immunofluorescence micrographs of cross sections of normal rat testes (A-C) and rat testis at 7 hours (D-F), 12 hours (G-I) and 24 hours (J-O) after $CdCl_2$ treatment. Occludin appears as red fluorescence (Cy-3, white arrowheads in A), ZO-1 as green fluorescence (FITC, arrowheads in B). (C,F,I,L,O) Merged images of the corresponding immunofluorescence micrographs for occludin and ZO-1, in which co-localization appears orange (arrowheads in C). White broken lines in M-O indicate the approximate location of the basement membrane of the seminiferous epithelium. Scale bar: 20 μm.

BTB-damage-mediated cathepsin L induction (Fig. 7). Yet SB202190 pretreatment failed to affect the level of α_2-MG during $CdCl_2$-induced BTB damage (Fig. 7). The observations reported herein (Fig. 7) were subsequently confirmed using immunohistochemistry to locate occludin, ZO-1, N-cadherin and α_2-MG in the seminiferous epithelium with and without SB202190 pretreatment during the $CdCl_2$-induced BTB damage (Fig. 8). For occludin, ZO-1 and N-cadherin, the immunoreactive belts of staining at the site of the BTB were weakened after $CdCl_2$ treatment (positive controls) (Fig. 8B,E,H compared with A,D,G, respectively), yet pretreatment of rat testes with SB202190, to block the downstream TGF-β3 action, effectively blocked the BTB damage-associated protein loss from the BTB sites (Fig. 8C,F,I compare with A,D,G and B,E,H). However, the staining of α_2-MG in testes pretreated with SB202190 before $CdCl_2$ administration (Fig. 8L) did not show any observable differences when compared with vehicle pretreatment followed by $CdCl_2$ (Fig. 8L versus Fig. 8K), suggesting that the changes in α_2-MG during the BTB damage was not regulated by TGF-β3, or if it was, it was not mediated via the p38 MAP kinase pathway.

Discussion

Can the use of immunofluorescence microscopy and immunoblotting to monitor the levels of occludin and ZO-1 at the site of the BTB be a reliable indicator of BTB integrity?

Cadmium is a major industrial pollutant and environmental toxicant that can cause significant damage to many organs, including the lung, liver, kidney and testis in humans and rodents (for a review, see Prozialeck, 2000). Its damaging effects to the testis have been known for decades, including disruption of the Sertoli cell TJ-barrier function and the vascular system, failure of spermiation, germ cell loss from the seminiferous epithelium, tissue necrosis and apoptosis (Aoki and Hoffer, 1978; Gunn and Gould, 1970; Hew et al., 1993a; Mason et al., 1964; Xu et al., 1996), some of which can be prevented by zinc (Parizek, 1957). More recent studies have suggested that its primary site of action in the testis is the Sertoli cell, perturbing the TJ-barrier function in vitro (Chung and Cheng, 2001; Janecki et al., 1992) and in vivo (Hew et al., 1993b; Setchell and Waites, 1970). As such, the injection of $CdCl_2$ in

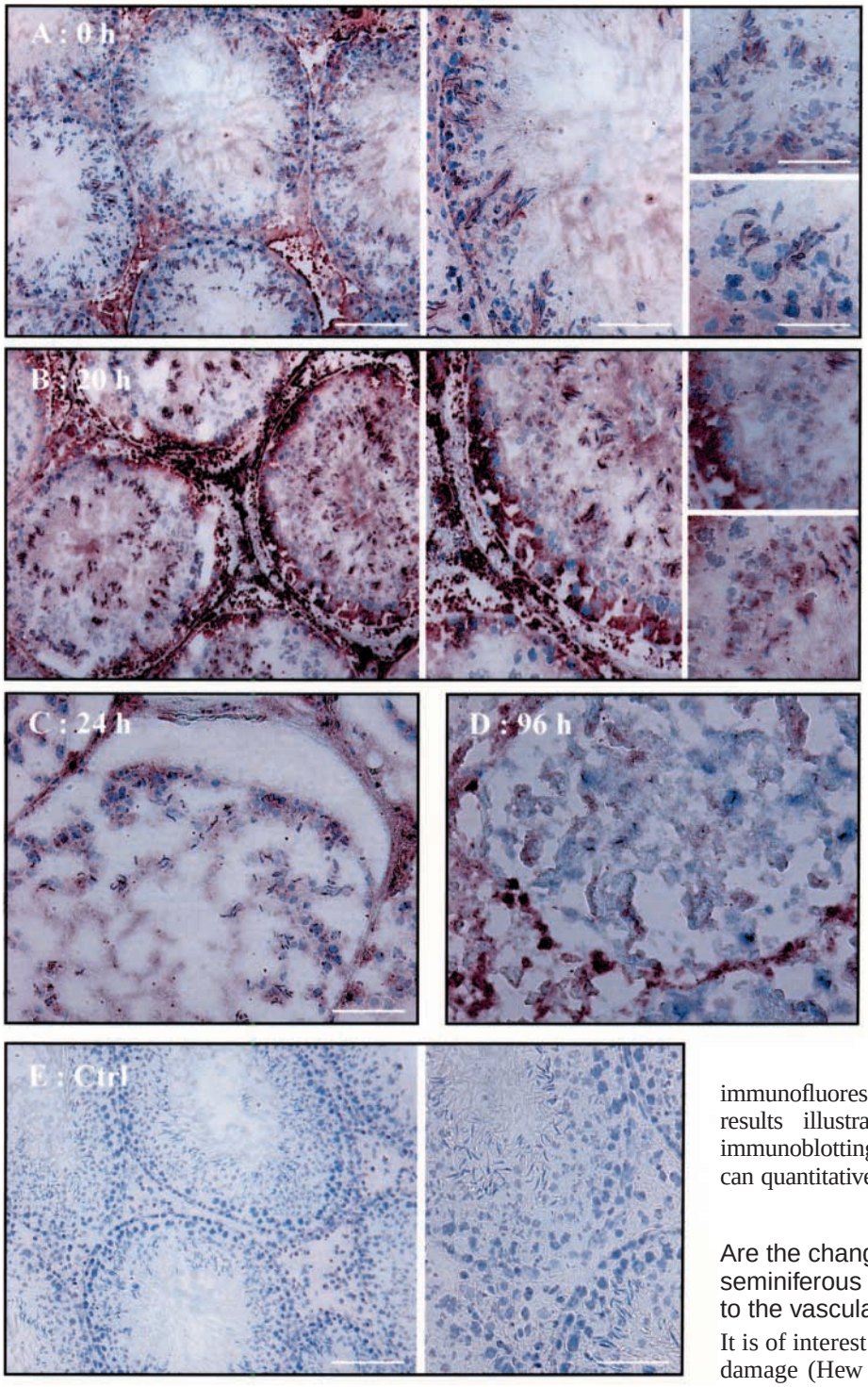


Fig. 5. Immunohistochemical localization of protease inhibitor α_2-MG in the seminiferous epithelium of rat testes at the BTB site during $CdCl_2$-induced BTB damage. (A-D) Immunoreactive α_2-MG in the seminiferous epithelium after treatment of rats with $CdCl_2$ (3 mg/kg b.w., i.p.). Shown are different magnifications of cross sections of testes at time 0 hours, 20 hours, 24 hours and 96 hours. Immunoreactive α_2-MG appears as reddish-brown precipitates. (E) Control in which normal rabbit serum was substituted for the primary antibody, illustrating that the staining for α_2-MG shown in A-D is specific. The smaller images in A, B and E are higher magnifications of the representative micrographs shown on the left panel that illustrate the cellular localization of α_2-MG in the seminiferous epithelium. Bars in left panel of A and E, 200 μm; bars in C, in middle panel of A and right panel of E, 80 μm; bar in right panel of A, 20 μm. Bars shown in A are applicable to B, and bar in C is applicable to D.

vivo to induce BTB damage is regarded as one of the best models to study TJ dynamics in the testis (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003b). Yet the techniques used in earlier studies to monitor BTB integrity are tedious and time consuming, requiring expensive equipment and the use of radioisotopes, confocal microscopy, electron microscopy, and micropuncture techniques (Chung et al., 2001; Eng et al., 1994; Hew et al., 1993b; Wiebe et al., 2000). By using dual immunofluorescence microscopy we have shown that both occludin and ZO-1 are localized at the same site at the basal compartment of the seminiferous epithelium, consistent with their localization at the BTB, forming an almost superimposable yet continuous fluorescent ring encircling the entire base of each tubule. By 20 hours after $CdCl_2$ treatment, a significant reduction in both ZO-1 and occludin proteins was detected by immunoblotting, consistent with results of immunohistochemistry. More importantly, the dual fluorescent belt-like structure at the site of BTB was badly disrupted when visualized by immunofluorescence miscroscopy. Taken collectively, these results illustrate unequivocally that the use of both immunoblotting and fluorescence microscopy techniques can quantitatively assess the BTB integrity.

Are the changes in target proteins in the seminiferous epithelium reported herein secondary to the vascular damage induced by $CdCl_2$?

It is of interest to note that $CdCl_2$ can also induce vascular damage (Hew et al., 1993b; Setchell and Waites, 1970), which was detected in some rats in our experiments at 24 hours after $CdCl_2$ exposure, when red blood cells were found in the interstitium. Thus, it is possible that some of the changes seen in Sertoli cells are secondary effects to anoxia. While such a possibility exists, it is not likely to be the case for the following reasons. First, a disruption of the seminiferous epithelial barrier function was detected by both immunohistochemistry and fluorescence microscopy by 7 hours when red blood cells were not yet detected in the interstitium, suggesting that the Sertoli cell-based BTB function was disrupted prior to the damage of the vascular

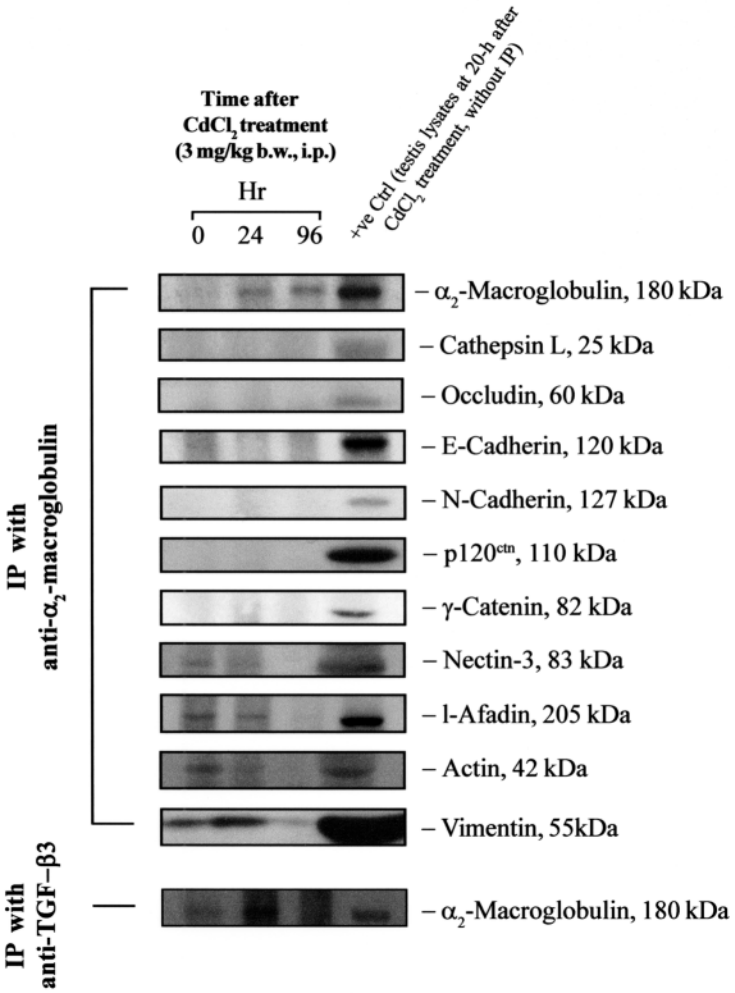
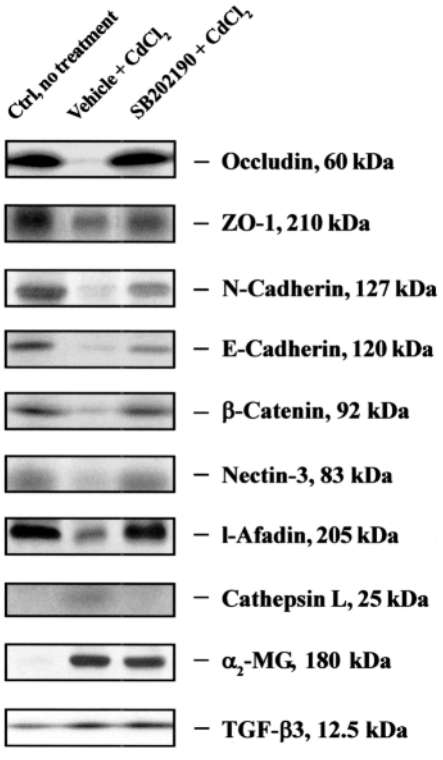


Fig. 6. Studies by co-immunoprecipitation to investigate the association of the protease inhibitor α_2-MG with TGF-β3, cathepsin L, and TJ-associated, AJ-associated and cytoskeletal proteins during $CdCl_2$-induced BTB damage. Immunoprecipitation was performed using either anti-α_2-MG or anti-TGF-β3 antibody and lysates of testes from rats that had received $CdCl_2$ (3 mg/kg b.w., i.p.) at 0, 24 and 96 hours earlier. For positive controls, lysates of testes from rats (~150 μg protein per lane) at 20 hours after $CdCl_2$ treatment *without* immunoprecipitation were used since at this time most target proteins pertinent to our studies were induced (see Fig. 2), confirming the specificity of the antibodies. Only a set of representative data is shown, but two other experiments using different rat testis lysates yielded identical results.



barrier function. This result is also consistent with an earlier study by Setchell and Waites (Setchell and Waites, 1970) indicating that the BTB function is compromised by $CdCl_2$ prior to the vascular damage. Obviously, in order to clarify this, further experiments must be conducted to assess the kinetics of vascular and BTB damages following treatments of rats with 0.5-1 versus 3 mg $CdCl_2$/kg b.w., i.p., using more sensitive markers, such as proteins that specifically constitute these TJs. Second, when a single dose of 1 mg/kg b.w. of $CdCl_2$ was used, it failed to induce visible vascular lesions in rat testes, i.e. blood cells were not detected in the testes of any of the animals examined (n=4 for each time point), which is consistent with an earlier report that the damaging effects of $CdCl_2$ on the vascular barrier in testes was not visible at 0.5 or 1 mg/kg b.w. (Hew et al., 1993a). However, we can still detect similar patterns of induction of α_2-MG and reduction of occludin in all animals treated with 1 mg/kg b.w. $CdCl_2$ (and visible damage to the seminiferous epithelial barrier in some rats at 48 and 72 hours, seen by immunofluorescence microscopy and immunohistochemistry), consistent with our postulate that changes in these target proteins take place before any visible damage to the BTB function. In essence, BTB dynamics are regulated by the intricate interactions of cytokines, proteases, protease inhibitors and TJ-integral membrane proteins. Third, and perhaps the most important of all, the use of SB202190, a specific inhibitor of p38 MAP kinase, indeed blocked the $CdCl_2$-induced loss of ZO-1 and occludin from the site of the BTB, abolishing the disruptive effects of TGB-β3 on the Sertoli cell-TJ barrier function as reported earlier (Lui et al., 2003a; Lui et al., 2003d). This clearly illustrates the physiological significance of TGF-β3 in BTB dynamics.

Fig. 7. A study to assess if a blockade of the p38 MAP kinase by SB202190 can delay the $CdCl_2$-induced target protein losses from the sites of BTB and AJs in the testis during BTB damage. Normal rat testes (negative controls) (Ctrl), and testes injected with 1% DMSO in saline (vol/vol, intratesticular administration) followed by $CdCl_2$ treatment served as positive controls ($CdCl_2$/vehicle). For test groups (n=3-5 rats), testes of rats were pre-treated with 8 nmol SB202190 in 200 μl saline with 1% DMSO (i.e. 5 μM assuming a volume of 1.6 ml per testis), followed by $CdCl_2$ treatment (3 mg/kg b.w.i.p.) ($CdCl_2$ + SB202190), and rats were sacrificed 16 hours later. Lysates were prepared from these samples for immunoblotting with antibodies against occludin, ZO-1, N-cadherin, E-cadherin, β-catenin, nectin-3, l-afadin, cathepsin L, α_2-MG, and TGF-β3.

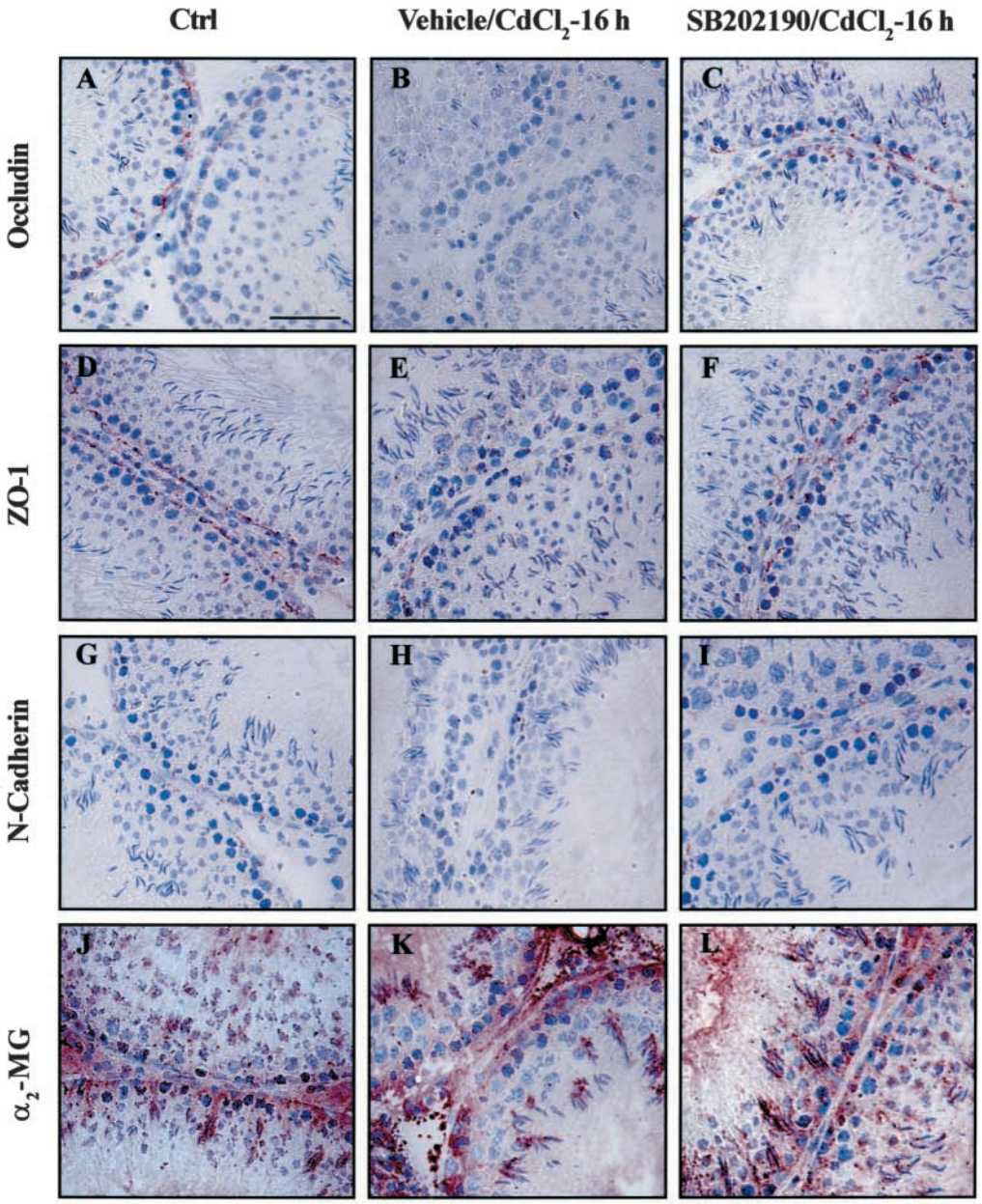


Fig. 8. Immunohistochemical localization of occludin, ZO-1, N-cadherin and α_2-MG in the seminiferous epithelium of rat testes with and without pretreatment with either SB202190 or vehicle (1% DMSO) (via intratesticular injection) prior to $CdCl_2$ administration to induce BTB damage. (A-L) Micrographs of seminiferous epithelium of normal rats (A,D,G,J, negative controls), rats pretreated with vehicle (1% DMSO) followed by $CdCl_2$ (B,E,H,K; positive controls), and rats pretreated with 5 μM SB202190 followed by $CdCl_2$ (C,F,I,L; test). A-C, D-F, G-I and J-L show the immunohistochemical localization of occludin, ZO-1, N-cadherin and α_2-MG, respectively. It is noted that after the pre-treatment of testes with SB202190, the $CdCl_2$-induced losses of occludin (C compared with B), ZO-1 (F compared with E) and N-cadherin (I compared with H) were blocked. However, the induction of α_2-MG expression by $CdCl_2$ was not affected (L compared with K). Scale bar in A: 80 μm, applicable to B-L.

Furthermore, this inhibitor also prevented the $CdCl_2$-induced loss of several AJ proteins, such as N- and E-cadherin, β-catenin, nectin-3 and afadin from the seminiferous epithelium, suggesting that AJ dynamics are regulated via the TGF-β3/p38 MAP kinase pathway. Undoubtedly, changes of target proteins detected in the testis in particular at ~24 hours onwards could be somewhat affected by the disruption of the vascular barrier function. Yet this cannot negate the significance of our finding that a primary disruption of the Sertoli cell-barrier function can contribute to a secondary loss of Sertoli-germ cell adhesive function, because the TGF-β3/p38 MAP kinase pathway was shown to be used by the seminiferous epithelium to regulate both TJ and AJ dynamics. Our findings also illustrate the shortcoming of this $CdCl_2$ model to study BTB dynamics since it is not entirely clear how much of the current data stems from changes in blood vessel barrier function in the testis, unless $CdCl_2$ targets itself specifically to a TJ-integral membrane protein at the site of the BTB, which is not found in vascular endothelial junctions.

Does the primary disruption of TJs also lead to secondary loss of cell adhesion function at the site of AJs? If it does, what is the signaling pathway that regulates AJ disruption?

The loss of BTB integrity in the testis by 20-24 hours following $CdCl_2$ treatment is accompanied by a subsequent loss of germ cells from the seminiferous epithelium, which became clearly visible by 24-96 hours. For instance, by 24 hours, ~50% of the tubules were devoid of elongate/elongating and round spermatids. Greater than 90% of the tubules examined were devoid of germ cells by 96 hours. The damaging effect of $CdCl_2$ on the BTB were *irreversible* since immunohistochemistry studies have shown that the BTB

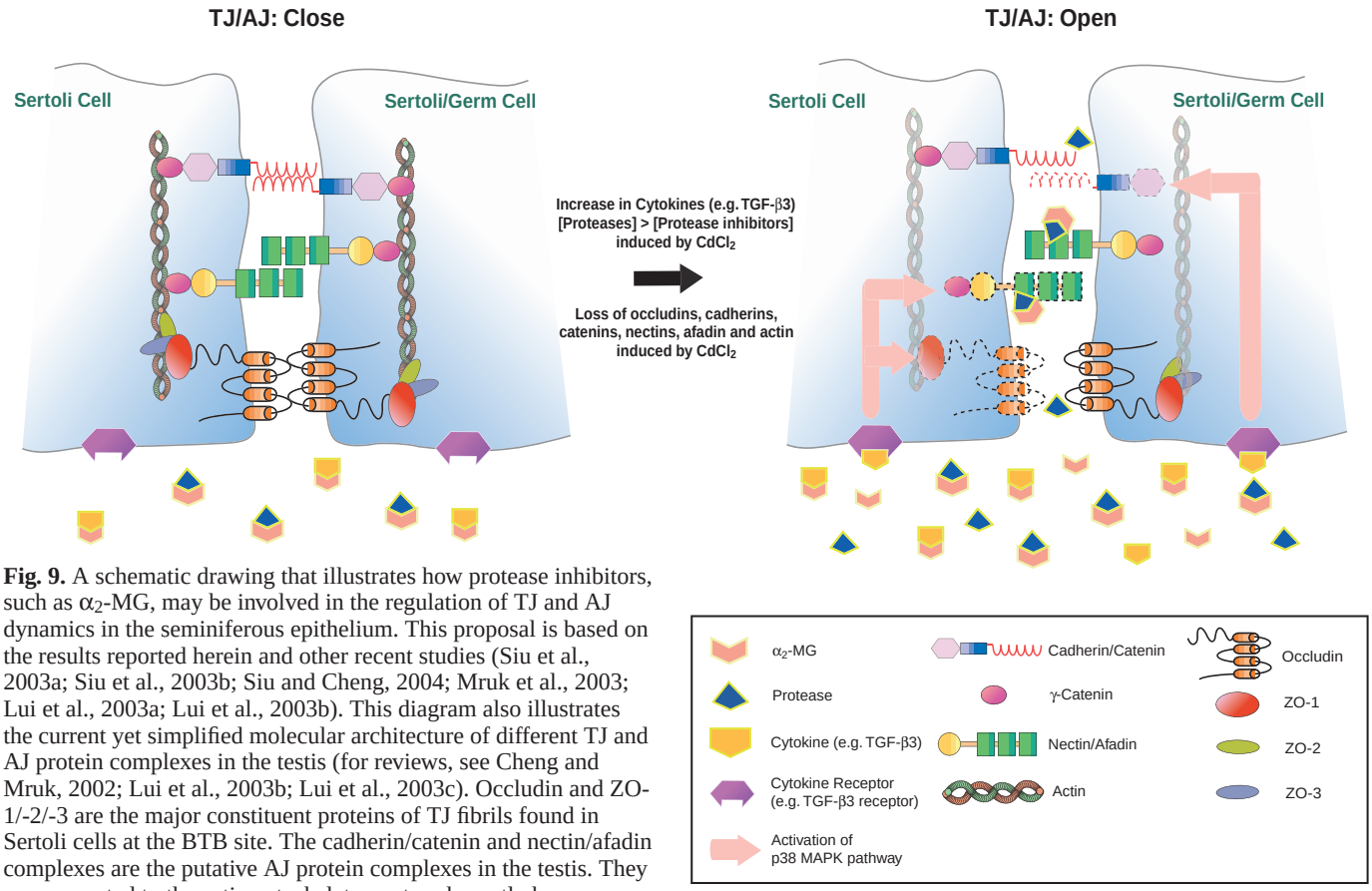


Fig. 9. A schematic drawing that illustrates how protease inhibitors, such as α_2-MG, may be involved in the regulation of TJ and AJ dynamics in the seminiferous epithelium. This proposal is based on the results reported herein and other recent studies (Siu et al., 2003a; Siu et al., 2003b; Siu and Cheng, 2004; Mruk et al., 2003; Lui et al., 2003a; Lui et al., 2003b). This diagram also illustrates the current yet simplified molecular architecture of different TJ and AJ protein complexes in the testis (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003b; Lui et al., 2003c). Occludin and ZO-1/-2/-3 are the major constituent proteins of TJ fibrils found in Sertoli cells at the BTB site. The cadherin/catenin and nectin/afadin complexes are the putative AJ protein complexes in the testis. They are connected to the actin cytoskeleton network, partly by α-catenin. When TJs and AJs are 'closed', the junction-associated multiprotein complexes of two apposed cells interact with each other, whereas the level and/or activity of cytokines and proteases apparently are kept low by α_2-MG. However, when TJ and AJ disassemble (or 'open'), such as when treated with $CdCl_2$, junction functionality is perturbed. This may be mediated by cytokines, such as TGF-β3 and TNFα that bind to their receptors and lead to the rapid turnover (possibly undergoing proteolysis) of junction components (represented by broken lines) via the p38 MAP kinase signal pathway. Proteases activities may also be induced, facilitating junction dissociation. At this time, the amount of the protease inhibitor α_2-MG in the seminiferous tubules increases, binding to the excessive cytokines to limit their action, or inhibiting proteases to protect the integrity of the BTB. As such, protease inhibitors may act as 'rescuers' during junction remodeling in the testis, regulating the homeostasis of proteins that constitute both TJs and AJs.

remained 'broken' by 4 weeks (data not shown), consistent with earlier reports of the permanent damaging effects of $CdCl_2$ on BTB function and spermatogenesis (Hew et al., 1993a; Setchell and Waites, 1970). These results suggest that either the damaging effect of $CdCl_2$ on Sertoli cells is permanent or all the spermatogonia are depleted from the epithelium, or both. This observation is somewhat similar to the effects of glycerol on the BTB in rats, which also induces irreversible germ cell loss from the epithelium (for a review, see Wiebe et al., 1986). Collectively, these data suggest that a primary loss of BTB function can lead to a secondary loss of cell adhesion function that induces the loss of germ cells.

Studies by immunoblotting and immunohistochemistry have shown that the disappearance of occludin and ZO-1 from the site of TJs is indeed accompanied by a loss of cadherin/catenin and afadin/nectin proteins, two putative protein complexes that constitute the ES in the testis (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003c; Ozaki-Kuroda et al., 2002; Vogl et al., 2000), from the site of AJs. This time frame of losses of AJ integral membrane proteins in the cadherin/catenin and nectin/afadin protein complexes coincides with the disappearance of germ cells from the seminiferous epithelium. It is not likely that the loss of these four AJ proteins is entirely the consequence of germ cell disappearance from the epithelium, although recent studies have shown that Sertoli and germ cells contribute almost equally to the pool of these proteins (Lee et al., 2003). This is because the amount of these AJ proteins during $CdCl_2$-induced BTB damage is 4- to 5-fold less than that in normal rat testes by 24 hours after $CdCl_2$ treatment, several orders of magnitude greater than the 50% drop that would be anticipated (note: assuming cadherins or nectins residing in Sertoli and germ cells interact with each other as N-cadherin:N- or E-cadherin and nectin-2:nectin-2/-3 using a 1:1 ratio) if germ cell disappearance from the epithelium *alone* accounted for all the losses. Besides, the use of SB202190 to inhibit p38 MAP kinase activity, downstream of TGF-β3, blocked the loss of N- cadherin, E-cadherin, β-catenin, l-afadin and nectin-3 (but not α_2-macrogloublin), together with occludin and ZO-1, from the site of ES and/or BTB. This clearly illustrates that the function of both cadherin/catenin and afadin/nectin complexes are regulated,

at least in part, by TGF-β3 via the p38 MAP kinase signaling pathway during AJ disruption. The experiments using SB202190 to prevent the loss of TJ and AJ integral membrane proteins from the seminiferous epithelium have demonstrated unequivocally for the first time that the TGF-β3/p38 MAP kinase pathway is being used to regulate both TJ and AJ dynamics in the testis in vivo.

Moreover, AJ disruption mediated by $CdCl_2$ is likely to involve $p120^{ctn}$ and c-Src, two putative signaling and regulatory AJ peripheral proteins (for reviews, see Anastasiadis and Reynolds, 2000; Cheng and Mruk, 2002; Thomas and Brugge, 1997). Their inductions were detected at 12-24 hours and 7-16 hours after $CdCl_2$ treatment, respectively. This coincides with the time frame of germ cell loss from the epithelium, suggesting that the AJ signaling network has been activated to elicit AJ disruption. The activation of c-Src, an upstream activator of $p120^{ctn}$, may lead to an induction, and possibly the phosphorylation, of $p120^{ctn}$ (for a review, see Daniel and Reynolds, 1997). This in turn causes the loss of cell adhesion function in the cadherin/catenin complex, as recent findings have suggested that $p120^{ctn}$ is an inhibitory regulator of this complex (for a review, see Anastasiadis and Reynolds, 2000). In cells with over-expression of $p120^{ctn}$, or with normal expression of $p120^{ctn}$ but having mutations at selected phosphorylation sites, or with hyperphosphorylation of $p120^{ctn}$, the cadherin-mediated adhesion is defective; but the inclusion of Ser/Thr protein kinase inhibitors can restore cell adhesive function (Aono et al., 1999; Daniel and Reynolds, 1997).

In contrast, the regulatory signaling molecules that trigger the loss of adhesion function in the nectin/afadin complex are not entirely known. Nonetheless, the nectin/afadin complex was shown to tightly associate with the cadherin/catenin complex and the phosphorylation of nectin-2 was detected in response to nectin-afadin-mediated cell-cell adhesion (Kikyo et al., 2000). But it is not known whether c-Src, a protein tyrosine kinase, can regulate the phosphorylation status of nectins. It is also possible that the disruption of the nectin/afadin-based AJs can be a direct consequence of the TJ disruption, since nectin is implicated in TJ organization, and afadin is physically linked to ZO-1, forming a 1:1 stoichiometric association (Fukuhara et al., 2002a; Fukuhara et al., 2002b). As such, a disruption of TJs can perturb the underlying nectin/afadin-based AJ structural function.

What roles do proteases and protease inhibitors have in junction dynamics in vivo?

Sertoli and germ cells are known to produce a number of proteases and protease inhibitors, and the interplay of these molecules regulates different testicular functions pertinent to spermatogenesis (for a review, see Fritz et al., 1993). For example, proteases produced by Sertoli cells remove apoptotic germ cells and other residual organelles during the epithelial cycle, possibly via the ubiquitin and lysosome pathways (for reviews, see de Kretser and Kerr, 1988; Russell, 1980). Yet their role(s), if any, in junction dynamics is not known. It is conceivable that the coordinate interactions of proteases and protease inhibitors are crucial not only to the 'opening' or 'closing' of junctions (Fig. 9) but also in removing unwanted junctional complex components. Indeed, it was shown that the presence of chloroquine, a protease inhibitor, in Sertoli cell cultures could facilitate the assembly and maintenance of TJ barrier function, making the TJ barrier 'tighter' than in control cultures (Okanlawon and Dym, 1996). Also, the assembly of the Sertoli cell-TJ barrier (Wong et al., 2000) or TJ barrier reassembly following its disruption by $CdCl_2$ treatment (Chung and Cheng, 2001) in vitro was associated with an induction of α_2-macroglobulin, implicating its significance in TJ dynamics. Furthermore, co-cultures of germ cells with a Sertoli cell epithelium in vitro, which mimics AJ reassembly events in vivo, induced protease activities in these cells (Mruk et al., 1997), while an intriguing expression pattern between proteases and protease inhibitors was shown during Sertoli-germ cell AJ assembly (Mruk et al., 1997). Addition of α_2-macroglobulin to Sertoli-germ cell cocultures can also facilitate the assembly of Sertoli-germ cell AJs as shown by an in vitro cell adhesion assay (Mruk et al., 2003).

As shown in the present study, the loss of the BTB integrity and cell adhesion function in the epithelium after $CdCl_2$ treatment was associated with an increase in cathepsin L. This protease has been implicated in junction restructuring events, such as facilitating spermiation (Chung et al., 1998; Penttila et al., 1995; Wright et al., 1995), suggesting that its induction may be necessary for junction cleavage. Unlike α_2-macroglobulin, cathepsin L is regulated by TGF-β3 via the p38 MAP kinase pathway since the presence of SB202190 can block the $CdCl_2$-induced cathepsin L production in the testis. Beside cathepsin L, MMP-2 was also induced transiently during the $CdCl_2$-induced BTB damage. Collectively, these data illustrate the significance of proteases in the junction dynamics of the testis.

α_2-Macroglobulin was initially isolated from Sertoli cell-conditioned media in the 1990s and was subsequently shown to be a product of Sertoli, but not germ cells. Its expression in the seminiferous epithelium behind the BTB is regulated quite differently from that produced by hepatocytes in the liver (Cheng et al., 1990; Stahler et al., 1991). Although α_2-macroglobulin is an acute-phase protein in the liver, in the testis it responds neither to glucocorticoids, cytokines, nor experimental induced inflammation (Braghiroli et al., 1998; Stahler et al., 1991), suggesting a specific role in the testis. The induction of α_2-macroglobulin during $CdCl_2$-induced BTB damage thus prompted us to investigate whether this protease inhibitor associates with proteins of the junctional complexes. While α_2-macroglobulin does not associate with either occludin, E-cadherin, N-cadherin, γ-catenin, or cathespin L (a cysteine protease), it does associate with the nectin-afadin protein complex and cytoskeletal proteins actin and vimentin, illustrating its possible function in AJ dynamics via the nectin/afadin complex. Equally important, α_2-macroglobulin was shown to associate with TGF-β3 structurally. α_2-Macroglobulin is found in the extracellular matrix (ECM) in other epithelia and is known to bind TGF-β, TNF-α and interleukins (for a review, see Feige et al., 1996). Both TGF-β3 (Lui et al., 2003a; Lui et al., 2003d) and TNF-α (Siu et al., 2003a) have recently been shown to be crucial regulators of TJ dynamics. Therefore, the demonstration of structural interactions between α_2-macroglobulin and cytokines, such as TGF-β3 reported herein, undoubtedly illustrates the physiological and clinical significance of this protease inhibitor

in TJ dynamics, i.e. maintaining the homeostasis of cytokines. Taken collectively, these findings suggest that α_2-macroglobulin apparently plays a crucial role in regulating both TJ and AJ dynamics via its interaction with TGF-β3. It maintains the pool of TGF-β3 that regulates both TJ-associated proteins and AJ multi-protein complexes in the testis, such as the ES.

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