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Hepatic Immunohistochemical Localization of the Tight Junction Protein ZO-1 in Rat Models of Cholestasis

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Structural alterations in hepatocyte tight junctions accompanying cholestasis were investigated using immunolocalization of ZO-1, the first known protein component of the tight junction. Disruption in the paracellular barrier function of the tight junction has been proposed to allow reflux of bile into the blood. Cholestasis was induced in 210 to 235 g male Sprague-Dawley rats either by five consecutive daily subcutaneous injections of 17-alpha-ethinyl estradiol (0.5 mg/kg/d in propylene glycol) or ligation of the common bile duct for 72 hours. The structural organization of the tight junction was assessed in each model by indirect immunofluorescent and immunoperoxidase staining for ZO-1 on frozen sections of liver and compared with controls. In control, sham-operated, and estradiol-injected animals, ZO-1 localizes in a uniform continuous manner along the margins of the canaliculi. In contrast, bile duct ligation results in the appearance of numerous discontinuities in ZO-1 staining accompanied by dilation or collapse of the lumenal space. Tissue content of the ZO-1 protein, as determined by quantitative immunoblotting, was unaffected in either cholestatic model compared with controls. These findings indicate that the molecular organization of the tight junction can be assessed from immunostaining patterns of ZO-1 in frozen sections of cholestatic livers. Under these experimental conditions, the organization of the tight junction at the level of the ZO-1 protein is altered by bile duct obstruction but not by ethinyl estradiol. (Am J Pathol 1989, 134:1055–1062)

Cholestasis represents a state of diminished hepatic bile secretion. Depending on the type of injury, the pathogenic mechanisms responsible may involve a defect in one or more steps in the transport of components from the blood to bile space.[1–5] The tight junction, or zonula occludens, seals the canalicular space and provides the only anatomic barrier maintaining the separation of bile from blood. Some types of cholestasis are thought to result from disruption of this barrier.[6,7] In this paper we use immunolocalization of ZO-1, the first protein known to be a specific component of the tight junction,[8] to investigate directly the structural state of tight junctions accompanying cholestasis produced in the rat by either common bile duct ligation or ethinyl estradiol administration.

In thin-sectioned electron microscopic images, the hepatocyte tight junction appears as a zone of close plasma membrane appositions bordering either side of the canaliculus.[6,9,10] Electron-dense tracer studies using ionic lanthanum-chloride demonstrate at the ultrastructural level that the barrier to paracellular solute movements is established at these contact points of the tight junction.[11] Freeze-fracture electron microscopic replicas of the tight junction reveal a band of anastamosing fibrils in the plane of the membrane, each composed of intramembrane particles that correspond to the points of membrane–membrane contact.[12] There is a good correlation between the number and organizational complexity of these fibrils and the resistance to paracellular permeability that can be measured between different cell and tissue types.[12,13]

Extrahepatic cholestasis, induced in the rat by common bile duct ligation, is accompanied by striking changes in the freeze-fracture replica electron microscopic appearance of the junction.[7,14,15] Fibrils that are normally positioned parallel to the canalicular lumen become irregular in number and organization, occasionally draping aberrantly onto the lateral cell surface. A concomitant increase in the paracellular permeability of electron-

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dense tracer proteins can be demonstrate by electron microscopy.[6,16] Similar ultrastructural changes have been documented in human liver biopsy samples taken from patients with extrahepatic obstruction.[14] The association between loss of the paracellular barrier and fibril integrity supports the notion that the fibrils are the resistive elements of the tight junction and that extrahepatic cholestasis is due, at least in part, to paracellular reflux of bile into the blood.

Alterations in fibril morphology also have been demonstrated in animal models of cholestasis induced by synthetic estrogens,[17,18] although numerous other estrogen-induced defects in the cellular mechanisms of bile secretion have been demonstrated and proposed as causes for the diminished bile production. These findings are of interest because similar pathophysiologic states can occur during human pregnancy[19] and in association with oral contraceptive use.[20,21] Other biochemical changes have been claimed to contribute to cholestasis by altering transport physiology, these include a 50% decrease in Na^+/K^+-ATPase activity,[22] as well as alterations in plasma membrane lipids leading to increased membrane viscosity.[23]

There are several limitations to previous structural studies of the role of the tight junction in cholestasis. Freeze-fracture techniques are prone to sampling errors and only a few investigators have applied any rigorous quantitative morphometric analysis to the data interpretation.[24] In addition, radiotracer permeability studies do not assess the physical extent of junction changes. In the present study we have assessed the structural organization of the tight junction using immunolocalization of the ZO-1 protein, the first protein known to be a specific component of the junction. Indirect immunofluorescence microscopy has localized this 225 kd protein to the tight junctions of all epithelial tissues. At the ultrastructural level, using immunogold techniques, ZO-1 is seen to localize precisely to the cytoplasmic surface of cell–cell contact points corresponding to freeze fracture fibrils (unpublished observations).[8] The number of copies of ZO-1 per cell has been estimated to be about the same as the number of freeze-fracture particles that compose the fibrils, suggesting that although ZO-1 as a peripheral membrane protein is not the integral protein responsible for fibrils, there exists a close relationship between them.[25] As a component of the tight junction, localization of ZO-1 reflects the structural state of the tight junction. Using antibodies to ZO-1, the present study investigated whether a visible alteration in tight junction structure accompanies cholestasis induced by ligation of the common bile duct or by administration of ethinyl estradiol.

Materials and Methods

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Models of Cholestasis

Male Sprague-Dawley rats (Charles River, Willmington, MA) weighing between 175 to 225 g were used in all experiments. Cholestasis resulting from common bile duct ligation was achieved as described by Easter and coworkers.[15] After inducing anesthesia with intraperitoneal injection of sodium pentothal (50 mg/kg), a 2 to 3 cm midline incision of the abdomen was made and the common bile duct exposed. The duct was doubly ligated midway to the duodenum and sectioned between the two ligatures in four animals. Sham operations were performed on four control animals, the common ducts of which were manipulated but not ligated or sectioned. Wounds were closed, and animals fed *ad libitum* and killed 72 hours after closure.

Cholestasis induced by ethinyl estradiol was produced as described by Boyer et al.[18] Five animals received five consecutive daily subcutaneous injections of 17-alpha-ethinyl estradiol (0.5 mg/kg/day) dissolved in 0.2 ml of propylene glycol; five control animals received propylene glycol alone on the same schedule. Animals were fed *ad libitum,* weighed daily, and killed on the sixth day. One rat of identical age and weight received no treatment and was killed and processed with the others.

Samples for immunohistochemical study were taken from the anterior portion of the left liver lobe, rapidly sliced into 3-mm cubes, and OTC embedded in a pool of liquid nitrogen-cooled isopentane. Samples were stored in liquid nitrogen until sectioning.[8]

Antibodies and Immunostaining

Production of rat IgG monoclonal antibodies (R40.76 and R26.4C) produced against mouse liver ZO-1 has been described previously.[8,25] Rabbit polyclonal antisera were produced against a 38 kd fragment of rat ZO-1 expressed as a beta-galactosidase fusion protein in *Esherichia coli.* Polyclonal anti-fusion protein antibodies were used for most of the studies described here; their tissue localization was identical to that of monoclonal antibodies (MAb) but staining intensity was significantly greater than that of MAbs. Briefly, a partial cDNA encoding ZO-1 was identified with MAbs by screening a rat kidney-derived cDNA library (Clonetech, Palo Alto, CA) constructed in lambda gt11.[26] A 1000 base pair sequence was identified, and used to produce beta-galactosidase fusion protein against which rabbit polyclonal antisera were produced. ZO-1-specific antibodies were purified on columns of fusion protein coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) and anti-beta-galactosidase antibodies removed on columns of immobilized beta-galac-

tosidase.[27] Detailed characterization of these antibodies will be published elsewhere (manuscript in preparation).

Indirect immunofluorescent staining was performed on 4 to 8-μ thick frozen sections of unfixed rat liver applied to gelatin-coated multi-spot slides as described previously.[8] Sections were permeabilized in acetone at −20 C for 2 minutes and washed with TRIS-buffered saline (TBS), 10 mM TRIS-Cl, pH 8.0, 150 mM NaCl, 0.02% NaN_3. Anti-ZO-1 MAbs produced in mouse ascites were used at a dilution of 1:100; secondary was fluorescein-conjugated rabbit anti-rat antibodies (Boehringer Mannhein Biochemicals, Indianapolis, IN) diluted 1:50 in TBS. Staining with affinity-purified anti-ZO-1 polyclonal antibodies (diluted to about 1 μg/ml) was performed in 10% normal goat serum in TBS for 1 hour at room temperature. After three 5-minute washes, samples were exposed to fluorescein-conjugated goat anti-rabbit IgG (Cappel), diluted 1:500 in TBS. Sections were mounted in 60% glycerol/TBS, 0.4% n-propyl gallate, and viewed on a Zeiss phase/epifluorescence microscope equipped with a ×63 planapo objective. Photographs were taken on Tri-X film (Kodak, Rochester, NY).

Immunoperoxidase staining was accomplished by the PAP method as described by Steinberger,[28] by successive incubations with optimally diluted affinity purified anti-ZO-1 polyclonal antibodies, swine anti-rabbit antibodies, and horseradish peroxidase complexed with rabbit anti-peroxidase (DAKO Corp., Santa Barbara, CA). Slides were developed with 3,3′-diaminobenzidine in 0.01% H_2O_2. Sections were counterstained with hemotoxylin to assess tissue preservation and then photographed on black and white film (Kodak Technical Pan, Rochester, NY) with bright-field illumination using a blue filter to remove the nonimmunostained detail. Photographs were taken with a ×25 planapo objective.

Quantitative Immunoblots

Samples for SDS-PAGE were taken from each animal's liver at the time of sacrifice. A piece of the left lobe (about 1 g) was removed, weighed, and rapidly dispersed by dounce homogenization in eight volumes (vol/wt) of 1 mM $NaHCO_3$. Two volumes of 10× SDS-PAGE gel sample buffer were added and the sample boiled for 3 minutes (1× is 1.25 mM TRIS, pH 6.8, 1% SDS, 2% mercaptoethanol, 2% sucrose). Samples were subjected to SDS-PAGE in triplicate by the method of Laemmli[29] on 7% acrylamide gels and electrophoretically transferred to nitrocellulose paper (Schleicher & Schuell Inc., Keene, NH) in 25 mM TRIS, pH 7.0, 192 mM glycine, 0.1% SDS, 20% methanol at 40 volts, 4 C for 24 hours. Nonspecific protein binding was blocked with TBS-BLOTTO (5% nonfat dry milk, Carnation Co., Los Angeles, CA)[30] at 37 C for 1 hour then blots were incubated with affinity-purified polyclonal anti-ZO-1 antibodies (about 1 μg/ml in TBS-BLOTTO) for 1 hour followed by [^{125}I]protein A (ICN, 2 μCi/ml, 92.3 μCi/μg). After extensive washing, blots were dried and autoradiographs (Kodak, X-OMAT AR film) produced that served as templates to cut out ZO-1 bands from the blots. [^{125}I]protein A bound to each band was quantified by liquid scintillation counting and counts per minute were shown to be a linear function of gel sample volume over the range analyzed.

Results

The hepatocyte's canalicular or apical membrane domain forms a 1.5 to 2 μ band that encircles the cell surface and whose parallel margins are demarcated by tight junctions. By immunolocalization the ZO-1 protein is clearly visualized as two parallel lines bordering the canaliculi in longitudinal section and as pairs of dots when cross-sectioned. This localization is demonstrated in normal liver by both immunoperoxidase staining (Figure 1a) and by immunofluorescence (Figure 2a, c). The distribution of ZO-1 along the junction appears to be uniform and continuous, disrupted only where through-focusing suggests the junction is passing near to or out of the tissue section. These findings are consistent with our previous observations in normal liver.[8] Based on these images and our previous studies, which localized ZO-1 to cell–cell contact points by immunogold electron microscopy, we assume that the position of ZO-1 reflects the actual position of the paracellular barrier sealing bile from the blood. Three days after bile duct ligation, ZO-1 localization at the tight junction is strikingly altered (Figures 1b and 2b, d to f). Staining is no longer uniform and continuous along the canalicular margin but shows variations in staining intensity and frank discontinuities. Local variations along the tight junction are most easily appreciated in immunofluorescent images, and although not quantifiable presumably, reflect a local variation in relative ZO-1 content or accesibility to antibody binding. A second characteristic change is found in the spacing of parallel junctions that define the width of the canalicular lumen. These spaces appear dilated, and in some areas reach a profile distance as much as 5 μ (Figure 2e). Within a single microscopic field, ie, a single lobule, the canalicular spaces can appear both dilated and collapsed. When the parallel tight junctions are viewed in superimposed profile, the lumen appears to be abolished. When extended stretches of junction are examined, however, as in Figure 2f, these regions are more likely to be explained by collapse of the canalicular space and approximation of its borders. Liver sections from ligated animals were always clearly distinguishable from controls, although there was some variation between the four experimental animals. Bile in the urine was

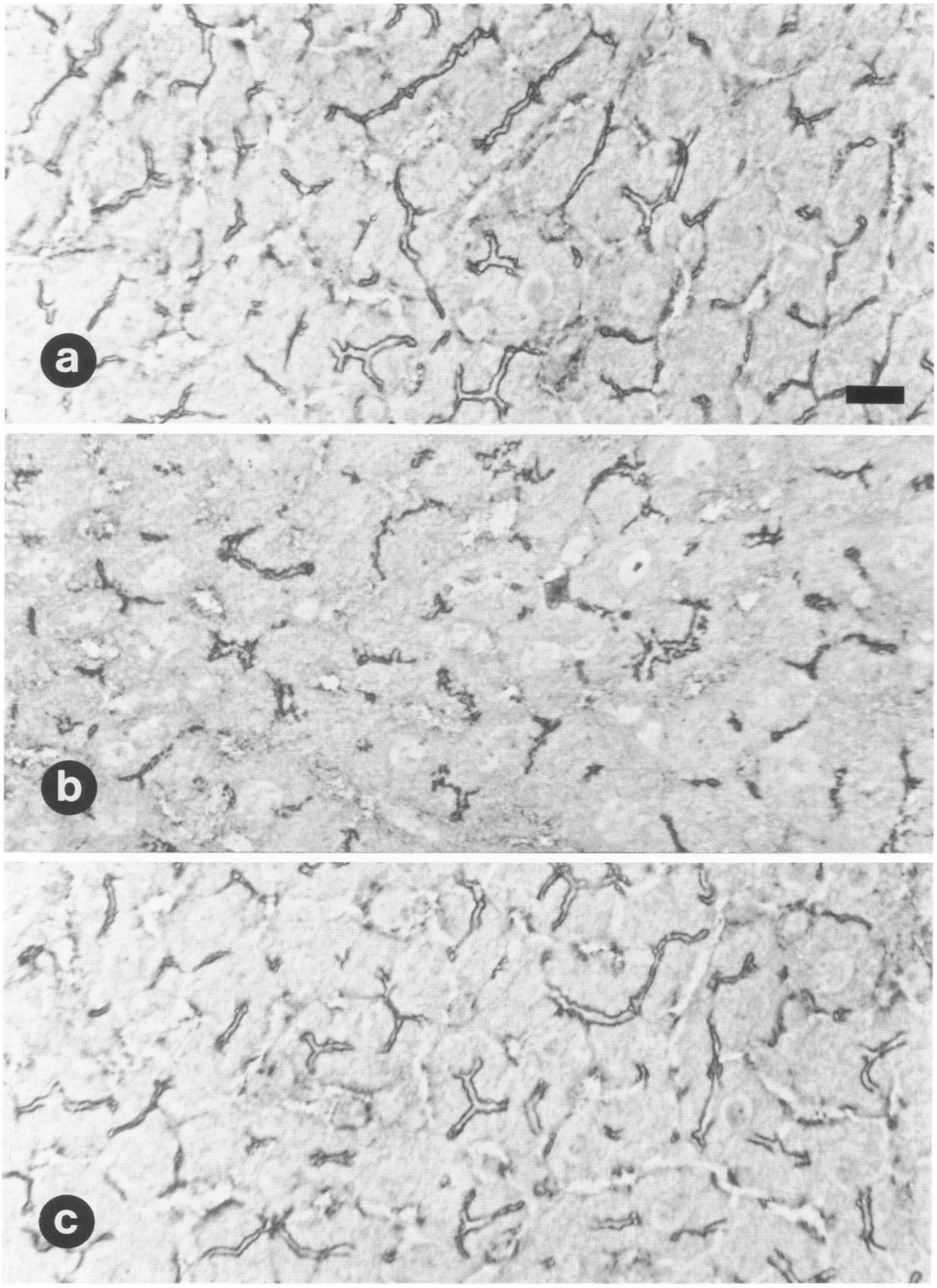

Figure 1. *Immunoperoxidase localization of ZO-1 in frozen sections of normal and cholestatic rat liver.* **a:** *Normal liver.* **b:** *Seventy-two hours after common bile duct ligation.* **c:** *After 5 days of ethinyl estradiol administration. In normal liver ZO-1 localizes continuously to the tight junctions bordering the canalicular surface. Extrahepatic obstruction is accompanied by dilatation and collapse of the canalicular space and fragmentation of junction staining. Estrogen-treated animals are not visibly different from controls. Sections are photographed through blue filters to eliminate most of the nonimmunologic stain resulting from hematoxylin counterstained cell structures. Bar, 10 μ.*

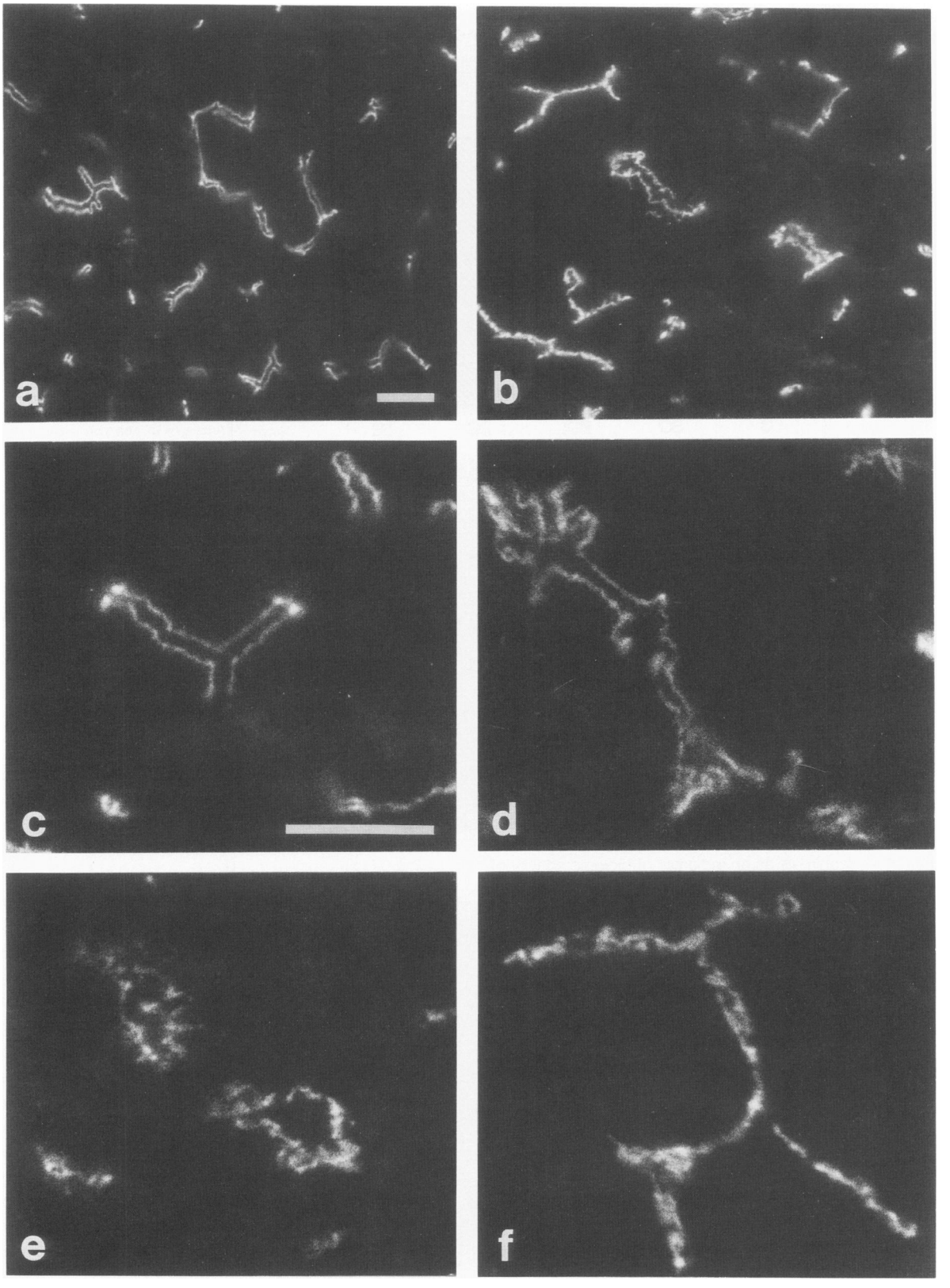

Figure 2. *Indirect immunofluorescent localization of ZO-1 in frozen sections of normal and bile duct-ligated rat livers.* **a** *and* **b** *are presented at lower magnification than* **c** *and* **d**. *Bar, 10 μ. Panels a and c are sections from normal untreated rat liver, notice the uniform staining and canalicular width. Panels b and d, and f are representative sections obtained 72 hours after common duct ligation. Notice the irregular junction margins, discontinuities, and dilitation, as well as apparent collapse of the canalicular spaces.*

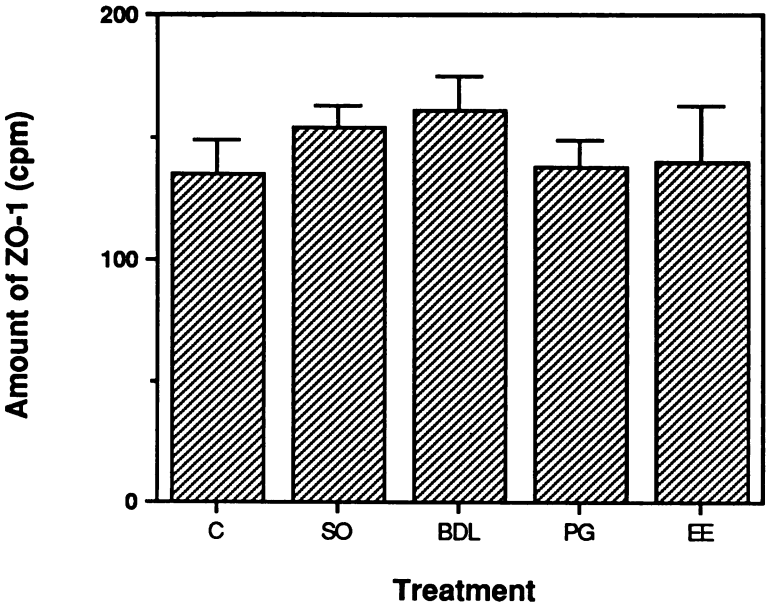

Figure 3. *Comparison of relative ZO-1 content per liver weight between cholestatic and control liver samples. ZO-1 content is presented as counts per minute of [^{125}I]protein A bound to immunoblot samples from each animal, determined in triplicate and averaged. Mean and standard error presented for each experimental group: C (N = 1, mean of multiple determinations), untreated control animal; SO (N = 4), sham-operated controls; BDL (N = 4), bile duct ligated; PG (N = 5), propylene glycol controls for estrogen treated, and EE (N = 5), ethinyl estradiol-treated animals. There are no significant differences in ZO-1 content.*

used as criteria that cholestasis was established at the time of death. No quantitative measures were made, however, so the degree of cholestasis could not be correlated with the degree of morphologic alterations. None of the sham-operated animals had dark urine and immunolocalization of ZO-1 in these animals was indistinguishable from that observed in untreated animals.

In contrast to the altered immunolocalization of ZO-1 induced by bile duct ligation, ethinyl estradiol administration induced no discernable changes in ZO-1 morphology. No changes were observed in five estradiol-treated animals as well as five control animals injectioned with the carrier liquid, propylene glycol, alone. Daily weights were taken on all animals as an indicator of biologic activity of the estrogen.[31] Propylene glycol control animals weighed an average of 217 ± 7 g (N = 5) on the first day and all grew to an average of 239 ± 4 g on the day of death. In contrast, estrogen-treated animals began at 220 ± 7 g (N = 5) and all declined to an average of 211 ± 6 g. In addition, all estrogen-treated animals but none of the propylene glycol animals developed darkly stained urine at the time of death. Thus, no morphologic change in ZO-1 was detected in estrogen-treated animals despite evidence of impaired bile secretion and systemic biologic effects.

Quantitative Analysis of ZO-1 Content

Quantitative immunoblotting was used to assess whether alterations in the barrier function of the tight junction accompanying cholestasis might be reflected in a change in the liver content of ZO-1. Figure 3 presents a comparison of the relative content of ZO-1 per weight of liver between all of the experimental groups studied. There are no statistically significant differences. This analysis does not, however, account for any potential changes in the intracellular location of ZO-1 not reflected in a net change in liver content.

Discussion

In the present study the immunolocalization of the tight junction protein ZO-1 has been used as a marker of the structural organization of hepatocyte tight junctions in two rat models of cholestasis, bile duct obstruction and estrogen-induced cholestasis. We have demonstrated a dramatic alteration in the organization of the tight junction accompanying cholestasis resulting from bile duct ligation. The normally uniform canalicular lumen was either dilated or collapsed, and the distribution of ZO-1 along the tight junctions was irregular and even disrupted. It has been proposed previously that canalicular dilation and loss of the junction barrier, as measured by permeability studies, are secondary to increased intrabiliary pressure.[3,6] Our results clearly demonstrate that the molecular organization of the tight junction is altered by extrahepatic obstruction in a way consistent with a route for paracellular reflux of bile contents as a contributing mechanism of cholestasis. The changes in junction morphology observed 72 hours after ligation of the bile duct were generalized and seen throughout the lobule. It is possible that at earlier times such changes might be focal or show a zonal distribution in the lobule. The ability to assess the extent of derangement in junction morphology throughout the lobule is of particular interest in the present report be-

cause previous studies using freeze-fracture electron microscopy could have been biased by sampling error and paracellular perfusion studies could not evaluate the physical extent or location of alterations in junctional structure. An additional function attributed to the tight junction is to limit the mixing of the biochemically distinct components of apical and basolateral plasma membranes.[32,33] If the breaks observed in junctional integrity were to allow mixing of apical and basolateral transport proteins, carriers, or lipids required for normal vectoral bile secretion, these changes in membrane polarity would provide an additional explanation for diminished bile flow. Such a rearrangement of membrane domain markers has been reported after bile duct obstruction.[34]

In contrast to obvious changes in ZO-1 that are produced by extrahepatic obstruction, we observed no recognizable alteration in ZO-1 localization in ethinyl estradiol-induced cholestasis. Although it is possible that our method for structural assessment of the junction is insufficiently sensitive to detect functionally significant changes, it is also possible that the paracellular barrier is unaffected by our treatment. The last conclusion seems inconsistent with published freeze-fracture studies showing alterations in fibril morphology; however, such changes are less extensive than those caused by bile duct obstruction.[17] In addition, the canalicular bile acid carrier protein has been reported to maintain its canalicular polarity during estrogen-induced cholestasis,[35] again in contrast to loss of polarity observed to accompany extrahepatic obstruction.[34] An additional confounding factor arises when comparing results from previous published experimental models of estrogen-induced cholestasis, because each has used a different dose of estrogen and interval of treatment. The difficulty with interpreting the role of paracellular permeability is illustrated in a recent report by Jaeschke and coworkers[31] in which both bile flow rates and paracellular tracer permeability were measured in isolated perfused livers from rats treated for many weeks with comparatively low doses of estradiol valerate. These doses were much lower than those used to induce ultrastructural changes with ethinyl estradiol,[17] and clearly demonstrated that the primary or temporally first effect of estrogen was to diminish basal and taurocholate-stimulated bile secretion, while an increased clearance of paracellularly routed tracers followed weeks later. Unfortunately, no studies of junction morphology were included to determine if junctions appeared normal at these early times despite diminished bile flow. The mechanism of cholestasis due to estrogens is certainly multifactorial and major alterations in plasma membrane viscosity as well as diminished Na^+/K^+-ATPase activity have been documented.[22,23] It remains to be convincingly demonstrated whether structural changes are primary or secondary to other estrogen-induced events and to what extent they contribute to cholestasis in animal models, human pregnancy, or oral contraceptive use.

As yet we do not know what role ZO-1 plays in the molecular organization of the tight junction. It is present precisely at cell–cell contacts and in nearly the same number per cell as the intramembranous particles that form the junction fibrils seen in freeze-fracture replicas.[25] Because ZO-1 is a peripherally-associated membrane protein, we presume that it contributes to a cytoplasmic plaque of protein such as has been identified on the cytoplasmic surface of the adherens junction and desmosome. Here it could potentially maintain the organization of fibril particles or link the junction to the cytoskeleton. Altered structural or functional states of the junction might then correlate with or result from altered cellular levels of ZO-1. We tested this simple hypothesis and found no significant differences in ZO-1 content per weight of liver with either experimental manipulation. This, of course, does not account for potential changes in the intracellular localization of ZO-1, such as a shift from a membrane-bound to cytoplasmic form of the protein. Recently, a second tight junction-specific protein, cingulin, has been identified, but at present its role in junction organization also is undefined.[36] A detailed description of the role of the tight junction in normal bile secretion and various forms of cholestasis will require further study of the molecular components, architecture, and regulation of the junction.

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