Histol Histopathol (2009) 24: 1463-1472

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Tight junction proteins and signal transduction pathways in hepatocytes

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Summary. Tight junctions of hepatocytes play crucial roles in the barrier to keep bile in bile canaliculi away from the blood circulation, which we call the blood-billiary-barrier (Kojima et al., 2003). Tight junction proteins of hepatocytes are regulated by various cytokines and growth factors via distinct signal transduction pathways. They are also considered to participate in signal transduction pathways that regulate epithelial cell proliferation, gene expression, differentiation and morphogenesis. This review focuses on recent findings about the relationship between tight junction proteins and signal transduction pathways in hepatocytes.

Key words: Hepatocyte, Tight junction, Signal transduction, Cytokine, Growth factor

Introduction

The polarization of hepatocytes involves the formation of functionally distinct sinusoidal (basolateral) and bile canalicular (apical) plasma membrane domains that are separated by tight junctions. Tight junctions, the most apically located of the intercellular junctional complexes, inhibit solute and water flow through the paracellular space (termed the "barrier" function) (Fig. 1A) (Gumbiner, 1993; Schneeberger and Lynch, 1992). They also separate the apical from the basolateral cell surface domains to establish cell polarity (termed the "fence" function) (Fig. 1A) (Van Meer and Simon, 1986; Cereijido et al., 1998). Recent evidence suggests that tight junctions also participate in signal transduction mechanisms that regulate epithelial cell proliferation, gene expression, differentiation and morphogenesis (Fig. 1A) (Matter and Balda, 2003).

Tight junctions are formed by not only the integral membrane proteins claudins, occludin, and JAMs, but also many peripheral membrane proteins, including the scaffold PDZ-expression proteins Zonula occludens (ZO)-1, ZO-2, ZO-3, multi-PDZ domain protein-1 (MUPP1) and membrane-associated guanylate kinase with inverted orientation-1 (MAGI)-1, MAGI-2, MAGI-3, and cell polarity molecules ASIP/PAR-3, PAR-6, PALS-1 and PALS-1 associated tight junction (PATJ) and the non-PDZ-expressing proteins, Cingulin, Symplekin, ZONAB, GEF-H1, aPKC, PP2A, Rab3b, Rab13, PTEN and 7H6 (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004). Zonula occludens-1 (ZO-1), ZO-2 and ZO-3 are members of the membrane associated guanylate kinase (MAGUK) family of proteins displaying a characteristic multidomain structure comprised of SH3, guanylate kinase-like (GUK) and multiple PDZ (PSD95-Dlg-ZO1) domains (Anderson, 1996). ZO-1 and ZO-2 are also closely associated with polymerization of claudins (Umeda et al., 2006). More recently, tricellulin was identified at tricellular contacts where there are three epithelial cells and was shown to have a barrier function (Ikenouchi et al., 2005).

The claudin family, consisting of 24 members, is solely responsible for forming tight junction strands and shows tissue- and cell-specific expression of individual members (Tsukita et al., 2001). Several lines of evidence point to claudins as the basis for the selective size, charge, and conductance properties of the paracellular pathway (Van Itallie and Anderson, 2006). The claudins have two extracellular loop domains (Furuse et al., 1998). The first extracellular loop influences the paracellular charge selectivity and the second

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extracellular loop is the receptor for a bacterial toxin (Van Itallie and Anderson, 2006).

Occludin, the first-discovered integral membrane protein of tight junctions, is most ubiquitously expressed at the apicalmost basolateral membranes, and is the most reliable immunohistochemical marker for tight junctions (Furuse et al., 1993; Tsukita et al., 2001). By contrast, only a single occludin transcript has been described, although an alternatively spliced form of occludin (termed occludin 1B) has been reported recently (Muresan et al., 2000). Overexpression of occludin increases the barrier function, indicated as an increase in transepithelial electric resistance (TER) increase in mammalian epithelial cells (Balda et al., 1996; McCarthy et al., 1996). However, TJ strands can be formed without occludin in some cell types, including occludin-deficient embryonic stem cells (Hirase et al., 1997; Saitou et al., 1998). Moreover, an occludin-deficient mouse model does not display a perturbation of epithelial barrier function, although a complex pathophysiological phenotype is observed with growth retardation, chronic inflammation and hyperplasia of the gastric epithelium, calcification in the brain, testicular atrophy, loss of cytoplasmic granules in striated duct cells of the salivary gland, and thinning of the compact bone (Saitou et al., 2000).

JAMs (JAM-A, -B, -C, -4) are immunoglobulin superfamily proteins expressed at cell junctions in epithelial and endothelial cells, as well as on the surfaces of leukocytes, platelets, and erythrocytes (Martin-Padura et al., 1998). They are important for a variety of cellular processes, including tight junction assembly, leukocyte transmigration, platelet activation, angiogenesis and adenovirus binding. Recently, in HepG2 cells and WIF-B cells, which have hepatic cell polarity, depletion of the integral tight junction protein JAM-A, which directly binds to the cell polarity protein PAR3, was found to inhibit formation of bile canaliculi (Ebnet et al., 2001; Itoh et al., 2001; Konopka et al., 2007; Braiterman et al., 2008).

The integral proteins, claudin, occludin and JAM bind to the domains of scaffold proteins ZO-1, PDZ1, GUK, and PDZ3, respectively (Fig. 1B) (Schneeberger and Lynch, 2004).

Expression of tight junction proteins in rodent and human livers

In murine livers, claudin-1, -2, -3, -5, -7, -8, -12, and -14 are detected together with occludin, JAM-A, CAR and tricellulin, and claudin-1, -2, and -3 are expressed in the bile canalicular region of hepatocytes (Fechner et al.,

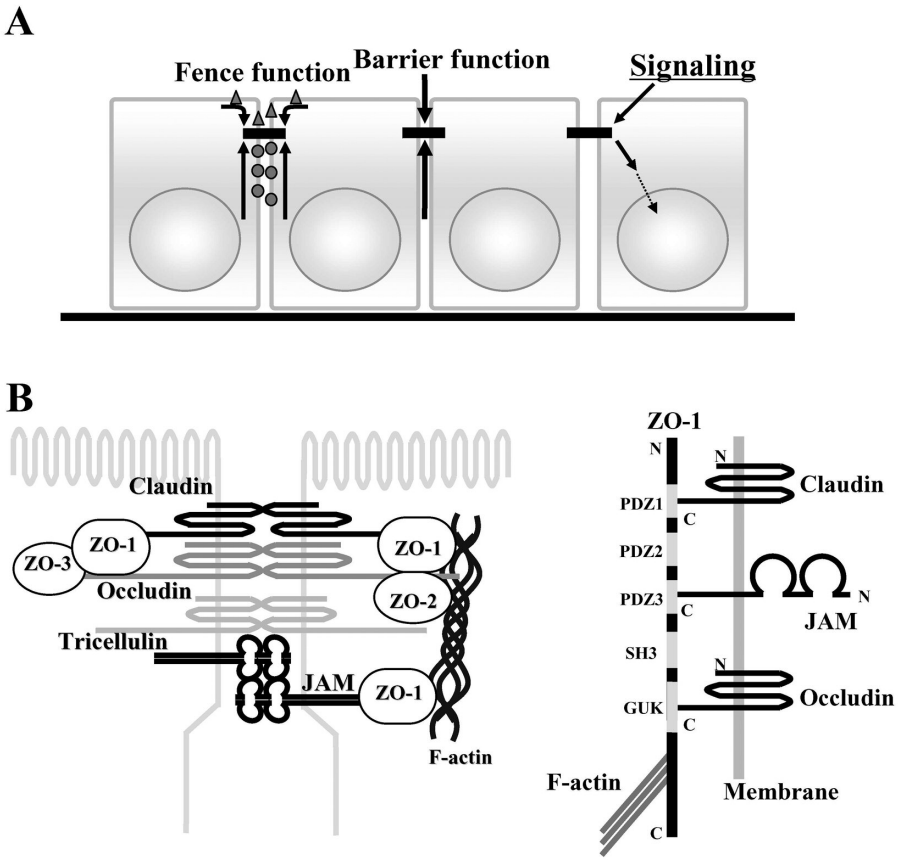

Fig. 1. Function and molecular components of tight junctions. **A.** Three major tight junctional functions (termed ''fence'', ''barrier'', ''signaling''). **B.** The major molecular components and the organization of tight junction proteins. **C.** The binding of proteins claudin, occludin and JAM to ZO-1.

1999; Tsukita et al., 2001; Wilcox et al., 2001; Kojima et al., 2003; Ikenouchi et al., 2005; Son et al., 2009). In the rat liver, claudin-2 shows a lobular gradient increasing from periportal to pericentral hepatocytes, whereas claudin-1 and -3 are evenly expressed in the whole liver lobule (Rahner et al. 2001; Yamamoto et al. 2005).

In the human liver, occludin, JAM-A, ZO-1, ZO-2, claudin-1, -2, -3, -7, -8, -12, -14 and tricellulin are detected together with well developed tight junction structures (Fig. 2A,C), and claudin-2 shows a lobular gradient increasing from periportal to pericentral hepatocytes as in the livers of rat and mouse, whereas claudin-1 is expressed in the whole liver lobule (Fig. 2B).

As a genetic disease of human tight junction protein, missense mutations in ZO-2 have been identified in patients with familial hypercholanemia (Carlton et al., 2003). In a syndrome associating ichthyosis and neonatal sclerosing cholangitis (NISCH syndrome), mutations of claudin-1 are also reported and the lack of claudin-1 may lead to increased paracellular permeability between bile duct epithelial cells (Hadj-Rabia et al., 2004). However, in the mouse claudin-1 KO model, the biliary disease was not detected, probably because the KO mice died at birth (Furuse et al., 2002).

HCV is an enveloped positive-stranded RNA hepatotropic virus and three host cell molecules are important entry factors or receptors for HCV

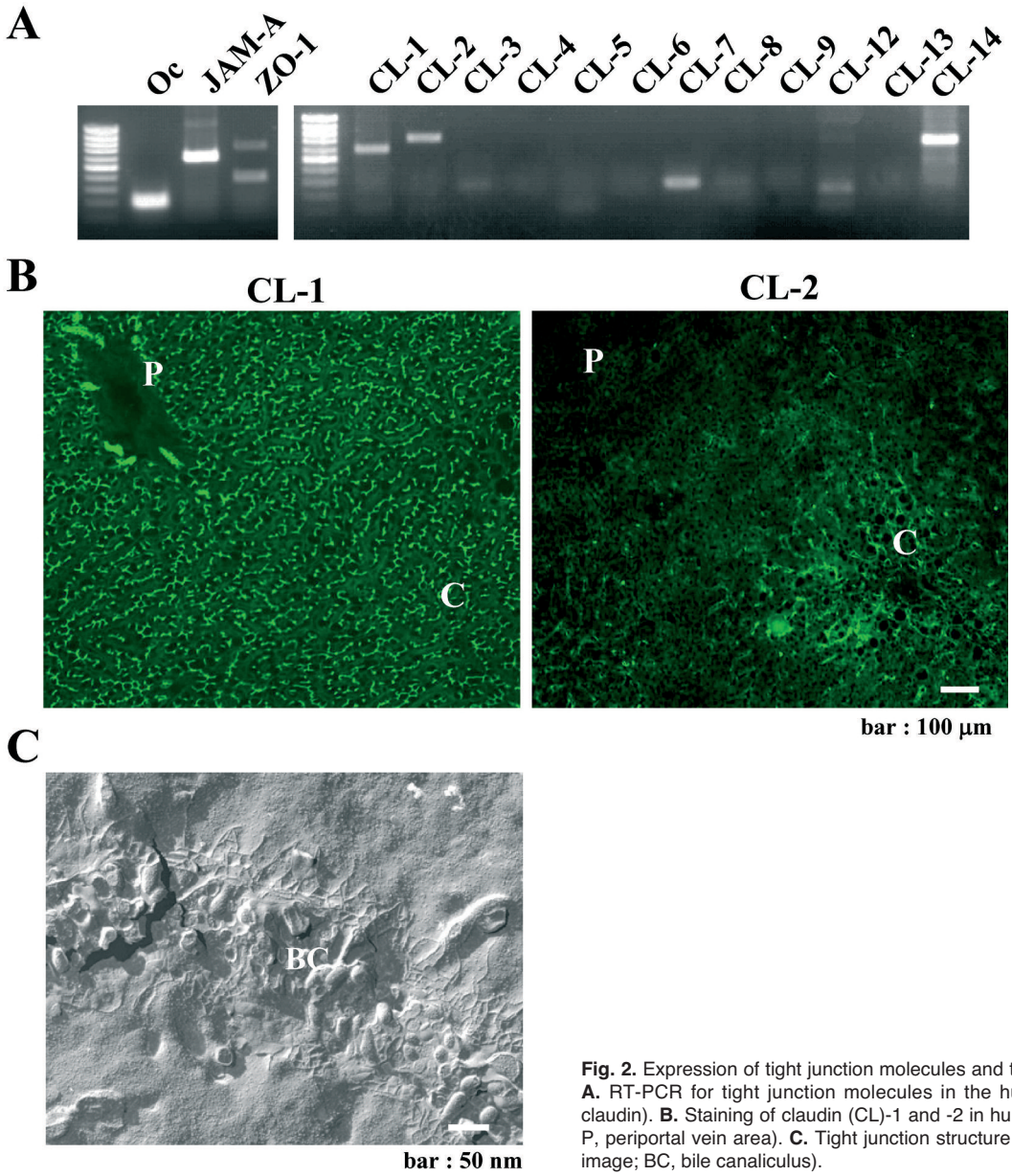

Fig. 2. Expression of tight junction molecules and the structure in the human liver. **A.** RT-PCR for tight junction molecules in the human liver. (Oc, occludin; CL, claudin). **B.** Staining of claudin (CL)-1 and -2 in human liver. (C, central vein area; P, periportal vein area). **C.** Tight junction structure in human liver. (freeze fracture image; BC, bile canaliculus).

internalization: scavenger receptor BI (SR-BI), tetraspanin CD81 and claudin-1 (Helle and Dubuisson, 2008). CD81 and claudin-1 act as co-receptors during late stages in the HCV entry process, and the first extracellular loop of claudin-1 in the liver is critical for the entry (Evans et al., 2007). Furthermore, not only claudin-1 but also claudin-6 and -9 work as cofactors for the entry of HCV (Meertens et al., 2008). Recently, occludin was also reported to be required for HCV entry (Benedicto et al., 2008; Liu et al., 2009). The tight junction proteins claudins and occludin are novel key factors for HCV and may be new targets for antiviral drugs.

Human hepatic stem cells, which are pluripotent precursors of hepatoblasts and hepatocytic and biliary epithelia, are located in ductal plates in the fetal liver and in the canal of Hering in the adult liver (Theise et al., 1999; Schmelzer et al., 2006). The stem cell phenotype expresses EpCAM, NCAM, CK19, c-kit, claudin-3, and weak levels of albumin, but not AFP or adult liver-specific proteins such as transferrin, Cx26, Cx32, PEPCK, DPPVI, or P450s (Schmelzer et al., 2007).

Tight junctions in primary cultures of rat hepatocytes using a 2% DMSO system

To investigate the regulation of hepatic tight junctions in detail, primary cultured rat hepatocytes were treated with 2% DMSO at day 4 after plating and maintained for 10 days (Fig. 3A). In primary rat hepatocytes cultured with 2% DMSO, tight junction proteins occludin-, claudin-1-, and ZO-1-immunoreactive lines were strongly observed on the most subapical plasma membrane of the cell borders (Fig. 3B), tight junction strands formed well-developed networks in a freeze fracture image of adluminal plasma membrane (Fig. 3C) and the fence function of tight junctions in the cells, as examined by diffusion of labeled sphingomyelin, was well maintained (Kojima et al., 2001, 2008; Yamamoto et al., 2004, 2005; Imamura et al., 2007). Although tight junctions in these differentiated cultured hepatocytes look like the distribution seen in simple polarized epithelial cells, this culture system provides a useful model in which to study hepatic tight junctions. Using this system, we also found a strong relationship between tight junctions and gap junctions in hepatocytes (Kojima et al., 2001, 2003)

Signal transduction to tight junction proteins in rodent hepatocytes

It is thought that tight junctions of hepatocytes may be regulated by various cytokines and growth factors produced by non-parenchymal cells (Fig. 4A). Therefore, we investigated the effects of cytokines and growth factors on tight junction proteins in primary cultured rat hepatocytes using a 2% DMSO system and immortalized mouse hepatocytic cell lines. In this system, downregulation of claudin-1 and upregulation of claudin-2 by growth factors, EGF, HGF and TGF-ß and by cytokines, IL-1ß and oncostatin M (OSM) are observed in the cultured rat hepatocytes (Fig. 4B) (Kojima et al., 2004, 2008; Yamamoto et al., 2004; Imamura et al., 2007). Furthermore, a decrease of tight junction strands, together with downregulation of claudin-1, is observed during DNA synthesis induced by EGF in primary cultured rat hepatocytes (Kojima et al., 1997, 1998, 2004). These findings suggest that growth factors and cytokines may affect bile canalicular sealing by tight junctions during regeneration and inflammation of the liver.

On the other hand, Snail is a transcription repressor that plays a central role in the epithelium-mesenchyme transition (EMT) by which epithelial cells lose their polarity. When Snail is overexpressed in cultured mouse epithelial cells, EMT is induced with concomitant repression of the expression of claudins and occludin, not only at the protein, but also at the mRNA level, and Snail binds directly to the E-boxes of the promoters of claudin and occludin genes like E-cadherin (Ikenouchi et al., 2003). In the oncogenic Raf-1-transfected mouse hepatic cell line, expression of occludin and claudin-2 and barrier function are downregulated during EMT (Lan et al., 2004, 2006). In mature rat hepatocytes in vitro, TGF-ß induces EMT by downregulation of claudin-1 and a decrease of the fence function by upregulation of Snail (Kojima et al., 2008).

A role for tight junctions in intracellular signaling has been proposed. In colonic epithelial cell line T84, MAPK/ERK activated by IL-17 is considered to upregulate claudin-1 and -2 expression (Kinugasa et al., 2000), while in MDCK cells treated with EGF and HGF, MAPK/ERK1/2 downregulates claudin-2 expression (Singh et al., 2004; Lipschutz et al., 2005). The expression of claudin-1 in osteoblast-like MC3T3-E1 cells after treatment with IGF-I is mainly upregulated via a MAP-kinase pathway and in part modulated by a PI3-kinase pathway (Hatakeyama et al., 2008).

To elucidate the mechanisms of signal transmission required for the regulation of tight junctions of hepatocytes, we have examined the effect of signaling pathways such as mitogen-activated protein (MAP) kinase, p38 MPK-kinase, PI3-kinase and PKC on the regulation of tight junctions (Fig. 4A). In primary rat hepatocytes, the changes of claudin-1 and -2 and occludin induced by growth factors (EGF, HGF and TGF-ß) and cytokines (IL-1ß and OSM) are regulated via distinct signal transduction pathways (Table 1) (Kojima et al., 2004, 2008; Yamamoto et al., 2004; Imamura et al., 2007). Furthermore, in immortalized mouse hepatocytes, upregulation of claudin-2 via PI3-kinase and PKC by treatment with OSM and downregulation of claudin-2 via MAP-kinase and p38 MAP-kinase by transfection with raf-1 are observed (Table 1) (Lan et al., 2004; Imamura et al., 2007). These indicate that in hepatocytes tight junction proteins claudin-1, -2 and occludin are regulated by various

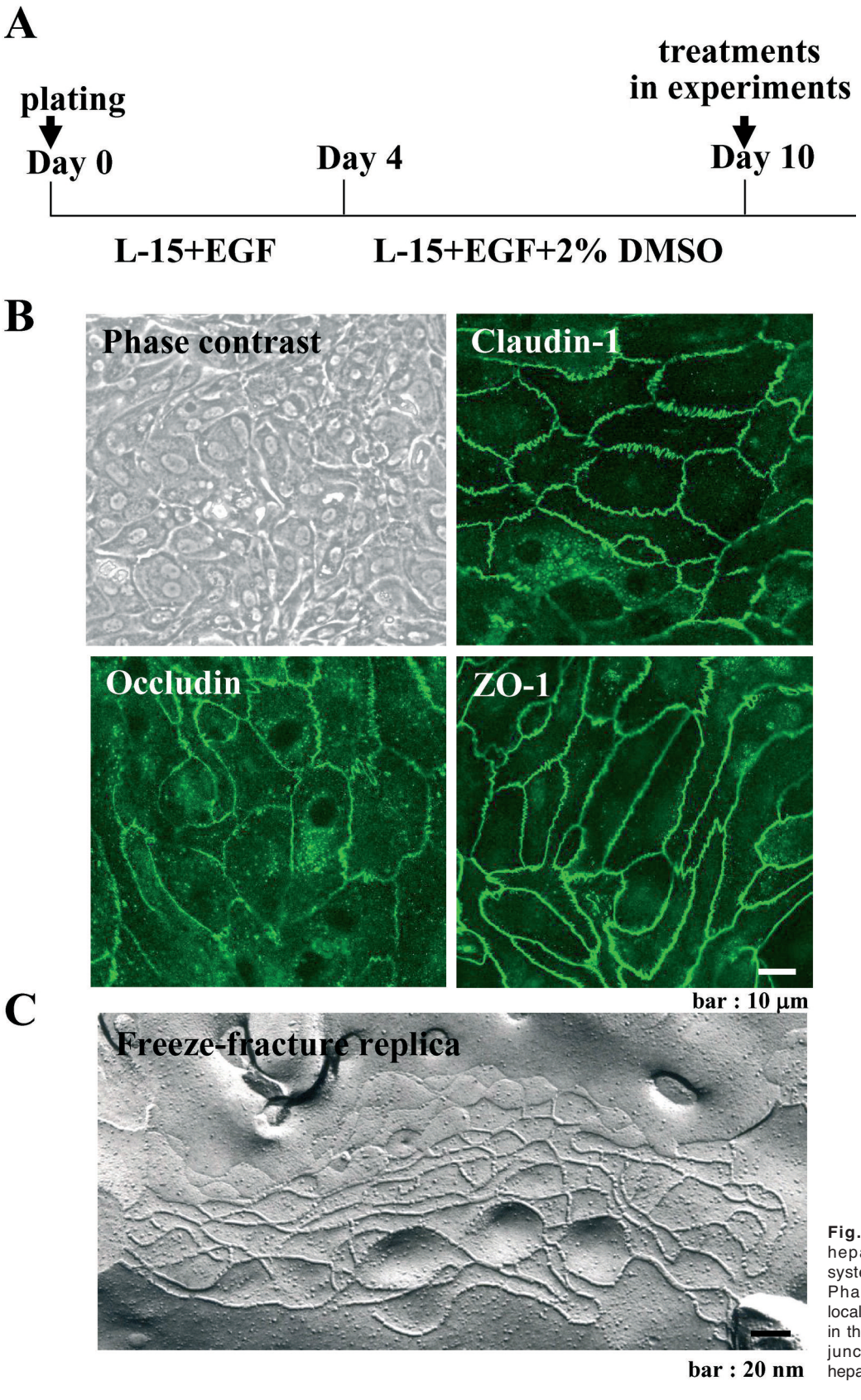

Fig. 3. Primary cultures of rat hepatocytes using 2% DMSO system. **A.** Protocol of cultures. **B.** Phase-contrast image and localization of tight junction proteins in the cultured hepatocytes. **C.** Tight junction structure in the cultured hepatocytes.

cytokines and growth factors via distinct signal transduction pathways.

In addition, it is thought that the p38 MAP-kinase pathway plays a crucial role in regulation of claudin-1 and -2 in hepatocytes. When primary cultured rat hepatocytes were treated with the p38 MAP-kinase activator anisomycin, downregulation of claudin-1 was observed (Kojima et al., 2003). Furthermore, a p38 MAP-kinase inhibitor prevented downregulation of claudin-1 by EGF in primary cultured rat hepatocytes (Table 1) (Yamamoto et al., 2005). In the regenerating rat liver, treatment with a p38 MAP-kinase inhibitor enhanced the upregulation of claudin-1 by partial hepatectomy (Table 1) (Kojima et al., 2003; Yamamoto et al., 2005). Although the reason for this discrepancy between the in vitro and in vivo is yet unclear, the p38

Table 1. Signal transduction to tight junction proteins in hepatocytes.

Signal	claudin-1	claudin-2	occludin
MAPK		EGF ↑; HGF *↑; IL-1ß ↑; raf-1 ↓; OSM[1] ↑; TGF-ß ↑	
p38MAPK	EGF ↓; HGF *↓ TGF-ß ↓; PH ↑	EGF ↑; HGF *↑; IL-1ß ↑; raf-1 ↓; OSM[1] ↑; TGF-ß ↑	
PI3K/Akt	EGF ↓; HGF* ↓	OSM[1,2] ↑; IL-1ß ↑; TGF-ß ↑	IL-1ß ↑; TGF-ß ↑
PKC		OSM[1,2] ↑	TGF-ß ↑

EGF, HGF*, IL-1ß, TGF-ß, oncostatin M (OSM)[1]: primary cultures of rat hepatocytes. OSM[2], raf-1: immortalized mouse hepatocytes. PH: partial hepatectomy in rat livers. *unpublished data.

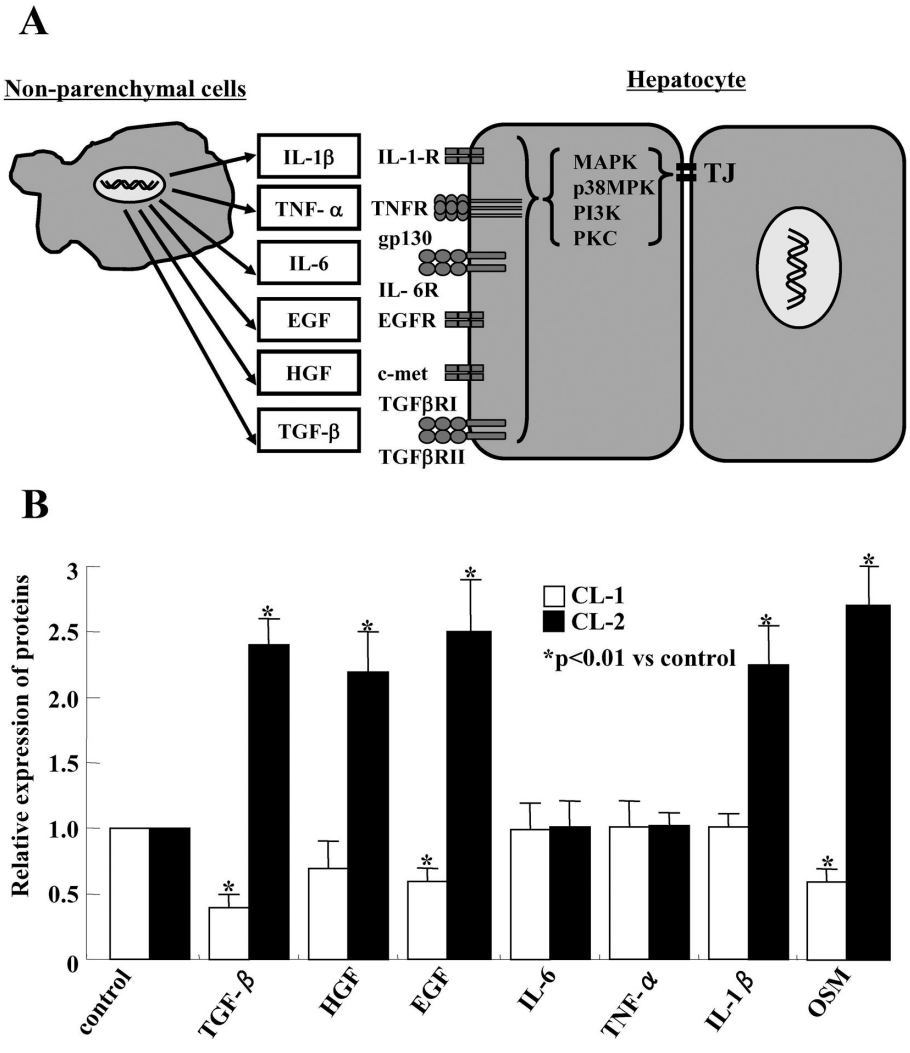

Fig. 4. Effectors of growth factors and cyokines on tight junction proteins in hepatocytes. **A.** growth factors and cytokines derived from non-parenchymal cells and the receptors and signaling pathways in hepatocytes. **B.** The changes in expression of claudin (CL)-1 and -2 proteins in hepatocytes after treatment.

MAP-kinase pathway may be important for formation of tight junctions during proliferation of hepatocytes and regeneration of the liver.

Signal transduction from tight junction protein occludin in hepatocytes

Occludin is an important regulatory component of signal transduction from tight junctions (Nusrat et al., 2000; Chen et al., 2002). The long carboxy-terminal domain of occludin is rich in serine, threonine, and tyrosine residues and a coiled-coil, which interact with c-Yes, PKC-ζ, Cx26, the regulatory subunit of PI3-kinase, and protein phosphatase 2A (PP2A), respectively (Fig. 3D) (Chen et al., 2002; Seth et al., 2007).

In primary cultures of occludin-deficient mouse hepatocytes, claudin-2 expression and apoptosis are induced by downregulation of the activation of MAP-kinase and Akt. In hepatic cell lines derived from occludin-deficient mice, claudin-2 expression and serum-free induced apoptosis are also increased by downregulation of the activation of MAP-kinase and Akt. Furthermore, in hepatic cell lines transiently transfected with mouse and rat occludin genes, induction of claudin-2 expression and apoptosis are inhibited, with increases in activation of MAP-kinase and Akt. These findings show that occludin plays a crucial role in claudin-2-dependent tight junction function and the apoptosis involving MAP-kinase and PI3-kinase/Akt signaling pathways in hepatocytes (Murata et al., 2005).

Signal transduction from tight junction protein claudin-2 in hepatocytes

Although it is thought that claudins may be key molecules in tight junctions of hepatocytes (Yamamoto et al., 2005; Imamura et al., 2007; Kojima et al., 2008), the physiological functions and regulation of claudins in hepatocytes remain unclear. To elucidate these questions we used WIF-B9 cells, which have the advantage of being well polarized and express a robust level of claudin-2.

WIF-B and its subclone, WIF-B9, are highly differentiated, polarized hepatoma cell lines (Ihrke et al., 1993; Shanks et al., 1994; Decaens et al., 1996). They were derived from segregated hybrid cells (Cassio et al., 1991) obtained by fusion of Fao rat hepatoma cells with WI38 human fibroblasts (Sellem et al., 1981). WIF-B9 cells develop morphological features that are close to those of primary hepatocytes (Shanks et al., 1994; Decaens et al., 1996), including functional bile canaliculus-like structures and secretion of bile acid derivatives (Bravo et al., 1998; Sai et al., 1999). Thus, this cell line has been used as a model for studying the mechanisms of bile canalicular formation, as have HepG2 cells. To investigate the role of tight junction proteins in bile canalicular formation, we used WIF-B9 cells after treatment with phenobarbital (PB), which causes an increase in bile flow in vivo (Okuda et al. 1988). PB preferentially induced expression of occludin and claudin-2 at the mRNA and protein levels, together with an increase of bile canalicular formation. Knockdown of claudin-2 using siRNA prevented bile canalicular formation in WIF-B9 cells treated with and without PB and induced expression of occludin, ZO-1, pLKB1, pp44/42 MAPK, pAkt and pp38 MAPK (Son et al., 2009).

Table 2. Signal transduction from tight junction proteins in hepatocytes.

Knockout of occludin	Knockdown of claudin-2
Primary cultures of mouse hepatocytes Immortalized mouse heptic cell line	WIF-B9 cells
claudin-2 ↑; pMAPK ↓; pAkt ↓	occludin ↑; ZO-1 ↑; pMAPK ↑; pAkt ↑; p38MAPK ↑; pLKB1 ↑
Apoptosis ↑	Bile canalicular formation ↓

Hepatocytic cell polarity development that results in bile canalicular formation is regulated by various kinases in response to extracellular signals. The serine/threonine kinase PAR1b/EMK1/MARK2 regulates bile canalicular formation in WIF-B9 cells, though the inhibition of bile canalicular formation by knockdown of PAR1b is weak (Cohen et al., 2004, 2007). Furthermore, Rho kinase, myosin-II and p44/42 MAPK, the first identified factors, are involved in hepatocyte-derived ECM-mediated multicellular patterning and bile canalicular luminal morphogenesis in HepG2 cells (Herrema et al., 2006). PI3K and p38 MAPK control tauro(ursodeoxy)cholate-induced trafficking of ATP-dependent transport to the canalicular surface in the rat liver, isolated hepatocytes and hepatic cell lines (Misra et al. 1998; Sai et al. 1999; Kubitz et al. 2004). LKB1/PAR4 is a serine/threonine kinase that is mutated in most cases of Peutz-Jeghers syndrome, in which benign hamartomas and a high frequency of malignant tumors develop (Cohen et al. 2004). The phosphorylation of LKB1 acts as a master kinase that activates PAR1 polarity kinase and AMPK (Baas et al., 2004; Xie et al., 2006). AMPK is known not only to act as a sensor of cellular energy status but also to regulate tight junction assembly and epithelial polarity (Zhang et al., 2006; Mirouse et al., 2007; Zheng and Cantley, 2007). By knockdown of claudin-2, upregulation of pLKB1, pp44/42 MAPK, pAkt and pp38 MAPK was unexpectedly observed, together with inhibition of bile canalicular formation (Son et al., 2009). These findings indicate that the signaling from tight junction proteins may be important as a subcellular system of bile canalicular formation.

Summary and perspective

It is thought that tight junctions of hepatocytes play crucial roles in the barrier to keep bile in bile canaliculi away from the blood circulation. The tight junction proteins are elaborately regulated by various cytokines and growth factors via distinct signal transduction

pathways. Furthermore, in this review we propose the idea that some tight junction proteins of hepatocytes may participate in signal transduction mechanisms that regulate apoptosis and bile canalicular formation. However, there are several transcriptional factors localized at tight junction areas (Matter and Balda, 2003, 2007), though we did not describe them. Thus, tight junctions of hepatocytes have not only barrier function, but also multiple functions, including signal transduction and gene expression (Balda and Matter, 2008). On the other hand, claudins and occludin are required for HCV entry (Evans et al., 2007; Benedicto et al., 2008; Liu et al., 2009). Although the detailed mechanisms of HCV entry via the tight junction proteins are still unclear, elucidation of the mechanisms involved in HCV entry into hepatocytes is urgently required.

Acknowledgements. We thank Dr. D. Cassio and Dr. C. Decaens (Univ Paris-Sud) for the WIF-B9 cells, and Ms. E. Suzuki (Sapporo Medical University) for technical support. This work was supported by Grants-in-Aid from the National Project "Knowledge Cluster Initiative" (2nd stage, "Sapporo Biocluster Bio-S"), the Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labour and Welfare of Japan.

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Accepted April 29, 2009