Department of Pharmaceutics and Biopharmaceutics[1], Showa Pharmaceutical University, Machida, Tokyo; Department of Bio-Functional Molecular Chemistry[2]; Department of Toxicology[3], Graduate School of Pharmaceutical Sciences, Osaka University, Suita-shi, Osaka; Department of Biopharmaceutics[4], School of Pharmaceutical Sciences, Teikyo University, Tsukui-gun, Kanagawa, Japan

Effect of forskolin on the expression of claudin-5 in human trophoblast BeWo cells

M. Harada[1], M. Kondoh[1,2], A. Masuyama[1], M. Fujii[1], T. Nakanishi[3], N. Utoguchi[4], K. Yagi[2], Y. Watanabe[1]

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Masuo Kondoh, Ph.D., Department of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita-shi, Osaka 565-0871, Japan
masuo@phs.osaka-u.ac.jp

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Trophoblasts, a cell type found in the placenta, play a pivotal role in the function of the placenta as a barrier between the maternal fluid and the fetus. Recently, claudin, a 24-kDa transmembrane protein, was identified as being responsible for the barrier function of epithelia. In the present study, we investigated the expression profiles of claudin and the changes in expression during the differentiation of BeWo human trophoblast cells. Reverse transcriptase-polymerase chain reaction and immunoblotting demonstrated the expression of claudin-1, -3, -4, and -5 in BeWo cells. Forskolin, which induces the differentiation of BeWo cells from cytotrophoblast-like cells into syncytiotrophoblast-like cells, reduced slightly the expression of claudin-5. This is the first report to show changes in claudin-5 in forskolin-treated BeWo cells.

1. Introduction

Placenta is a vital tissue for the development of the embryo during pregnancy. It plays an essential role in the selective delivery of oxygen and nutrients from the maternal fluid to the fetus and acts as a selective barrier against harmful stimuli in the maternal circulation. The placenta consists of several types of trophoblast cell lineages, including cytotrophoblasts and syncytiotrophoblasts. Syncytiotrophoblasts are polarized, epithelial-like cells that make up the outermost layer of the placental villi and form a physical and metabolic barrier between the fetal and maternal blood circulation (Stulc 1989). However, the barrier function of trophoblasts is not fully understood.

Epithelia are sheets of epithelial cells, and they act as borders between tissues (Powell 1981). In the skin, epidermal cell sheets play a pivotal role in formation of an epidermal barrier (Nemes and Steinert 1999); in the intestine, epithelial sheets control absorption of nutrients by means of their transporters (Cheeseman 1992); and in the brain, endothelial cell sheets comprise the blood-brain barrier (Matter and Balda 2003). The tight junction (TJ) is a complex apparatus made up of several kinds of protein, and it mediates cell-cell interactions in the epithelial sheet (Anderson 2001; Anderson and Van Itallie 1995). Extracellular protein components of the TJ include occludin, junctional adhesion molecule, and claudin; intracellular proteins include ZO-1, ZO-2, cingulin, and 7H6 (Anderson and Van Itallie 1995).

Several lines of evidence indicate that, of these proteins, claudin, a four-transmembrane protein with a molecular weight of approximately 24 kDa, is critical for the barrier function of the TJ (Anderson 2001; Tsukita and Furuse 2000; Tsukita et al. 2001). Interestingly, the barrier functions of the claudin family members differ, and there is tissue specificity with respect to the barrier function. For instance, claudin-1-deficient mice lose barrier function in epidermis (Furuse et al. 2002). Also, a claudin-4 modulator has been shown to enhance drug absorption in the jejunum but not the colon of rats (Kondoh et al. 2005). Furthermore, a deficiency of claudin-5 results in loss of barrier function in the blood-brain barrier (Nitta et al. 2003). Thus, claudin plays a pivotal role in the barrier function of TJs in epithelia.

Epithelial-like syncytiotrophoblasts are responsible for the barrier function of the placenta, and they regulate the transport of substances between the maternal fluid and the fetus. However, the expression of claudins in the placenta has never been investigated. Furthermore, because there are differences in placental function among species, it is difficult to predict the properties of human placenta from the *in vivo* data on placenta in mice or rats (Nakanishi et al. 2002). Instead, many investigators have attempted to understand the properties of human placenta by *in vitro* studies using human trophoblast cell lines, including BeWo, JEG-3, and JAR. Of these cell lines, BeWo cells are expected to be a good model for studying the function of human placenta because treatment with forskolin (FK) differentiates them from cytotrophoblast-like cells to syncytiotrophoblast-like cells (Liu et al. 1997; Nakanishi et al. 2002; Utoguchi and Audus 2000; Utoguchi et al. 2000). For these reasons, we investigated the expression profiles of claudins in BeWo cells, and we found that the expression of claudin-5 changed upon treatment with FK.

2. Investigations and results

2.1. Expression profiles of the claudin family in BeWo cells

Although human choriocarcinoma BeWo cells have been widely used as an *in vitro* model of human placenta (Asano et al. 2006; Liu et al. 1997; Nakanishi et al. 2002; Utoguchi and Audus 2000; Utoguchi et al. 2000), expression of claudin in these cells has not been investigated. Therefore, we first investigated the expression of the claudin family in BeWo cells by RT-PCR. As shown in Fig. 1A, mRNAs for claudin-1, -2, -3, -4, -5, -7, and -11 were detected in BeWo cells. Because the commercial availability of antibodies for the claudin family is limited, we evaluated expression of only the claudin-1, -2, -3, -4, and -5 proteins by Western blotting. As shown in Fig. 1B, all of these claudin proteins were observed in BeWo cells. We also evaluated expression of claudin proteins in two other human trophoblast cell lines, JAR and JEG-3. We found that the expression profiles of claudin proteins in JEG-3 cells were similar to those of BeWo cells, but JAR cells were deficient for expression of claudin-3.

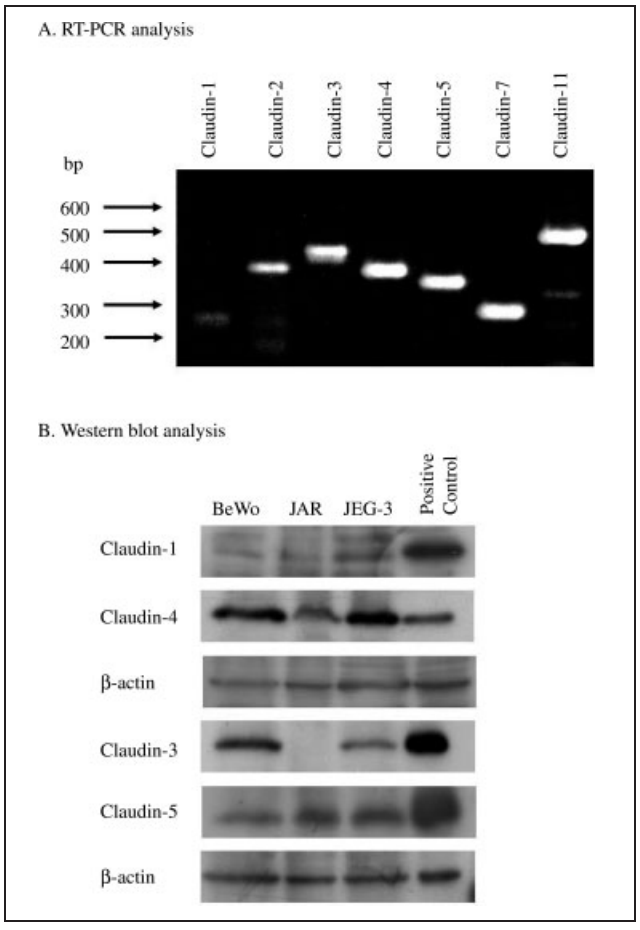

Fig. 1: Expression profiles of claudin family members in BeWo cells A) BeWo cells were seeded on a culture dish at a sub-confluent density. After 3 days, the cells were harvested, and the expression of mRNA for claudin family members was evaluated by RT-PCR. The putative sizes of PCR products were summarized in Table 1. B) BeWo, JAR and JEG-3 cells were seeded on a culture dish at a sub-confluent density. After 3 days of culture, the cells were harvested, and the expression of claudin family members was detected by Western blot analyses. Caco-2 lysates were used as a positive control for claudin-1 and -4, and mouse lung lysates were used for claudin-3 and −5. β-actin was detected as an internal control. Data are representative of three independent experiments

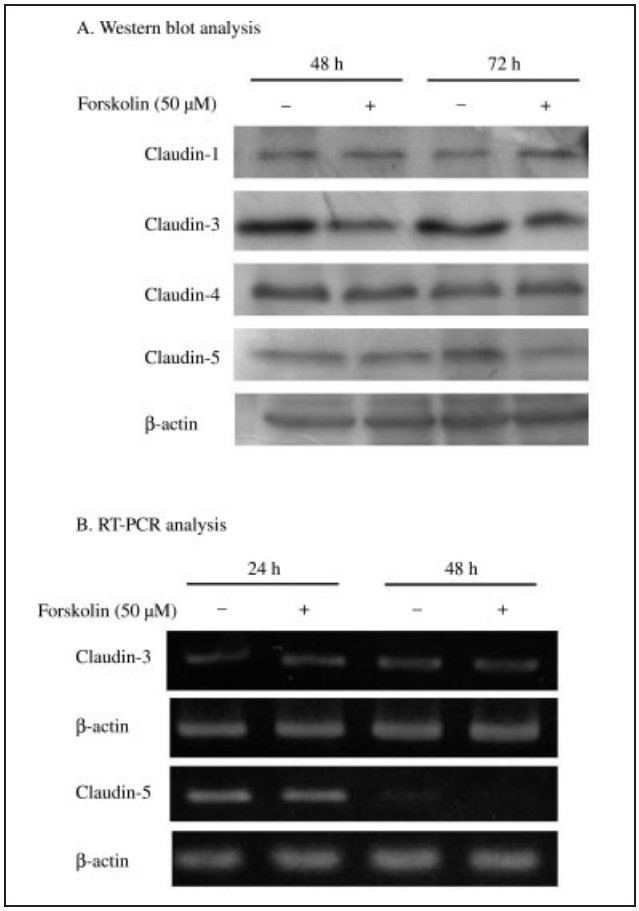

Fig. 2: Expression of claudin family members in forskolin-treated BeWo cells BeWo cells were seeded on a culture dish at a sub-confluent density. After 1 day, forskolin (50 μM) or vehicle was added to the culture medium. The medium was refreshed every 24 h. After 24–72 h of treatment, the cells were harvested, and expression of claudin family proteins and mRNA was evaluated by Western blot (A) and RT-PCR analysis (B), respectively. β-actin was detected as an internal control. Data are representative of at least three independent experiments

2.2. Effect of FK on expression of the claudin family

The polarized epithelial-like syncytiotrophoblasts constitute the outer layer of the placental villi and form a physical and metabolic barrier between the maternal blood and the fetus. In physiological situations, human cytotrophoblasts spontaneously differentiate into syncytiotrophoblasts. In this regard, BeWo cells are a good *in vitro* system for studying human placenta because treatment with FK causes the differentiation from a cytotrophoblast-like to a syncytiotrophoblast-like cell type (Wice et al. 1990). Indeed, hallmarks of the differentiation of BeWo cells into syncytiotrophoblast-like cells, including cell-cell fusion and the secretion of human chorionic gonadotropin, are observed in BeWo cells treated with 50 μM FK (Asano et al. 2006). As shown in Fig. 2A, the expression of claudin-5 changed in BeWo cells during FK-induced differentiation. To evaluate changes in claudin-5 in mRNA expression, we performed RT-PCR analysis, and we found that the expression of claudin-5 mRNA decreased after seeding the cells (Fig. 2B).

3. Discussion

Expression profiles of claudin family members have not been previously characterized in human trophoblast cell lines. Here we examined the expression profiles of some

claudin members in human trophoblast cell lines, and we showed that, in BeWo cells, the expression of claudin-5 is altered by treatment with forskolin (FK).

FK treatment caused a decrease in the expression of claudin-5 in the BeWo cells, but we did not address why or how claudin-5 were decreased in the present study. We also found that cell-cell fusion occurred during FK-induced differentiation of BeWo cells, resulting in the disappearance of TJs (Asano et al. 2006). As in trophoblast differentiation, TJs disappear during epithelium-mesenchyme transition (Hay 1995). Therefore, cell-cell fusion may be one reason for the changes in claudin levels. Indeed, we confirmed that expression of claudin-5 also decreased during spontaneous differentiation of rat trophoblast rcho-1 cells to trophoblast giant cells (data not shown).

Ohkubo and Ozawa (2004) found that snail, a transcription repressor that plays a pivotal role in the epithelium-mesenchyme transition, down-regulates some TJ proteins, including claudin-1 and occludin but that the transcription of occludin but not claudin-1 is attenuated by overexpression of snail. Also, Ikenouchi reported that snail is a potent regulator of some claudin family members, including claudin-3, -4 and -7, *via* regulation of claudins transcription (Ikenouchi et al. 2003). It will therefore be interesting to investigate the role of snail in the regulation of claudin-5 by FK.

The level of E-cadherin, an adherent junction protein, has also been shown to be decreased during differentiation of BeWo cells (Coutifaris et al. 1991). Very recently, Tunggal et al. reported that conditional inactivation of E-cadherin results in dysregulation of proper recruitment of claudin as a result of the loss of the function of atypical protein kinase C (Tunggal et al. 2005). Thus, the reduction of claudin-5 by FK in BeWo cells may also be due to regulation of E-cadherin. In future studies, we will investigate the involvement of atypical protein kinase C in FK-induced changes in claudin levels.

A deficiency or inhibition of claudin generally results in the attenuation of the barrier function of TJs in the epithelia. Syncytiotrophoblasts separate the maternal fluid and the fetus and therefore act as a placental barrier. Therefore, it is unexpected that the expression of some claudins decreased during the differentiation of BeWo cells. One possible explanation for the reduction in claudin-5 is that they contribute to the formation of leaky junction in undifferentiated BeWo cells. Indeed, Van Itallie et al. (2003) indicated that the properties of TJs, including the regulation of paracellular ionic transport and the barrier function, varied among epithelia. For example, exogenous expression of claudin-11 elevates the barrier function of TJs in MDCK cells but lowers it in LLC-PK1 cells. Also, exogenous expression of claudin-2 causes a decrease in TJ integrity, and the specific mixture and ratios of claudin subtypes is thought to determine the barrier properties of TJs (Furuse et al. 1999, 2001). Therefore, it is important to determine the combination of claudin species responsible for the barrier function of TJs in BeWo cells. Finally, other claudins bound to claudin-5 could play a pivotal role in the barrier function of syncytiotrophoblasts.

Claudin-3 was not expressed in JAR cells, which may be due to differences among cell lines. Mitchell et al. (1995) indicated that the JAR cell line is unlikely to be a suitable model for studies of transepithelial transport in the placenta because they lack cell polarity. Thus, the idea that JAR cells are an epithelial cell line should be reconsidered.

In summary, this is the first report to show the change of claudin family members during the differentiation of BeWo trophoblast cells. Although this is only a preliminary report, these data should be useful for investigations into the barrier function of syncytiotrophoblasts in the placenta.

4. Experimental

4.1. Chemicals

Forskolin (FK) was obtained from Wako Pure Chemicals (Osaka, Japan). Anti-claudin antibodies were purchased from Zymed laboratories (San Francisco, CA, USA). All reagents used in this study were of research grade.

4.2. Cell culture

Human choriocarcinoma BeWo, JAR, and JEG-3 cells were used in this study. BeWo cells were cultured in Dulbecco's modified eagle's medium supplemented with 10% fetal calf serum (FCS), 19 mM $NaHCO_3$, 19.4 mM D-glucose, 4 mM L-glutamine, and 1% nonessential amino acid solution (Invitrogen, Gaithersburg, MD). JAR cells were cultured in RPMI 1640 medium with 10% FCS, 17.8 mM $NaHCO_3$, 1 mM pyruvate, 25 mM D-glucose, and 10 mM HEPES. JEG-3 cells were cultured in minimum essential medium eagle medium containing 10% FCS, 17.8 mM $NaHCO_3$, 4 mM L-glutamine, 1 mM pyruvate, and 1% nonessential amino acids. All of the cell lines were maintained in a 5% CO_2 atmosphere at 37 °C.

4.3. Treatment of BeWo cells with FK

FK was dissolved in ethanol at 10 mM and stored at −20 °C before use. BeWo cells were seeded on culture dishes at a sub-confluent density, and the culture medium was replaced with medium containing vehicle (0.5% ethanol) or FK (50 μM). The medium was refreshed every 24 h, and after 48 or 72 h of treatment with FK, the cells were harvested with a scraper and washed once with phosphate buffered saline. The resulting cells were used for reverse transcriptase-polymerase chain reaction (RT-PCR) or Western blot analyses.

4.4. RT-PCR analysis

BeWo cells were seeded on a culture dish at a sub-confluent density. After a few days of culture, confluent cells were harvested with a cell scraper. Total RNA was extracted from the cells with TRIzol (Invitrogen, Gaithersburg, MD). RT-PCR was carried out using a TaKaRa RNA PCR kit (AMV) Version 3.0 using Oligo dT primer according to the manufacturer's instructions (TaKaRa, Shiga, Japan). Primers used here and the sizes of the putative PCR products are listed in the Table. The primers were designed using Oligo 4.0 (Molecular Biology Insights, Inc.). The PCR conditions were as follows: Claudins: 60 s at 95 °C, 60 s at 60 °C, 60 s at 72 °C for 36 cycles; β-actin: 30 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C for 20 cycles. RT-PCR products were not detected without total RNA (data not shown).

4.5. Immunoblotting

Cells were harvested with a cell scraper and lysed in lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.2% sodium dodecyl sulfate, 2 mM ethylenediaminetetraacetic acid, and 1% protease inhibitor cocktail (Sigma, St. Louis, MO). Protein was quantified using a protein assay kit with bovine serum albumin as a standard (Bio-Rad, Hercules,

Table: Primers used in this study

Gene		Sequence (5′ to 3′)	Product size (bp)
Claudin-1	forward	CCTCCTGGGAGTGATAGCAA	240
	reverse	CCAGTGAAGAGAGCCTGACC	
Claudin-2	forward	GGGCTACATCCTAGGCCTTC	395
	reverse	GAAGATTCCAGGCAACAGGA	
Claudin-3	forward	CATCACGTCGCAGAACATCT	469
	reverse	CTTGGTGGCCGTGTACTTCT	
Claudin-4	forward	AAGGTGTACGACTCGCTGCT	392
	reverse	GCGGAGTAAGGCTTGTCTGT	
Claudin-5	forward	CTGGACCACAACATCGTGAC	352
	reverse	GCACAGACGGGTCGTAAAAC	
Claudin-7	forward	GGGGAGACGACAAAGTGAAG	262
	reverse	TACCCAGCCTTGCTCTCATT	
Claudin-11	forward	GACCACCTCCACCAATGACT	536
	reverse	CATGAGTCGGGGAGCTAGAG	
β-Actin*	forward	GACCACCTCCACCAATGACT	536
	reverse	CATGAGTCGGGGAGCTAGAG	

CA). Equal amounts of protein (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blot analysis using anti-claudin or β-actin antibodies. Immunoreactive bands were detected with a peroxidase-labeled secondary antibody, followed by chemiluminescence reagents (Amersham Bioscience, NJ).

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