FULL PAPER *Physiology*

Expression of MAP Kinases and Connexins in the Differentiation of Rat Mammary Epithelial Cells

Min-Soo SEO[1,2)]**, Joon-Suk PARK[1,2)]**, Se-Ran YANG[1,2)], Ki-Su PARK[1,2)], In-Sun HONG[1)], Eun-Hye JO[1)], Kyung-Sun KANG[1)] and Yong-Soon LEE[1,2)]*

[1)]*Department of Veterinary Public Health, College of Veterinary Medicine, Seoul National University, Seoul and* [2)]*School of Agricultural Biotechnology, Seoul National University, Seoul 151–742, Korea*

(Received 1 June 2005/Accepted 7 February 2006)

ABSTRACT. Gap junctional intercellular communication (GJIC) is involved in the regulation of many cellular processes. MAP kinases are known to affect GJIC and phosphorylation of connexin (Cx). MAP kinases can also be a regulator of cell proliferation and growth. This study was undertaken to show the relevance between expression patterns of Cxs and MAP kinases in rat mammary epithelial cells (RMECs). In order to characterize the RMECs, they were stained with Peanut lectin, which indicates most alveolar epithelial cells, and Thy-1.1 was used as a marker of luminal epithelial cells or myoepithelial cells, respectively. We studied the expression patterns of major gap junction proteins, Cx 26, 32, and 43 in RMECs. Western blot analysis demonstrated that Cx26 gradually decreased from day 2, while Cx32 was expressed constantly from day 1 to 14. Cx43 dramatically increased on day 5 and decreased thereafter. The expression patterns and phosphorylation of ERK1/2 and JNK were similar to Cx43, but expression of p38 was like that of Cx32. These results showed that the MAP kinases that comprise ERK1/2, p38, and JNK were involved in regulation of Cxs. Our data suggests that GJIC plays an important role during rat mammary differentiation and that MAP kinases may be closely related functionally to regulate the gap junction.

KEY WORDS: connexin, gap junction, MAP kinase, rat mammary epithelial cell.

J. Vet. Med. Sci. 68(6): 567–571, 2006

Usually, rat mammary epithelial cells (RMECs) are comprised of luminal epithelial cells and myoepithelial cells. Recently, immunocytochemical stains have been used to distinguish the ductal epithelial, myoepithelial, and alveolar epithelial cell types in the rat. PNA will stain most of the luminal alveolar cells of the rat [18]. Thy-1.1 antigen has been described as a differentiation marker of potential rat mammary myoepithelial cells *in vitro* [10]. Gap junctions are often abundant in glands [5]. Expression patterns of major gap junction proteins, including connexin (Cx) 26, 32, and 43, have already been demonstrated *in vivo* in the rat mammary gland [23]. Cx activation is often accompanied by changing in the level of GJIC. In particular, it has been well documented that phospho-extracellular signal-regulated protein kinases (ERK) (activated form) can inhibit gap junctional intercellular communication (GJIC) in several kinds of cell lines, including WB cells, by Cx43 phosphorylation [19, 20]. The p38 group kinase has been found to be involved in inflammation, cell growth, cell differentiation, the cell cycle, and cell death [14]. Therefore, MAPKs might play important roles in the regulation of GJIC by phosphorylation of Cxs [2, 3]. MAPKs, which include ERK, c-Jun N-terminal kinase (JNK), and the p38 subfamilies, are important regulatory proteins that transduce various extracellular signals into intracellular events [13]. This means that MAPKs are regulated by separate signal transduction pathways that control many aspects of mammalian cellular physiology, including cell growth, differentiation, and cell death [7, 9, 13, 22]. The purpose of this study was to characterize the cell types of RMECs and examine the expression patterns of Cx26, 32, and 43 in RMECs during cell culture by immunostaining and western blot analysis. Furthermore, we showed the expression patterns of MAPKs in RMECs during differentiation.

* CORRESPONDENCE TO: LEE, Y.-S., Department of Veterinary Public Health, College of Veterinary Medicine, Seoul National University, San 56–1, Sillim-9 dong, Gwanak-gu, Seoul 151–742, Korea.

**These authors contributed equally to this work as the first author.

MATERIALS AND METHODS

Cell culture: The methods for preparation of RMECs for primary culture have been described previously [4]. To culture RMECs from intact glands, we used virgin, 50- to 55-day-old Sprague-Dawley CD rats (Crl:CDBR) purchased from Charles River (Wilmington, MA) as the only source of mammary organoids in the primary culture. The rat mammary glands were finely minced, resuspended in digestion solution [collagenase type III (2 mg/m*l*); Worthington Biochemical, Freehold, NJ] and shaken at 37°C for approximately 3 hr. After incubation, the suspension was washed in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12; 1:1) medium and centrifuged at 350 × g for 10 min. The pellet was resuspended in DMEM/F-12 medium that consisted of 50 μg/m*l* gentamicin, 10 μg/m*l* human EGF, 5 μg/m*l* insulin, 0.5 μg/m*l* hydrocortisone, 2 μg/m*l* human transferrin, and 0.005 μg/m*l* 17β-estradiol (Sigma, St. Louis, MO, U.S.A.). The suspended rat mammary digest was distributed into 10-cm polystyrene culture dishes in DMEM/F-12 with 5% FBS (Fetal Bovine Serum;

Gibco BRL Life Technologies, Inchinnan, UK) and incubated at 37°C for 2 hr to attach rapidly adhering cells, which are predominatly fibroblasts and other mesenchymal cells. Then, the supernate, which contained free epithelial cells, cell clumps, and multicellular organoids, was collected and washed by centrifugation at 350 × g for 5 min. Subsequently, the pellet was resuspended and distributed in culture dishes into DMEM/F-12 with 5% FBS. The RMECs were incubated for 1 to 14 days at 37°C in a humidified 5% CO_2/air atmosphere. DMEM/F-12 with 5% FBS was used until day1 of culture, at which point the FBS was removed. The medium was changed three times weekly.

Immunofluorescence staining: To characterize luminal epithelial cells and myoepithelial cells, we used immunostaining for 1 to 14 culture days. The cells were fixed in 4% paraformaldehyde (in PBS, pH 7.2) for 10 min at room temperature and then washes 3 times in PBS (pH 7.2). The cells were then permeabilized with 0.3% Triton X-100 in PBS for 10 min. Afterwards they were washed in PBS and blocked in PBS containing 10% normal goat serum (NGS, Zymed) overnight. After blocking, the cells were incubated in PNA-FITC (Vector Laboratories, 1:100) and/or phycoerythrin-conjugated anti-Thy-1.1 monoclonal antibody (Bioproducts for Science, 1:100), mouse monoclonal anti-Cx43, anti-Cx32 antibody (Chemicon, 1:200), and mouse monoclonal anti-Cx26 (Zymed, 1:200) diluted in PBS containing NGS for 2 hr at room temperature. They were then washed in PBS 3 times and incubated in FITC goat anti-rabbit secondary antibody for 30 min at room temperature. The fluorescence-stained sites were observed with an inverted fluorescent microscope (Olympus Ix70, Okaya, Japan).

Western blot analysis: Protein extracts were prepared using 20% SDS solution containing 1 mM phenylmethylsulfonyl fluoride (PMSF; a protease inhibitor), 1 μM leupeptin, 1 μM antipain, 0.1 μM aprotinin, 0.1 mM sodium orthovanadate, and 5 mM sodium fluoride. Protein content was determined using a DC assay kit (Bio-Rad, CA) and separation on 12% SDS-polyacrylamide gels using a standard SDS-PAGE protocol. After electrophoresis, the gels were transferred onto nitrocellulose (Bio-Rad, CA). After blocking with 5% skim milk in PBS containing 0.1% Tween-20, the membranes were incubated with mouse monoclonal anti-Cx43, anti-Cx32 antibody (Chemicon, 1:1,000), mouse monoclonal anti-Cx26 (Zymed, 1:1,000), rabbit anti-Map kinase (Zymed, 1:1,000), rabbit anti-active p38, anti-active MAPK antibody (Promega, 1:1,000), mouse monoclonal anti-p38, anti-pJNK, and rabbit anti-JNK (Santa Cruz, 1:1,000), followed by anti-mouse or anti-rabbit IgG horseradish peroxidase-linked antibody (Zymed, 1:2,000). The bound antibody was visualized using an ECL detection kit (Amersham).

RESULTS

Isolation and characterization of RMECs: RMECs were isolated and cultured in DMEM/F-12 medium for 1 to 14 days. Monolayer cells were suspended out from the organoids. Immunostaining of RMECs with PNA-FITC and Thy-1.1-PE showed distinctive cell growth patterns. PNA-FITC was positive for luminal epithelial cells, whereas Thy-1.1-PE was positive for myoepithelial cells. Morphologically, small cuboidal, cobblestone-appearing epithelial cells that spread out from the organoid were tightly packed luminal epithelial cells. Relatively, the myoepithelial cells were elongated cells that were found surrounding the luminal epithelial cells or often between the epithelial colonies (Fig. 1). On day 1, most of the out-growth cells were PNA+ cells and Thy-1.1+ cells. At this time, the cell types were not distinguished clearly (Fig. 1A). Most of the monolayer cell colonies were established by day 3. PNA+ cells had a cuboidal cobblestone appearance and Thy-1.1+ cells had larger or elongated shape. Most of the tightly packed small cuboidal epithelial cells located near the center of the colonies were positive for PNA-FITC. Larger epithelial cells located at the colony boundaries or elongated cells present in the areas between epithelial colonies were positive for Thy-1.1-PE (Fig. 1B). The cell states of the RMECs remained at the same on day 5 (Fig. 1C). After day 7, the luminal epithelial cells were converted into myoepithelial cells [8]. The location of PNA+ cells was similar to Thy-1.1+ cells, and the shape of PNA+ cells changed and became similar to Thy-1.1+ cells (Fig. 1D).

Immunofluorescence staining for Cx26, 32, and 43 in RMECs: Immunofluorescence staining was used to show the existence of major gap junction proteins in RMECs on day 3. Cx32 was expressed at filament in cytoplasm of RMECs (Fig. 2A). In comparison, Cx43 was expressed in cell membrane like as plaque (Fig. 2B). It was difficult to show the immunoreactivity of Cx26 compared with Cx32 and 43 (data not shown). But Cx26 was detected by West-

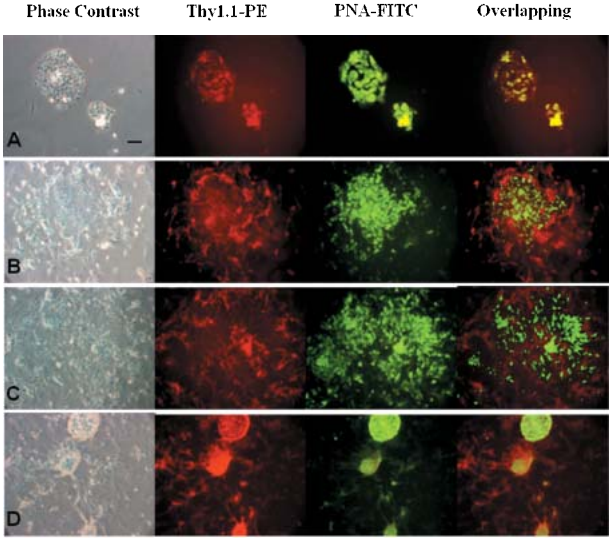

Fig. 1. Immunoreactivity of PNA and Thy-1.1 in RMECs on day 1 (A), 3 (B), 5 (C), 7 (D). PNA (PE, red) was positive for luminal epithelial cells, whereas Thy-1.1 (FITC, green) was positive for myoepithelial cells. The control was a negative primary antibody, that did not express. Scale bar, 400 μm.

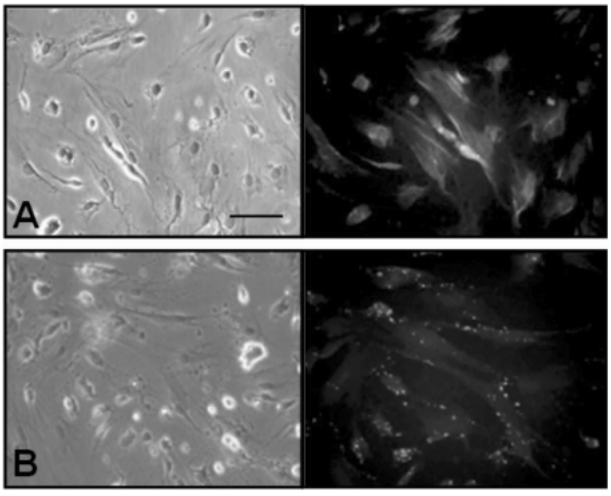

Fig. 2. Immunoreactivity of Cx32 and 43 in RMECs on day 3. The majority of the RMECs show Cx32 and 43 immunostaining. The control was a negative primary antibody, that did not express. Scale bar, 200 μm.

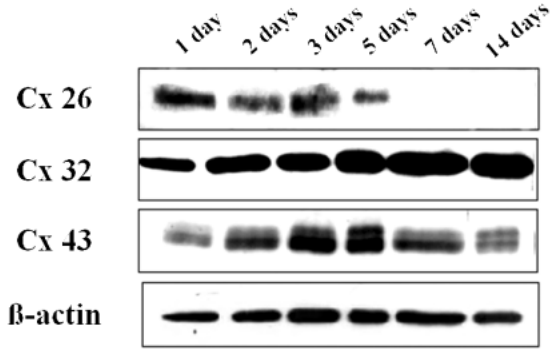

Fig. 3. Expression of Cx26, 32, and 43 during differentiation of RMECs by western blot analysis. This resulted in several species of Cx43, which could be detected by Western blotting (NP and P1) (NP, non-phosphorylated; P1, phosphorylated). Total cellular protein extracts were prepared and Western blot analysis was performed with 20 μg of protein using Cx26, 32, and 43 antibodies.

ern blot analysis, as was Cx32 and 43 (Fig. 3).

Expression patterns of gap junction proteins (Cx26, 32, and 43) in RMECs: Western blot analysis was used to examine changes in gap junction proteins (Cx26, 32, and 43) in RMECs on culture days 1, 3, 5, 7, and 14. Cx26 was detected strongly on early culture days (1 to 3 days), and gradually increased on day 5. After day 7, expression of Cx26 was barely detected or not at all. However, expression of Cx32 remained from day 1 to day 14. Phosphorylation of Cx43 gradually increased up to day 5, whereas after day 7, it decreased (Fig. 3).

Expression patterns of MAPKs (ERK1/2, JNK, p38) in RMECs: ERK1/2, pERK1/2, JNK, pJNK, p38, and pp38 were evaluated by Western blot analysis to discover the expression and phosphorylation patterns of MAPKs in

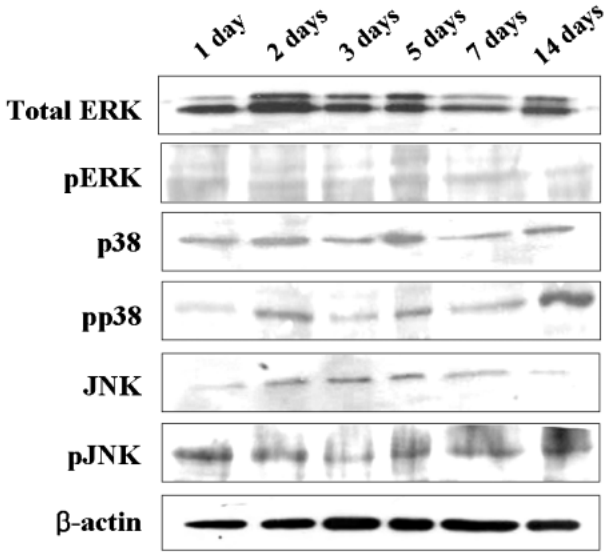

Fig. 4. Western blot analysis of MAPKs (ERK1/2, pERK1/2, JNK, pJNK, p38, and pp38) during differentiation of RMECs by western blot analysis. Total cellular protein extracts were prepared, and Western blot analysis was performed with 20 μg of protein using ERK1/2, pERK1/2, p38, pp38, JNK, and pJNK antibodies.

RMECs during culture days. The protein expression and phosphorylation patterns of ERK1/2 and JNK were similar to that of Cx43. ERK1/2 and JNK gradually increased up to day 5, whereas after day 7, they decreased. The expression and phosphorylation pattern of p38 was similar to that of Cx32 (Fig. 4).

DISCUSSION

This study was undertaken to show the relevance between expression patterns of Cxs and MAPKs in RMECs. RMECs were cultured for 1 to 14 days *in vitro*. The RMECs spread out slowly from the organoids. Morphologically, two major cell types were observed by phase-contrast microscopy. One of the cell types was luminal epithelial cells, which are small cuboidal-shaped epithelial cells, and the other was elongated myoepithelial cells. In order to characterize RMECs, PNA, which is a marker used to identify luminal epithelial cells, and Anti-Thy-1.1 antibodies bind to the cell-surface of Thy-1.1 antigen on normal myoepithelial cells and myoepithelial-like cells [1, 18]. On day 1, the RMECs were positive for both PNA and Thy-1.1. At this time, characterization of the RMECs cell types was not clear. But, from day 3 to 5, the RMECs were distinguishable between luminal epithelial cells and myoepithelial cells. After day 7, the luminal epithelial cells were gradually converted into myoepithelial cells (Fig. 1).

In vivo, Cx26 and 32 appear in the luminal epithelial population of the rat mammary gland [16]. Cx32 protein transcripted into the luminal epithelium of the BALB/c mouse and SD rat mammary gland has only been detected during

lactation. No Cx26 has been detected during pregnancy by immunocytochemistry, but during parturition and lactation, Cx26 has been detected by Western blot analysis [23]. Cx26 and 32 have been shown in the virgin gland and during all stages of mammary development in CD1 mice and Wistar rats. Within the myoepithelial cell population, the only Cx identified thus far is Cx43, which can be detected in virgin, pregnant, and lactating mammary glands [11]. Furthermore, GJIC is considered to be necessary for regulation of growth, embryonic development, and tissue differentiation [5, 21].

In this study, we examined the expression patterns of major gap junction proteins, Cx26, 32, and 43, in RMECs. Western blot analysis demonstrated that Cx26 gradually decreased from day 2, while Cx32 was expressed constantly from day 1 to 14. Cx43 dramatically increased on day 5 and decreased thereafter (Fig. 3). Because Cx26 and 32 were abundantly located in luminal epithelial cells, the luminal epithelial cells increased during early culture days, and then were converted into myoepithelial cells, which can be a relative expression pattern of Cx26. In the case of Cx32, the existence of Cx32 protein continuously during culture day 5 suggested to us that Cx32 may be relative to not only luminal epithelial cells, but also myoepithelial cells. Usually Cx43 exists in myoepithelial cells. Expression of Cx43 increased gradually, and then decreased. The inverted U-shaped time-response pattern of Cx43 means the differentiations of myoepithelial cells.

Activation of p38 and JNK is related to stress response, growth arrest, and apoptosis, while ERK is important in mitogenesis and differentiation [6, 7, 9, 22]. Growth factors like EGF, PDGF, FGF-2, and others (like insulin) have been known to affect GJIC in a number of cell types [12, 15]. The above-mentioned growth factors activate the ERK pathway (one of several MAPKs pathways; two other well-characterized MAPKs pathways are the p38 and the JNK pathways). In rat liver epithelial cells, EGF causes a transient, but strong, decrease in GJIC [17]. EGF profoundly affects the phosphorylation state of Cx43 in these cell systems. In contrast to the response to EGF described above, GJIC increased in a human kidney cell line after exposure to EGF [18]. This also appears to be dependent on the ERK pathway, but protein synthesis was needed [3]. It is presently unknown as to why two cell types are stimulated with the same growth factors, utilizing the same transduction pathway, and having the same Cx protein, yet have opposite responses with regard to GJIC.

In this study to discover the relationship between MAPKs and GJIC in RMECs, Western blot analysis showed that the MAPKs were involved in ERK 1/2, p38 and JNK. In addition, we showed that activation of MAP kinases during RMECs differentiation might play an important role in regulation of Cxs. The expression patterns of ERK1/2 and JNK were similar to Cx43, but expression of p38 was similar to Cx32. We also found pERK1/2, pp38, and pJNK, activation forms of ERK1/2, p38 and JNK. Therefore, ERK1/2 and JNK may have an effect on Cx43, and p38 may have an affect on Cx32. These results suggest that MAP kinases may be closely related functionally to regulate the gap junction during rat mammary differentiation.

ACKNOWLEDGMENT. This work was supported by a Korea Research Foundation grant (KRF-005-E00076), Brain Korea 21 Project grant, and the Korea Health 21 R&D Project grant, Ministry of Health & Welfare, Republic of Korea (02-PJ10-PG4-PT02-0015).

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