Expression of Gap Junction Proteins Connexin 26 and 43 Is Modulated During Differentiation of Keratinocytes in Newborn Mouse Epidermis

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We examined the expression of the gap junction proteins connexin 26 (Cx26), 32 (Cx32), and 43 (Cx43) in keratinocytes of newborn mouse epidermis to elucidate which connexins are expressed in keratinocytes in intact skin of newborn mice, and whether the expression of connexins is modulated during terminal differentiation of keratinocytes. Immunofluorescent staining using antibodies against Cx26, Cx32, and Cx43 combined with type-specific anti-keratin immunohistochemistry showed that Cx26 was expressed in keratinocytes in the granular layer and in the upper part of the squamous layer, whereas Cx43 was localized in keratinocytes in the basal layer and in the lower part of the squamous layer. No specific staining of Cx32 was found in mouse epidermis. Double staining of Cx26 and Cx43 revealed that some keratinocytes in the squamous layer expressed both connexins, but that in most cases localization of the two kinds of connexins was different, i.e., Cx26 was localized on the upper surface, whereas Cx43 was present on the lower surface of the plasma membrane of keratinocytes. Northern and Western blot analyses confirmed that Cx26 and Cx43, but not Cx32, were expressed at mRNA and protein levels in newborn mouse skin. These results suggest that the modulation of connexin expression from Cx43 to Cx26 takes place during terminal differentiation of keratinocytes in mouse epidermis. Key words: intercellular communication/mouse skin. *J Invest Dermatol 101:773–778, 1993*

Gap junctions are the transmembrane channels that directly link neighboring cells and mediate reciprocal exchange of low-molecular-weight metabolites and ions, including second messengers such as cyclic adenosine monophosphate (cAMP), inositol trisphosphate, and Ca^{++}, between the cells. Gap junctional intercellular communication is thought to play a crucial role in the maintenance of homeostasis, morphogenesis, cell differentiation, and growth control in multicellular organisms [1–4].

Like most other epithelial cells, keratinocytes in epidermis are connected by gap junctions. Gap junctions in keratinocytes in intact skin were detected electron microscopically [5], by using electron-dense tracers such as lanthanum [6], and by using freeze-fracture replicas [7]. Existence of gap junctional intercellular communication in keratinocytes *in vivo* or *in vitro* was demonstrated by electrophysiologic-techniques [8–10] and by dye-transfer assay [10–12]. Involvement of gap junctional intercellular communication in the differentiation and cell growth of keratinocytes in epidermis was suggested by the studies using the dye transfer method [11,12]. These studies showed that gap junctional intercellular communication in mouse and human skin is restricted to vertical compartments, which could be related to the organization of the epidermal proliferative unit [13].

The gap junction channels are composed of hexagonal arrangements of oligomeric proteins called connexins (Cx) [14]. Thus far, approximately ten different homologous connexin sequences have been cloned and characterized in the mouse or rat genome [15]. Most organs and many cell types have been shown to express multiple connexins. Expression of connexins in keratinocytes in intact skin or in culture has been investigated by immunofluorescence and Northern and Western blot analyses [16–19]. However, the results of these investigations have sometimes been contradictory. For example, with respect to expression of connexins in mouse primary keratinocytes, Jongen *et al* [17] showed the expression of Cx43, but not Cx26, whereas Brissette *et al* [16] detected both Cx26 and Cx43. Although a freeze-fracture study revealed that gap junctions exist in keratinocytes from the basal to the granular layers [7], few studies have centered on what kinds of connexins are expressed in keratinocytes in different layers in intact skin, except that Guo *et al* [18] reported Cx43-immunoreactive plaques in regions of cell/cell contact of suprabasal cells in human epidermis.

In this study, we performed immunofluorescence studies of newborn mouse epidermis using antibodies against Cx26-, Cx32-, and Cx43-specific peptides combined with immunohistochemistry for type-specific keratin expression to elucidate which Cxs are expressed in keratinocytes in intact skin and whether the expression of connexins is modulated during terminal differentiation of keratinocytes.

MATERIALS AND METHODS

Animals Newborn CD-1 mice, 24–48 h old, were used. Dorsal skin was rapidly frozen in Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN) for immunofluorescence study. Additional portions were quick-frozen in liquid nitrogen for RNA extraction.

Immunofluorescence Frozen sections about 4-μm thick were cut on a cryostat and placed on poly-L-lysine-coated slides. The sections were fixed with acetone for 10 min at −20°C and dried. After rinsing in phosphate-

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Abbreviations: Cx, connexin; RT, room temperature.

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buffered saline (PBS) containing 5% skim milk for 20 min, the sections were first incubated with one of the following antibodies for 1 h at room temperature (RT): a rabbit polyclonal antiserum against a Cx26-specific peptide (amino acid residues 101–119) [20] (1/6000 dilution), a rabbit anti-J-peptide antiserum against Cx32 [21] (1/100 dilution), or a rabbit polyclonal antiserum against Cx43-specific peptide (amino acid residues 360–376) (1/8000 dilution). After washing in PBS, the sections were reacted with swine fluorescein-conjugated anti-rabbit immunoglobulins (1/50 dilution; DAKO, Copenhagen, Denmark) for 1 h and examined under a fluorescence microscope. Some sections were double-stained for either Cx26 or Cx43 and keratins 5/6 (K5/6), keratin 10 (K10), or keratin 14 (K14), in which mouse anti-K5/6 monoclonal antibody (1/20 dilution; Boehringer Mannheim, Mannheim, Germany), mouse anti-K10 monoclonal antibody (1/100 dilution; ICN ImmunoBiologicals, Costa Mesa, CA), or mouse anti-K14 monoclonal antibody (1/1000 dilution; Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK) and horse Texas Red–conjugated anti-mouse immunoglobulins (1/40 dilution; Vector Laboratories, Inc., Burlingame, CA) were utilized. Some sections were double-stained for Cx26 and Cx43 by utilizing mouse monoclonal antibody against Cx43 (1/1000 dilution; Zymed Laboratories, Inc., San Francisco, CA). As positive controls, mouse liver and heart sections were used for Cx26 and for Cx43, respectively.

Northern Blot Analysis Total RNA was isolated from newborn mouse skin and adult mouse heart and liver using a single-step thiocyanate-phenol-chloroform extraction method [22]. The concentration of RNA was determined by measuring absorption at 260 nm and 10 μg of total RNA was run on a 1% agarose/formaldehyde gel containing 0.2 μg/ml of ethidium bromide. Gels were capillary-blotted in 20 × standard saline citrate (SSC) onto nylon membranes (Hybond N; Amersham, Buckinghamshire, UK) and fixed by ultraviolet (UV) light. The digoxigenin (DIG) luminescent detection system (Boehringer Mannheim) was used and the protocol described by Höltke *et al* [23] was followed exactly. Transcriptions for the generation of DIG-labeled antisense RNA probes for Cx26 [24], Cx32 [25], and Cx43 [26] were performed with SP6, SP6, and T3 RNA polymerase (Boehringer Mannheim), respectively, after linearization of the template DNAs. Membranes were pretreated with 50% (v/v) formamide, 5 × SSC (pH 7.0), 2% (wt/vol) blocking reagent (Boehringer Mannheim), 0.1% (wt/vol) *N*-lauroyl-sarkosine, 0.02% (wt/v) sodium dodecyl sulfate (SDS) at 68°C for 1 h. RNA was then hybridized overnight in the same buffer with the DIG-labeled probes. Stringent washes were performed for 5 min at RT with 2 × SSC and 0.1% SDS twice, followed by 15 min at 68°C with 0.1 × SSC and 0.1% SDS twice. X-ray films were exposed at RT for 2–30 min.

Western Blot Analysis For Western blot analysis, whole-tissue fractions were prepared and gap junction proteins of tissue homogenates were enriched by alkali treatment according to the method of Herzberg *et al* [27]. Newborn mouse skin and adult mouse liver were immediately excised from the animal, placed in 20 mM $NaHCO_3$, and homogenized using a Potter-Elvehjem homogenizer. After brief sonication, homogenates were centrifuged at 48,400 × *g* at r_{max} for 20 min. The pellets were washed with 20 mM NaOH and then 1 mM $NaHCO_3$, using the same centrifugation conditions, and resuspended in bicarbonate buffer. The pellets were solubilized in Laemmli sample buffer without dithiothreitol (DTT) for at least 30 min at RT.

Approximate protein concentration was determined by a bicinchonic acid protein assay reagent (Pierce Chemical Co., Rockford, IL). Approximately 20 μg of protein for each sample was treated with DTT (final concentration of 100 mM) for 15 min at RT and then loaded on 10–20% SDS-polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Proteins were electrically transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA) using a semi-dry blotting for 2 h (0.65 mA/cm^2), stained with Ponceau S (Sigma, St. Louis, MO), and then photographed. The membranes were saturated overnight at 4°C with a blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20, 4% skim milk, 0.1% Na azide) and incubated with rabbit anti-Cx26 or anti-Cx43 at the dilution of 1/5000 in the blocking buffer at RT for 2 h. The membranes were washed with the buffer three times and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (1/7500 dilution) (Promega, Madison, WI) at RT for 1 h. After washing three times, bound antigens were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as chromogenic substrates. The total lysate of primary neonatal rat cardiac myocytes cultured for 7 d was used as a positive control for Cx43. Primary neonatal rat cardiac myocytes were lysed in a buffer (25 mM Tris-HCl, 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP-40, 0.2% SDS, 1 mM phenylmethanesulphonyl fluoride, aprotinin 50 μg/ml, pH 7.4), centrifuged at 15000 × *g* for 15 min at 4°C and the supernatant was used for Western blot analysis.

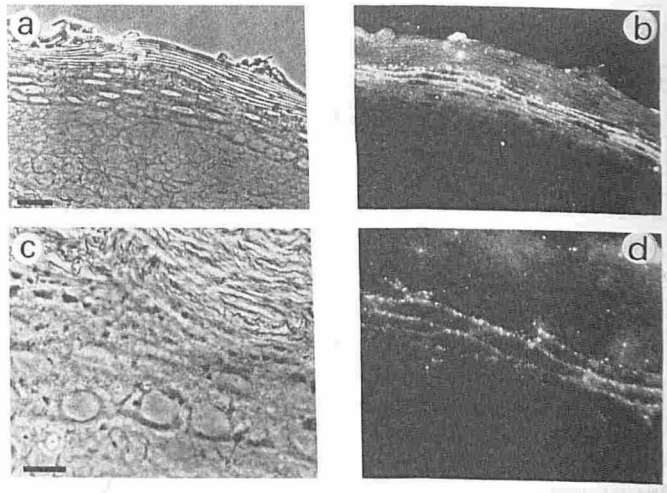

Figure 1. Immunofluorescent staining of Cx26 in newborn mouse skin. *a)* Phase-contrast image. *Bar,* 20 μm. *b)* Same section under epifluorescent optics. *c)* Phase-contrast image with a higher magnification. *Bar,* 10 μm. *d)* Same section under epifluorescent optics.

RESULTS

Immunofluorescence Newborn mouse epidermis was thick enough that the different layers of stratified keratinizing tissues could be clearly recognized, whereas adult back epidermis was considerably thinner, so that the squamous and granular layers were only slightly recognizable (data not shown). Therefore, the present immunofluorescence study was performed on sections from newborn mouse skin using specific antibodies to Cx26, Cx32, and Cx43.

The polyclonal antibody against Cx26-specific peptides revealed macular staining on membranes between adjacent keratinocytes in the granular layer and in the upper part of the squamous layer (Fig 1). Cx26 was more abundant in the granular layer than in the squamous layer. No fluorescent spots were detected in basal keratinocytes with the anti-Cx26 antibody.

The polyclonal antibody against Cx43-specific peptides showed macular fluorescent plaques on membranes between keratinocytes in the basal layer and in the lower part of the squamous layer (Fig 2). In addition to the localization of Cx43 on the plasma membrane, some Cx43 fluorescent spots seemed to be localized in cytoplasm, in accordance with the findings reported by Berthoud *et al* [28], Naus *et al* [29], and Puranam *et al* [30]. Cx43-positive spots were scarcely found in keratinocytes in the granular layer. Cx43 was observed on the lateral surfaces of basal keratinocytes, including attachment sites to the basal lamina, but whether Cx43 is expressed in the plasma membrane at the undersurface of basal keratinocytes was not clarified in the present study. The result obtained by use of the mouse monoclonal antibody against Cx43 was essentially the same as that with the rabbit antibody against Cx43 (Fig 2*f*). Hair follicles were immunolabeled with the anti-Cx43 (Fig 2*b*) and anti-Cx26 antibodies (data not shown), in agreement with the report of Risek *et al* [31].

With the polyclonal antibody against Cx32-specific peptides no specific staining was observed throughout the mouse skin (Fig 3*c*). In the absence of the first antibody, only a little unspecific fluorescence was noted in the mouse skin (Fig 3*d*). The anti-Cx26 and anti-Cx43 antibodies demonstrated the expected punctate pattern of gap junctional plaques in liver and in heart, respectively (Figs 3*e,f*).

To elucidate the localization of the Cxs in more detail, we performed double staining of Cx26 or Cx43 and different kinds of keratins, of which expression depends on the differentiation of keratinocytes in epidermis [32]. We used anti-K5/6 and anti-K14

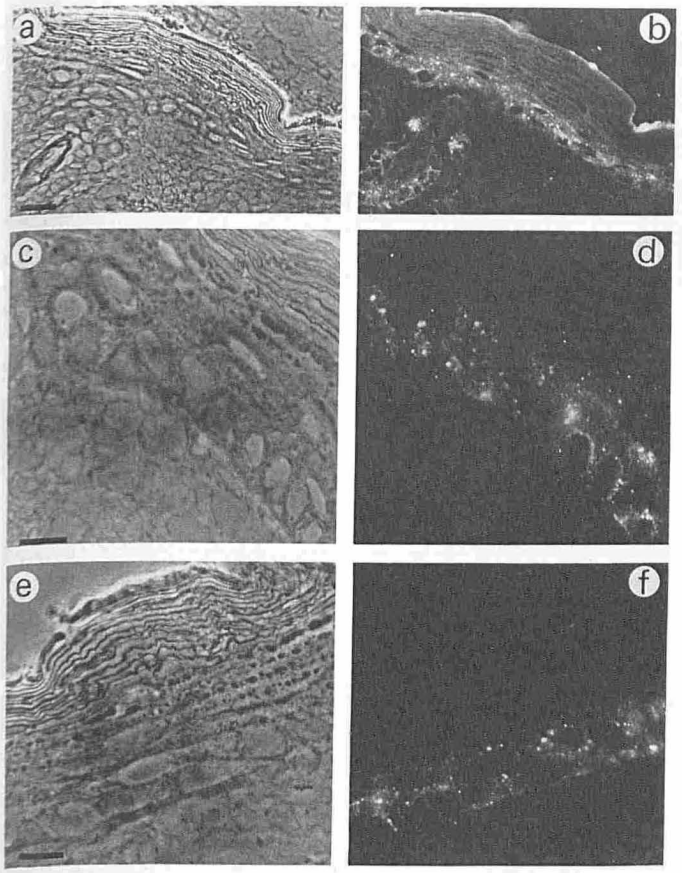

Figure 2. Immunofluorescent staining of Cx43 in newborn mouse skin. *a)* Phase-contrast image. *Bar,* 20 μm. *b)* Same section stained with the rabbit polyclonal antiserum against Cx43. *c)* Phase-contrast image with a higher magnification. *Bar,* 10 μm. *d)* Same section stained with the rabbit polyclonal antiserum against Cx43. *e)* Phase-contrast image. *Bar,* 10 μm. *f)* Same section stained with the mouse monoclonal antibody against Cx43.

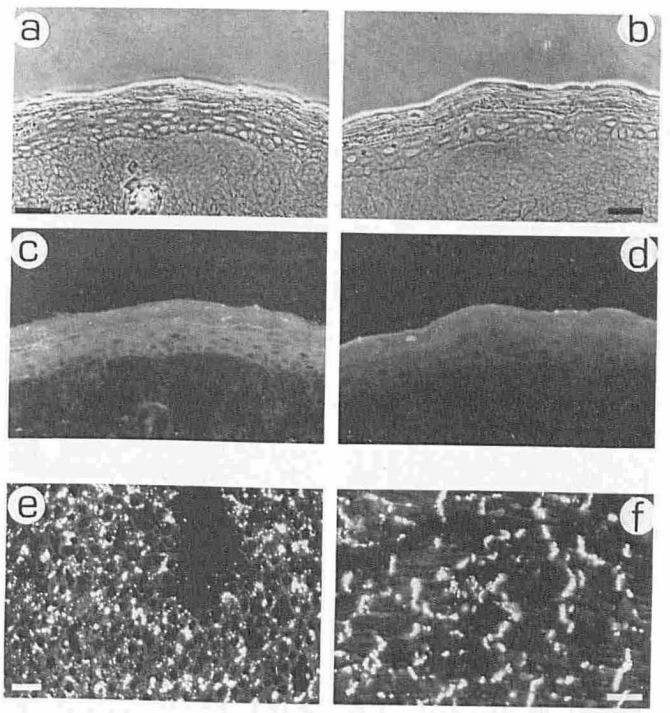

Figure 3. Immunofluorescent controls. *a,b)* Phase-contrast images of newborn mouse skin. *Bar,* 30 μm. *c)* Same section as *a* stained with anti-Cx32 antibody. *d)* Same section as *b* in the absence of the primary antibody. *e)* Cx26 localization in mouse liver. *Bar,* 50 μm. *f)* Cx43 localization in mouse heart. *Bar,* 30 μm.

antibodies to label basal keratinocytes, whereas anti-K10 antibody was utilized for the labeling of suprabasal keratinocytes. The anti-K5/6 (Figs 4*e,f*) and anti-K14 (data not shown) antibodies revealed strong cytoplasmic staining in basal cells as well as weak staining in keratinocytes in the lower part of the squamous layer, whereas K10 was negative in basal keratinocytes (Figs 5*e,f*). These data were in accordance with previously reported findings [33]. Keratinocytes that were positively stained with anti-Cx26 expressed K10 (Fig 5*c*), but did not express K5/6 (Fig 4*c*) or K14 (data not shown). On the other hand, the expression of Cx43 was basically restricted to keratinocytes that expressed K5/6 (Fig 4*d*) and K14 (data not shown), with the exception that on rare occasions Cx43 was localized on the upper surface of keratinocytes in the upper part of the squamous layer that were negative for K5/6, but positive for K10. These data revealed that Cx26 is expressed in keratinocytes in the granular layer and in the upper part of the squamous layer, whereas Cx43 is localized in keratinocytes in the basal layer and in the lower part of the squamous layer.

To assess whether individual keratinocytes express both Cx26 and Cx43, and if this is the case, whether both connexins colocalize at the same gap junction plaques, double staining of Cx26 and Cx43 was performed using rabbit anti-Cx26 antibody and a mouse monoclonal antibody against Cx43. About 50% of keratinocytes in the upper part of the squamous layer expressed both Cx26 and Cx43 (Fig 6), but in most cases the localization of connexins was different. Cx26 was localized on the upper surface, whereas Cx43 was present on the lower surface. However, on rare occasions, it seemed that Cx26 and Cx43 were colocalized in the same gap junction plaques at a light microscopic level.

Northern Blot Analysis Total RNA from newborn mouse skin was examined for Cx26, Cx32, and Cx43 using specific DIG-labeled RNA probes. Under stringent conditions, clear bands of 2.5 kilobase pair (kb) with the Cx26 probe and 3.1 kb with the Cx43 probe were detected in mouse skin (Fig 7). These are in accordance with the reported transcript sizes for Cx26 and Cx43 mRNAs [24,26]. No bands were observed in mouse skin with the Cx32 probe even after longer exposure.

Western Blot Analysis Alkali-treated tissue homogenates of newborn mouse skin were examined by Western blot analysis using the same anti-Cx26 and anti-Cx43 antibodies utilized for immunofluorescence. As positive controls, we used mouse liver and rat cardiac myocytes for Cx26 and Cx43, respectively. The anti-Cx26 antibody showed an ~ 24-kilodalton (kD) band in mouse skin and mouse liver, whereas the anti-Cx43 antibody reacted with an ~ 43-kD band in mouse skin and rat cardiac myocytes (Fig 8). None of these bands were found in the absence of the primary antibodies under otherwise similar conditions (data not shown).

DISCUSSION

The present immunofluorescence study has clearly shown that, in newborn mouse epidermis, keratinocytes express Cx26 and Cx43 and that different connexins are expressed during differentiation of keratinocytes. Cx26 was demonstrated only in keratinocytes in the granular layer and in the upper part of the squamous layer. On the other hand, the expression of Cx43 was limited to keratinocytes in the basal layer and in the lower part of the squamous layer. Although some keratinocytes in the squamous layer expressed both Cx26 and Cx43, Cx26 was localized on the upper surface, whereas Cx43 was present on the lower surface of the plasma membrane of those keratinocytes. The Northern and Western blot analyses con-

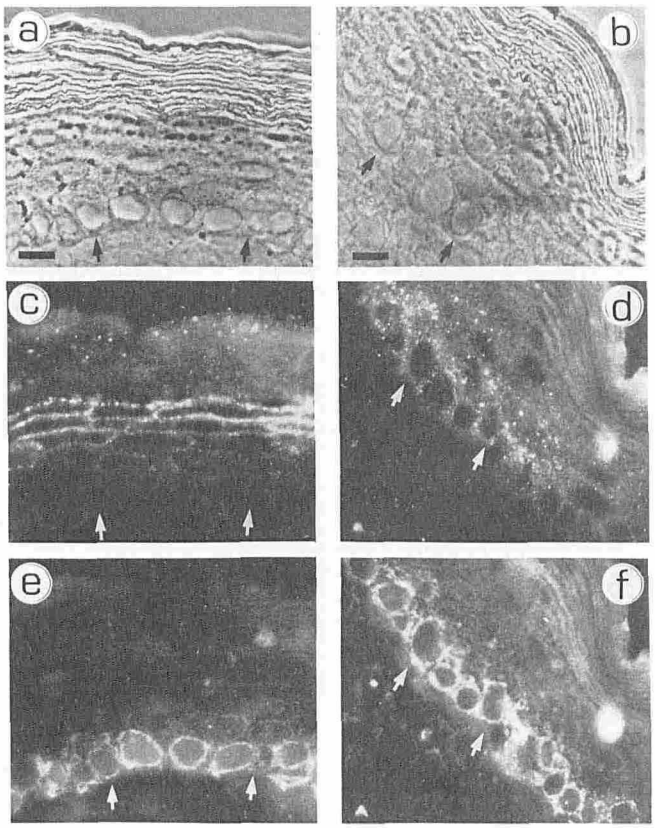

Figure 4. Double immunofluorescent staining of Cx26 or Cx43, and keratins K5/6 in newborn mouse skin. *a,b)* Phase-contrast images. *Bars,* 10 μm. *c)* Same section as *a* stained with anti-Cx26 antibody. *d)* Same section as *b* stained with anti-Cx43 antibody. *e)* Same section as *a* stained with anti-K5/6 antibody. *f)* Same section as *b* stained with anti-K5/6 antibody. *Arrows,* borders between epidermis and dermis.

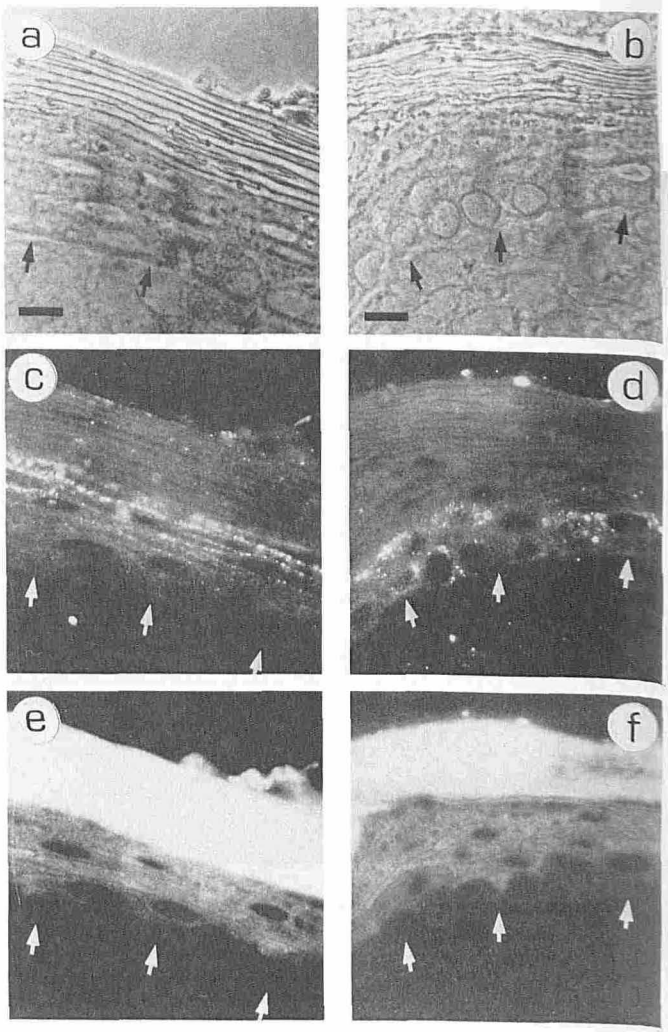

Figure 5. Double immunofluorescent staining of Cx26 or Cx43, and keratin K10 in newborn mouse skin. *a,b)* Phase-contrast images. *Bars,* 10 μm. *c)* Same section as *a* stained with anti-Cx26 antibody. *d)* Same section as *b* stained with anti-Cx43 antibody. *e)* Same section as *a* stained with anti-K10 antibody. *f)* Same section as *b* stained with anti-K10 antibody. *Arrows,* borders between epidermis and dermis.

firmed that Cx26 and Cx43, but not Cx32, were expressed at mRNA and protein levels in newborn mouse skin that included not only epidermis but also dermis.

With regard to the presence of both Cx26 and Cx43 in keratinocytes, the data we obtained in the present study are consistent with reports that mouse primary keratinocytes in culture express both Cx26 and Cx43 [16], and that Cx43 and Cx26 are expressed in keratinocytes of the rat epidermis [31,34]. In contrast, our data are inconsistent with those of Guo *et al* [18] and Meda *et al,** who reported that Cx43 is localized in regions of cell/cell contact of suprabasal cells, but not in basal or lower spinous keratinocytes in normal adult human skin and neonatal foreskin. They could find no Cx26 in any cell types in the skin. The most probable explanation for the discrepancy is species difference, not age difference, because newborn mouse skin differs markedly from adult human skin from both the developmental and the histologic viewpoints, and we found Cx26 in keratinocytes in adult mouse epidermis (data not shown).

Nishi *et al* [35] demonstrated that gap junctional gene expression can be modulated during the differentiation of a single cell type. They found that during differentiation of F9, teratocarcinoma cell line Cx32 was detected only in the visceral endoderm population, although all the different cell types expressed Cx43 mRNA at very high levels and expressed Cx26 mRNA at different levels. Stutenkemper *et al* [36] also found that the Cx phenotype is sensitive to the differentiated state of the cell, that is, the hepatocyte-specific phenotype of murine liver cells correlates with high expression of Cx32 and Cx26 but with very low expression of Cx43. In the present study, we found that during terminal differentiation of keratinocytes in mouse epidermis, the Cx expression is modulated from Cx43 to Cx26 and that some keratinocytes express both connexins. Our results are in agreement with the findings obtained by Risek *et al* [31] for rat skin development. They observed differential expression of Cx43 and Cx26 in the newly formed epidermal layers, coinciding with the differentiation of the rat epidermis.

The mechanisms underlying normal keratinocyte differentiation involve the regulation of expression of a number of structural and non-structural genes [37]. Among them, keratins are the most prominent proteins of keratinocytes and the major differentiation product of skin. In the basal layer, keratinocytes synthesize keratins K5 and K14. In suprabasal cells, K5 and K14 gene expressions are repressed and synthesis of a new subset of differentiation-specific keratins, K1 and K10, is initiated. These become the quantitatively predominant proteins of differentiated keratinocytes. It has been

* Meda P, Masgrau E, Saurat J-H, Salomon D: Identification of connexin 43, a gap junction protein, of human epidermis (abstr). *J Invest Dermatol* 95:480, 1990

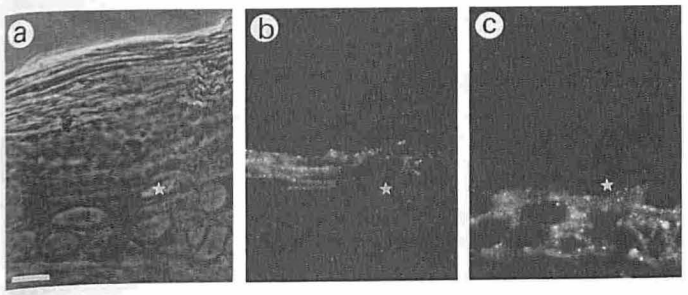

Figure 6. Double immunofluorescent staining of Cx26 and Cx43 in newborn mouse skin. *a)* Phase-contrast image. *Bar,* 10 μm. *b)* Same section stained with anti-Cx26 antibody. *c)* Same section stained with mouse monoclonal antibody against Cx43. The same cell is marked by *asterisks.*

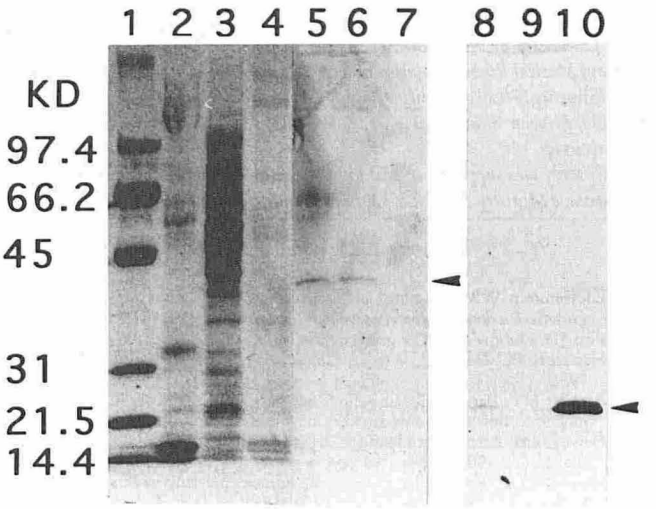

Figure 8. Western blot analyses for Cx43 and Cx26 in newborn mouse skin, rat cardiac myocytes, and adult mouse liver. Proteins extracted as described in *Materials and Methods* (about 20 μg/*lane*) were separated by SDS-PAGE and transferred to nitrocellulose. *Lanes 1–4* were stained with Ponceau S. *Lanes 5–7* were reacted with anti-Cx43 antibody. *Lanes 8–10* were reacted with anti-Cx26 antibody. *Lane 1,* molecular weight marker; *lanes 2, 5,* and *8,* newborn mouse skin; *lanes 3, 6,* and *9,* rat cardiac myocytes; *lanes 4, 7,* and *10,* adult mouse liver. The anti-Cx43 antibody shows an ~ 43-kD band *(arrowhead)* in mouse skin *(lane 5)* and rat cardiac myocytes *(lane 6).* The anti-Cx26 antibody reacts with an ~ 24-kD band *(arrowhead)* in mouse skin *(lane 8)* and adult mouse liver *(lane 10).*

shown that keratin expression is largely regulated at the transcriptional level by hormones, vitamins, and calcium [37,38] and that keratinocyte-specific expression is controlled complexly through interactions among various hormones, vitamins, their nuclear receptors, and upstream regulatory regions of keratin genes [37,39–41].

The turn-on of Cx43 in the basal layer, its subsequent shut-off in the suprabasal layers, and the turn-on of Cx26 in the suprabasal layers are expected to be regulated at the level of transcription, as in the case of keratin gene expression, but little is known about the molecular mechanisms underlying tissue-specific and differentiation-dependent gene expression of connexins. Hennemann *et al* [42] very recently reported sequences of mouse Cx26 and Cx32 promoter regions. In spite of the fact that both genes show similar genomic organization, consensus-binding sites for transcription

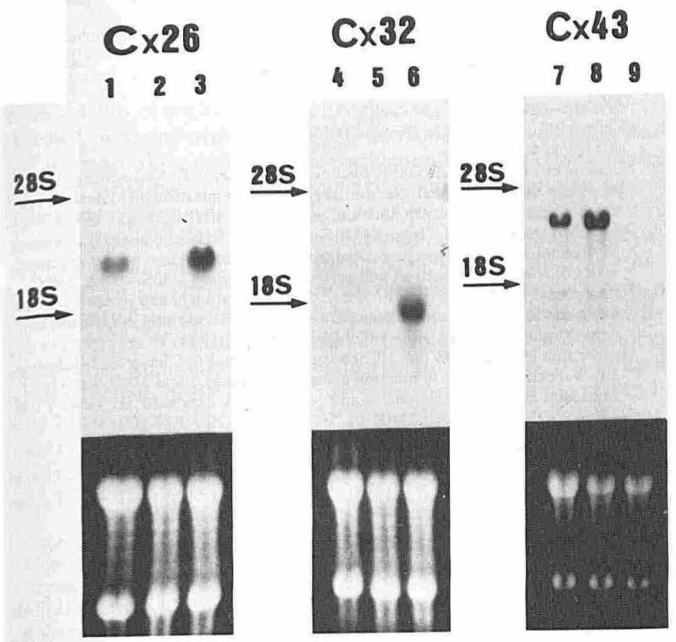

Figure 7. Northern blot analyses for Cx26, Cx32, and Cx43 in newborn mouse skin, adult mouse heart, and liver. Total RNAs (20 μg) were fractionated by electrophoresis in a 1% agarose-formaldehyde gel and hybridized with digoxigenin-labeled Cx26 *(lanes 1–3),* Cx32 *(lanes 4–6),* and Cx43 cRNA probes *(lanes 7–9). Lanes 1, 4,* and 7, newborn mouse skin; *lanes 2, 5,* and *8,* adult mouse heart; *lanes 3, 6,* and *9,* adult mouse liver. The lower half of the figure shows ethidium bromide staining of ribosomal RNAs before transfer to membranes.

factors in the putative promoter regions of both genes revealed few common denominators. The distinct features of promoter sequences of the two Cxs could explain the phenomenon of keratinocytes expressing Cx26, but not Cx32. However, any of the possible common regulatory elements involved in keratinocyte-specific gene expression have been identified in the mouse Cx26 promoter region [42]. To date, no reports have appeared concerning the promoter region of mouse Cx43 gene. Bertram and colleagues [18,43] reported that retinoids or retinoic acid increased Cx43 mRNA and Cx43 protein in intact human skin, cultured keratinocytes, and C3H 10T1/2 cells, at least at a certain concentration. Retinoic acid may directly regulate Cx43 expression by interaction with its receptor and the Cx43 gene, or indirectly by negative control of transcription of a number of differentiation-specific epidermal genes. Further studies on regulatory regions of the Cx43 gene and functional analysis of the putative regulatory elements reported earlier by gel-shift assays and by reporter gene constructs are necessary to understand the molecular mechanisms of differentiation-specific Cx gene expression in epidermis.

Recent molecular cloning studies have shown that new members of the connexin family, that is, two Cx31 transcripts of 1.9 and 2.3 kb [44], the 1.6-kb Cx31.1 mRNA, and two Cx30.3 transcripts of 1.9 and 3.2 kb [45], are expressed in mouse skin and keratinocyte-derived cell lines. When appropriate antibodies against these connexins become available, it would be of interest to examine whether expression of these Cx genes is related to terminal differentiation of keratinocytes.

In conclusion, we have immunohistochemically studied the expression of connexins in keratinocytes in newborn mouse epidermis. We have demonstrated that Cx26 was expressed in keratinocytes in the granular layer and in the upper part of the squamous layer, whereas Cx43 was localized in keratinocytes in the basal layer and in the lower part of the squamous layer. The modulation of connexin expression from Cx43 to Cx26 appears to occur during differentiation of keratinocytes in mouse epidermis.

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REFERENCES

1. Loewenstein WR: Junctional intercellular communication and the control of growth. *Biochim Biophys Acta* 560:1–65, 1979
2. Pitts JD, Finbow ME: The gap junction. *J Cell Sci* 4 (suppl):239–266, 1986
3. Hertzberg EL, Johnson RG (eds.): *Gap Junctions.* Alan R. Liss, Inc., New York, 1988
4. Bennett MV, Barrio LC, Bargiello TA, Spray DC, Hertzberg E, Sáez JC: Gap junctions: new tools, new answers, new questions. *Neuron* 6:305–320, 1991
5. Dewey MM, Barr L: A study of the structure and distribution of the nexus. *J Cell Biol* 23:553–585, 1964
6. Hashimoto K: Intercellular spaces of the human epidermis as demonstrated with lanthanum. *J Invest Dermatol* 57:17–31, 1971
7. Caputo R, Peluchetti D: The junctions of normal human epidermis. A freeze-fracture study. *J Ultrastruct Res* 61:44–61, 1977
8. Van Heukelom JS, Slaaf DW, Van Der Leun JC: Cell communication in the basal cells of the human epidermis. *Biophys J* 12:1266–1284, 1972
9. Cavoto FV, Flaxman BA: Communication between normal human epidermal cells in vitro. *J Invest Dermatol* 59:370–374, 1972
10. Salomon D, Chanson M, Vischer S, Masgrau E, Vozzi C, Saurat J-H, Spray DC, Meda P: Gap junctional communication of primary human keratinocytes: characterization by dual voltage clamp and dye transfer. *Exp Cell Res* 201:452–461, 1992
11. Kam E, Melville L, Pitts JD: Patterns of junctional communication in skin. *J Invest Dermatol* 87:748–753, 1986
12. Salomon D, Saurat JH, Meda P: Cell-to-cell communication within intact human skin. *J Clin Invest* 82:248–254, 1988
13. Allen TD, Potten CS: Fine-structural identification and organization of the epidermal proliferative unit. *J Cell Sci* 15:291–319, 1974
14. Beyer EC, Paul DL, Goodenough DA: Connexin family of gap junction proteins. *J Membr Biol* 116:187–194, 1990
15. Willecke K, Hennemann H, Dahl E, Jungbluth S, Heynkes R: The diversity of connexin genes encoding gap junctional proteins. *Eur J Cell Biol* 56:1–7, 1991
16. Brissette JL, Kumar NM, Gilula NB, Dotto GP: The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. *Mol Cell Biol* 11:5364–5371, 1991
17. Jongen WM, Fitzgerald DJ, Asamoto M, Piccoli C, Slaga TJ, Gros D, Takeichi M, Yamasaki H: Regulation of connexin 43-mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. *J Cell Biol* 114:545–555, 1991
18. Guo H, Acevedo P, Parsa FD, Bertram JS: Gap-junctional protein connexin 43 is expressed in dermis and epidermis of human skin: differential modulation by retinoids. *J Invest Dermatol* 99:460–467, 1992
19. Wilgenbus KK, Kirkpatrick CJ, Knuechel R, Willecke K, Traub O: Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues. *Int J Cancer* 51:522–529, 1992
20. Kuraoka A, Iida H, Hatae T, Shibata Y, Itoh M, Kurita T: Localization of gap junction proteins, connexin 32 and 26, in rat and guinea pig liver as revealed by quick-freeze, deep-etch immunoelectron microscopy. *J Histochem Cytochem* 41:971–980, 1993
21. Milks LC, Kumar NM, Houghten R, Unwin N, Gilula NB: Topology of the 32-kd liver gap junction protein determined by site-directed antibody localization. *EMBO J* 7:2967–2975, 1988
22. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
23. Höltke HJ, Sagner G, Kessler C, Schmitz G: Sensitive chemiluminescent detection of digoxigenin-labeled nucleic acids: a fast and simple protocol and its applications. *BioTechniques* 12:104–113, 1992
24. Zhang JT, Nicholson BJ: Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. *J Cell Biol* 109:3391–3401, 1989
25. Paul DL: Molecular cloning of cDNA for rat liver gap junction protein. *J Cell Biol* 103:123–134, 1986
26. Beyer EC, Paul DL, Goodenough DA: Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J Cell Biol* 105:2621–2629, 1987
27. Hertzberg EL, Skibbens RV: A protein homologous to the 27,000 dalton liver gap junction protein is present in a wide variety of species and tissues. *Cell* 39:61–69, 1984
28. Berthoud VM, Ledbetter MLS, Hertzberg EL, Sáez JC: Connexin43 in MDCK cells: regulation by a tumor-promoting phorbol ester and Ca^{2+}. *Eur J Cell Biol* 57:40–50, 1992
29. Naus CCG, Hearn S, Zhu D, Nicholson BJ, Shivers RR: Ultrastructural analysis of gap junctions in C6 glioma cells transfected with connexin43 cDNA. *Exp Cell Res* 206:72–84, 1993
30. Puranam KL, Laird DW, Revel J-P: Trapping an intermediate form of connexin43 in the Golgi. *Exp Cell Res* 206:85–92, 1993
31. Risek B, Klier FG, Gilula NB: Multiple gap junction genes are utilized during rat skin and hair development. *Development* 116:639–651, 1992
32. Fuchs E, Green H: Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell* 19:1033–1042, 1980
33. Roop DR, Cheng CK, Toftgard R, Stanley JR, Steinert PM, Yuspa SH: The use of cDNA clones and monospecific antibodies as probes to monitor keratin gene expression. *Ann NY Acad Sci* 455:426–435, 1985
34. Risek B, Gilula NB: Spatiotemporal expression of three gap junction gene products involved in fetomaternal communication during rat pregnancy. *Development* 113:165–181, 1991
35. Nishi M, Kumar NM, Gilula NB: Developmental regulation of gap junction gene expression during mouse embryonic development. *Dev Biol* 146:117–130, 1991
36. Stutenkemper R, Geisse S, Schwarz HJ, Look J, Traub O, Nicholson BJ, Willecke K: The hepatocyte-specific phenotype of murine liver cells correlates with high expression of connexin32 and connexin26 but very low expression of connexin43. *Exp Cell Res* 201:43–54, 1992
37. Fuchs E: Epidermal differentiation: the bare essentials. *J Cell Biol* 111:2807–2814, 1990
38. Roop DR, Krieg TM, Mehrel T, Cheng CK, Yuspa SH: Transcriptional control of high molecular weight keratin gene expression in multistage mouse skin carcinogenesis. *Cancer Res* 48:3245–3252, 1988
39. Leask A, Byrne C, Fuchs E: Transcription factor AP2 and its role in epidermal-specific gene expression. *Proc Natl Acad Sci USA* 88:7948–7952, 1991
40. Ohtsuki M, Tomic-Canic M, Freedberg IM, Blumenberg M: Nuclear proteins involved in transcription of the human K5 keratin gene. *J Invest Dermatol* 99:206–215, 1992
41. Oshima RG: Intermediate filament molecular biology. *Curr Opin Cell Biol* 4:110–116, 1992
42. Hennemann H, Kozjek G, Dahl E, Nicholson B, Willecke K: Molecular cloning of mouse connexins26 and -32: similar genomic organization but distinct promoter sequences of two gap junction genes. *Eur J Cell Biol* 58:81–89, 1992
43. Rogers M, Berestecky JM, Hossain MZ, Guo H, Kadle R, Nicholson BJ, Bertram JS: Retinoid-enhanced gap junctional communication is achieved by increased levels of connexin 43 mRNA and protein. *Mol Carcinog* 3:335–343, 1990
44. Hennemann H, Schwarz HJ, Willecke K: Characterization of gap junction genes expressed in F9 embryonic carcinoma cells: molecular cloning of mouse connexin31 and -45 cDNAs. *Eur J Cell Biol* 57:51–58, 1992
45. Hennemann H, Dahl E, White JB, Schwarz HJ, Lalley PA, Chang S, Nicholson BJ, Willecke K: Two gap junction genes, connexin 31.1 and 30.3, are closely linked on mouse chromosome 4 and preferentially expressed in skin. *J Biol Chem* 267:17225–17233, 1992