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Cutting Edge: Lack of Evidence for Connexin-43 Expression in Human Epidermal Langerhans Cells

Simone C. Zimmerli,* Florence Masson, *José Cancela, † Paolo Meda, † and Conrad Hauser**

A provocative study has shown that viral peptides may be transferred in vitro from epithelial cells to APC through connexin-43 gap junction channels. In support of this cross-presentation pathway, the study also reported that human dendritic cells, including Langerhans cells of skin, express connexin-43. In this report we show that if this was the case, the levels of connexin-43 are below those detectable by immunofluorescence, flow cytometry, quantitative PCR of purified CD1a+ cells, and electron microscopy, raising questions about the relevance of the connexin-43-dependent mechanism for Langerhans cells of noninflamed human skin. The Journal of Immunology, 2007, 179: 4318–4321.

Gap junctions (GJ) are assemblies of channels that permit the diffusion-driven exchange of molecules ≤1000 Da between coupled cells (1). Connexins (Cx) are the structural proteins of GJ (1, 2). Cx43 is broadly expressed in tissues and is the most prominent Cx of keratinocytes (2), which intercommunicate through GJ within a normal epidermis (3). Its presence has also been reported in immune cells, including T and B cells, neutrophils, mast cells, monocytes, and macrophages (4, 5). Cx43 may mediate the coupling of human monocyes (6, 7), monocyte-derived dendritic cells (DC) (8), and human bone marrow cell cultures in GM-CSF following in vitro exposure to TNF-α (8), and murine bone marrow cells cultured in GM-CSF of human monocytes (6, 7), monocyte-derived dendritic cells, and macrophages (4, 5). Cx43 may mediate the coupling of human monocyes (6, 7), monocyte-derived dendritic cells (DC) (8), and human bone marrow cell cultures in GM-CSF following in vitro exposure to TNF-α (8), and murine bone marrow cells cultured in GM-CSF of human monocytes (6, 7), monocyte-derived dendritic cells, and macrophages (4, 5).

CTL play a major role in the defense against viruses by killing infected cells after recognition of the viral peptides, which are derived in the cytoplasm of host cells and are associated with classical major MHC class I molecules. To become cytotoxic, CD8 T cells need to be activated by DC and/or macrophages (10, 11). A major issue is to understand how these cells acquire viral Ags if viruses primarily target the epithelial and not the immune cells. Recently, Neijssen et al. (7) have offered an innovative explanation by demonstrating in vitro that viral peptides pass from keratinocytes to activated monocytes via Cx43 GJ and subsequently activate CTL in the context of the MHC class I of recipient cells. Such a transfer represents a novel mechanism for Ag cross-presentation to CD8 T cells (12).

The same study reported that Cx43 is expressed in situ by intraepithelial DC, including human epidermal Langerhans cells (LC). Such an expression would allow the transfer of peptides from keratinocytes to LC, which may have major implications for the immune surveillance of skin, the role of LC being controversial in presentation of Ag to CD8 T cells (13–17). We have investigated this question in human and mouse skin. In this article, we report that immunolabeling, flow cytometry, and quantitative real-time RT-PCR do not support a native expression of Cx43 in LC, at least in control skin.

**Materials and Methods**

**Skin samples**

Control human and mouse skin was obtained at surgery and from C57B/6 mice, respectively, with the approval of the local ethical committees.

**Immunofluorescence**

Sections of intact skin and epidermal sheets (see below) were fixed in acetone/methanol (1:2) and labeled with a FITC-labeled mAb to CD1a (Sanquin) and with either one of two rabbit polyclonal Ab to human and murine Cx43 (Ab1 from Zymed Laboratories and Ab2 form Sigma-Aldrich), followed by a PE-labeled anti-rabbit secondary Ab (Molecular Probes). A blocking peptide for Ab1 was purchased from Zymed Laboratories. A blocking peptide for Ab2 was synthesized at Centre Medical Universitaire, Geneva, Switzerland. Images were acquired with a LSM 510 confocal laser microscope (Zeiss).

**Cell sorting**

The epidermis was separated from the dermis by dispase II (1.2 U/ml; Roche) or NH4SCN (0.5 M; Fluka). Epidermal cells were dispersed by incubating the separated epidermis for 30 min at 37°C in 0.5% trypsin (Sigma-Aldrich). Cells were washed, fixed, permeabilized with Cytofix/Cytoperm and Perm/Wash (BD Biosciences), immunolabeled, analyzed with a FACSCalibur flow cytometer (BD Biosciences), and data were processed with CellQuest software (BD Biosciences). For CD1a purification, cells were labeled with a CD1a FITC Ab and enriched through two successive sorting runs using a FACSVantage cell sorter (BD Biosciences).

**Western bloting**

Cells were lysed for 30 min in an ice-cold buffer containing 0.15 M NaCl, 0.05 M Tris (pH 6.8), 1 M EDTA, protease inhibitors (Roche) and 1% Triton X-100, followed by a 30 min centrifugation at 4°C at 13,000 × g (Eppendorf).

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3 Abbreviations used in this paper: GJ, gap junction; Cx, connexin; DC, dendritic cell; LC, Langerhans cell.

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Protein concentration of the supernatant was determined with a Bradford assay (Bio-Rad). Samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked in 0.1% Tween PBS (TBS) containing 5% milk for 2 h at room temperature on a rocking platform and incubated overnight at 4°C with either Ab1 or Ab2, both diluted to 2.5 μg/ml on a rocking platform. Goat anti-rabbit HRP served as secondary Ab. For the loading control, membranes were stripped, blocked with TBS containing 5% BSA for 2 h at room temperature, and incubated overnight at 4°C with β-tubulin Ab (Sigma-Aldrich) followed by anti-mouse IgG HRP Ab. Chemiluminescence detection used ECL detection reagents (Amersham Pharmacia). The membrane was exposed to a film and developed using a Kodak XP 2000 film developer device.

**Real-time PCR**

Total RNA was extracted from 10^5 to 10^6 FACS-enriched fractions of CD1a^+ and CD1a^- cells by using the RNAsodium-Micro kit (Ambion), treated with DNA-free (Ambion), and used to synthesize cDNA, which was synthesized using random hexamers and SuperScript II reverse transcriptase (Invitrogen Life Technologies) as per the manufacturer’s indications. Desmoplakin-1, Langerin (CD207), and Cx43 transcripts were amplified using the TaqMan gene expression assays Hs00189422-m1, Hs00210451-m1 and Hs00748445-s1, respectively (Applied Biosystems). Transcripts of the control genes EF1A1, GusB, and TBP were amplified using primers designed with Primer Express version 2.0 software (Applied Biosystems); PCR amplification, using 2X TaqMan Master Mix (Applied Biosystems) and either 1X diluted Applied Biosystems gene expression assays or 300 nM forward and reverse home-designed primers and 250 nM TaqMan probe (Eurogentec), was performed on an ABI PRISM® 7900HT sequence detection system (Applied Biosystems). Raw threshold cycle (Ct) values were obtained with SDS 2.2 (Applied Biosystems) and fold changes were calculated using the geNorm method (18).

**Immunoelectron microscopy**

Ultrathin cryosections of normal human skin were immunolabeled using either Ab1 or Ab2, and 15 nm protein A-coated gold particles as previously described (19).

**Results and Discussion**

**Cx43 is immunostained in the membranes of keratinocytes but not LC**

Skin sections and epidermal sheets were incubated with two polyclonal Abs against different C-terminal epitopes of Cx43, whereas LC were identified with a FITC-labeled mAb to CD1a, an LC marker in a steady-state epidermis (20–23). Ab1 revealed an abundant spotted Cx43 labeling on keratinocyte membranes, principally in the suprabasal layers that covered LC in skin (Fig. 1a) and epidermal sheets (Fig. 1c). No obvious co-localization of Cx43 and CD1a was seen (Fig. 1a). Preincubation of Ab1 with saturating amounts of the relevant blocking peptide abolished the Cx43 staining of keratinocytes but not the CD1a staining of LC cells (Fig. 1b). Analogous results were obtained with Ab2, the Ab used in the work by Neijssen et al. (7), with the exception that the labeling of keratinocyte membranes was less intense and that deposits were observed within LC in both epidermal sheets (Fig. 1c) and skin sections (not shown). These deposits were not abolished by preincubating Ab2 with its specific immunogenic peptide (Fig. 1d), suggesting nonspecific binding to LC.

**Cx43 is detected in isolated keratinocytes but not LC**

Because keratinocytes contact LC within the intact epidermis, Cx43 labeling cannot be unambiguously ascribed to one or the other cell type even under confocal microscopy. Thus, we immunostained cells dispersed from epidermal sheets and analyzed them using CD1a. Ab1 labeled a subpopulation of CD1a^- keratinocytes but not CD1a^+ LC (Fig. 2a), whereas Ab2 stained subpopulations of both keratinocytes and LC (Fig. 2d).

**FIGURE 1.** Immunofluorescence detects Cx43 in human keratinocytes but not LC. Sections of human skin (a and b) and epidermal sheets (c and d) were labeled for CD1a (green) and Cx43 (red) by using either Ab1 (a and b) or Ab2 (c and d) preabsorbed (b and d) at or near (a and c) the relevant immunogenic peptide. Using Ab1, Cx43 was evident in the membranes of keratinocytes but not those of CD1a^- LC cells. The keratinocyte labeling was abolished by preincubating Ab1 with its relevant immunogenic peptide (b). Using Ab2, the labeling of Cx43 was reduced at the keratinocyte membranes and deposits were observed in CD1a^- LC (c). These deposits were unchanged after the preabsorption of Ab2 with its relevant immunogenic peptide (d). Bars are 8.5 μm in a, 15 μm in b and c, and 8 μm in d. — peptide, Ab; + peptide, Ab preabsorbed with the relevant blocking peptide.

**FIGURE 2.** Flow cytometry does not identify Cx43 in CD1a^- cells. Dispersed human epidermal cells were fixed and permeabilized before labeling for CD1a (x-axes) and Cx43 (y-axes). The latter protein was revealed using either polyclonal Ab1 (a and b) or Ab2 (c and d), with (b and d) and without (a and c) preabsorption with the relevant blocking peptides. Ab1 labeled only CD1a^- keratinocytes (a), and this label was abolished by preincubation with the cognate immunogenic peptide (b). Ab2 labeled a larger number of keratinocytes as well as some CD1a^- LC (c). However, neither labeling was prevented by preincubation of Ab2 with its relevant peptide (d). — peptide, Ab; + peptide, Ab preabsorbed with the relevant blocking peptide.
Addition of the relevant blocking peptides inhibited the staining by Ab1 but not by Ab2 (Fig. 2, b and d). To check whether the reactivity of Ab1 and Ab2 was similarly blocked by the cognate immunogenic peptide, we ran Western blots of epidermal cell proteins. Ab1 and Ab2 revealed a prominent band at ~43 kDa that, in both cases, was blocked by the relevant peptide (Fig. 3). Thus, the flow cytometry data are in full agreement with the immunofluorescence observations and indicate the presence of Cx43 in keratinocytes but not in LC, as well as some nonspecific labeling by Ab2 under the conditions we used.

To assess whether LC may express minute levels of Cx43 that could have escaped detection by immunolabeling, we purified CD1a+ and CD1a- cells from a normal human epidermis. After two successive sorting runs, the purity of the LC fraction was 98.1 ± 0.62% as evaluated by the number of FITC+/FITC- cells, which resulted in a 67.7 ± 10.2-fold (n = 3) enrichment of Langerin mRNA, as evaluated by real-time PCR levels in CD1a+ cells/whole skin (Fig. 4). This method showed that the levels of Cx43 mRNA were high and similar in whole human skin, dispersed epidermal cells (not sorted), and keratinocytes of the CD1a- fraction but much lower in the CD1a+ fraction of LC (Fig. 4). The levels of Cx43 in the latter fraction were significantly (p < 0.01) smaller than those of desmoplakin-1, which were evaluated simultaneously in the same samples (Fig. 4) and reflect the small but unavoidable contamination of the CD1a+ fraction by some CD1a- cells. Thus, the minimal levels of Cx43 mRNA detected in purified LC are fully accounted for by their contamination with a few Cx43-expressing keratinocytes.

Electron microscopy of intact skin reveals Cx43 only at keratinocyte GJ

To bypass this problem, we immunolabeled ultrathin sections of steady-state skin for Cx43 using either Ab1 or Ab2. Protein A-coupled gold particles located Cx43 at GJ plaques but not at the desmosomes of suprabasal keratinocytes, showing the specificity of the ultrastructural labeling with Ab2 (Fig. 5). Identical results were obtained with Ab1 (not shown). Adjacent LC, which were identified by the presence of Birbeck granules, were not labeled within the very same sections (Fig. 5). Thus, immunoelectron microscopy confirmed the data obtained by immunofluorescence and flow cytometry despite a much higher spatial resolution and the specific characteristics of the labeling.

Using four independent methods, we could not demonstrate detectable levels of Cx43 in the LC of normal human skin within preparations (sections of intact skin, epidermal sheets, suspensions of dispersed epidermal cells, and purified suspensions of CD1a+ cells) that contained keratinocytes expressing easily detectable levels of this connexin. These findings, which were also made in control mouse skin (not shown) are consistent with the lack of dye coupling of keratin-negative cells observed within the intact human epidermis (3). They contrast,
however, with the report of Neijssen et al. (7), who documented the presence of Cx43 by immunolabeling of human skin samples. Several explanations could account for this difference, notably the type of skin used, the lot of Ab2 tested, the degree of resolution achievable by immunofluorescence microscopy, and the sensitivity of the detection systems used. Our experiments have confirmed the previous reports of high levels of Cx43 in human keratinocytes (2, 3). In this respect, two points are surprising in the putative Cx43 labeling of skin shown in Ref. 7. First, no labeling is visible in MHC class II cells, presumably keratinocytes that abut the illustrated LC. Second, the latter cell was not labeled at the cell membrane as is consistently the case in Cx43-expressing cells (1–3). Although it is not possible to unequivocally rule out that LC cells may transitorily express levels of Cx43 lower than the threshold for detection by the many methods we have used here, the quantitative PCR analysis of highly purified LC clearly indicates that such levels, if any, are significantly lower than that those that can be accounted for by the unavoidable contamination of CD1a cells by Cx43-expressing keratinocytes. Under these conditions it is questionable whether Cx43 could still ensure the proposed cross-presentation, because peptide transfer and CD8 T cell presentation were observed in vitro between cells that express sufficient Cx43 to be detected by immunofluorescence (7). In summary, our data do not support the involvement of Cx43 in the cross-presentation of Ag by LC of normal human skin.

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Disclosures
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