Organization and formation of the tight junction system in human epidermis and cultured keratinocytes

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Received November 14, 2001
Received in revised version January 29, 2002
Accepted February 2, 2002

Tight junctions – epidermis – keratinocytes – claudins – occludin

Occludin and several proteins of the claudin family have been identified in simple epithelia and in endothelia as major and structure-determining transmembrane proteins clustered in the barrier-forming tight junctions (TJ), where they are associated with a variety of TJ plaque proteins, including protein ZO-1. To examine whether TJ also occur in the squamous stratified epithelium of the interfollicular human epidermis we have applied several microscopic and biochemical techniques. Using RT-PCR techniques, we have identified mRNAs encoding protein ZO-1, occludin and claudins 1, 4, 7, 8, 11, 12, and 17 in both tissues, skin and cultured keratinocytes, whereas claudins 5 and 10 have only been detected in skin tissue. By immunocytochemistry we have localized claudin-1, occludin and protein ZO-1 in distinct plasma membrane structures representing cell-cell attachment zones. While claudin-1 occurs in plasma membranes of all living cell layers, protein ZO-1 is concentrated in or even restricted to the uppermost layers, and occludin is often detected only in the stratum granulosum. Using electron microscopy, typical TJ structures (“kissing points”) as well as some other apparently related junctional structures have been detected in the stratum granulosum, interspersed between desmosomes. Modes and patterns of TJ formation have also been studied in experimental model systems, e.g., during wound healing and stratification as well as in keratinocyte cultures during Ca2+-induced stratification. We conclude that the epidermis contains in the stratum granulosum a continuous zonula occludens-equivalent structure with typical TJ morphology and molecular composition, characterized by colocalization of occludin, claudins and TJ plaque proteins. In addition, cell-cell contact structures and certain TJ proteins can also be detected in other epidermal cell layers in specific cell contacts. The pattern of formation and possible functions of epidermal TJ and related structures are discussed.

Abbreviations. DAPI 4,6-Diamidino-2-phenylindole-dihydrochloride. – KGM Keratinocyte growth medium. – RT-PCR Reverse transcription-polymerase chain reaction. – SE Skin equivalents. – SKDM Serum-free keratinocyte-defined medium. – TJ Tight junction(s). – ZO-1, ZO-2, ZO-3 Zonula occludens proteins 1, 2, 3.

Introduction

Tight junctions play a central role in close cell-cell adhesion in simple epithelia and endothelia, connecting neighbouring cells in a controlled manner. They establish and maintain tissue barriers for the transport of particles and molecules between different compartments of the body (“barrier function”; such as the blood-brain barrier and the blood-testis barrier). Moreover, they separate the molecular components of the apical and basolateral portions of the plasma membrane (“fence function”). TJ are composed of specific transmembrane (claudin 1–20, occludin, junctional adhesion molecule JAM) and plaque (e.g. proteins ZO 1–3, symplekin) proteins (for reviews see e.g. (Stevenson and Keon, 1998; Tsukita et al., 2001)). Occludin (Furuse et al., 1993) and the family of claudins (Furuse et al., 0171-9335/02/81/05-253 $15.00/0
1998; Morita et al., 1999; Simon et al., 1999) contain four membrane-spanning regions whereas protein JAM (Naik et al., 1995) spans the membrane only once. Their exact roles in cell-cell adhesion and other possible functions are still under investigation. Furuse et al. (1998, 2001) have shown that the experimentally induced expression of Claudins 1 and 2 in cultured cells devoid of TJ results in the formation of characteristic TJ strands as demonstrable by freeze fraction electron microscopy and that the introduction of Claudin 2 in high-transperpeptidase-resistance MDCK cells converts the TJ of these cells from the tight to a leaky type, hinting on roles of Claudins in the formation and characterization of TJ and their functions. Moreover, the binding of Clostridium perfringens toxin to Claudins 3 and 4 of intestinal epithelia interferes with the barrier function of the TJ, followed by severe diarrhea (Katahira et al., 1997).

The TJ plaque proteins seem to play important roles in recruiting specific cytoplasmic proteins to TJ and in the anchoring of actin filaments to TJ (e.g. (Fanning et al., 1998; Itoh et al., 1999; Witten et al., 2000)). Functions of certain TJ proteins in cell signaling (Yamamoto et al., 1997; Balda and Matter, 2000) and vesicular transport (Lapiere et al., 1999) have also been proposed, and proteins ZO-1 and symplekin have been localized to both, TJ and cell nuclei (Stevenson et al., 1986; Gottardi et al., 1996; Keon et al., 1996).

TJ found in different tissues seem to differ considerably, notably in their Claudin complexity, probably reflecting different functions of these proteins (Mitic et al., 2000; Tsukita and Furuse, 2000; Tsukita et al., 2001). This is also indicated by the results of genetic alterations in some Claudins (e.g., (Gow et al., 1999; Simon et al., 1999)).

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The existence of TJ in the epidermis has been discussed controversially for decades, and most authors have concluded that true TJ are absent (see, e.g. (Hashimoto, 1971; Elias and Friend, 1975, 1976; Elias et al., 1977; Flick et al., 1989; Fawcett, 1994; Elias, 1996)). More recently, Morita et al. (1998) have been able to identify TJ-associated proteins occludin, ZO-1 and ZO-2 in rodent skin but did not detect typical TJ structures.

In the present report we demonstrate the occurrence of various Claudins, in addition to occludin and ZO-1, in human epidermis and cultured keratinocytes as well as typical TJ structures (“kissing points”) in the stratum granulosum, and compositionally related structures in various layers of the epidermis. Moreover, we show the pattern of TJ-protein expression and localization in cultured cells and in vitro model systems for keratinocyte stratification.

Materials and methods

Tissues and cell cultures

Skin samples of human trunk skin were obtained during the routine removal of epidermal cysts and tumors; the samples used were localized at least 2 cm from any lesion. The fetal skin samples were obtained from iatrogenic abortions performed for various medical and nonmedical reasons. The tissue samples were frozen at −120 °C in isopentane cooled with liquid nitrogen and stored at −80 °C.

The cultivation of primary keratinocytes and HaCaT cells was performed as described, using keratinocyte growth medium (CellSystems, St. Katharinen, Germany) and Dulbecco’s modified Eagle’s medium (DMEM) including 10% fetal calf serum (cf. (Moll et al., 1998; Boukamp et al., 1988; Demlehter et al., 1995)).

Skin equivalents (SE) are multilayers of keratinocytes (“epidermis”) grown in vitro on fibroblasts in a collagen matrix (“dermis”). The ongoing stratification can be investigated at different time points of cultivation. The in vitro reconstructed SE used in the present study were generated essentially as described by Stark et al. (1999). Briefly, 2.5 ml of a collagen solution containing 2.4 mg/ml bovine collagen type I (Vitrogen 100, Collagen Corporation, CA), 1× Hank’s buffered saline solution (Gibco BRL, Karlsruhe, Germany), 10% FCS and 1× 10^6 fibroblasts per ml, adjusted to pH 7.4 with 2 M NaOH, were poured into 24-mm cell-culture inserts ( Falcon, Becton Dickinsson, Schwechat, Austria) which were placed in special deep 6-well trays (Falcon). After gelation at 37 °C in a humidified atmosphere for 2 hours in the absence of CO₂, gels were equilibrated in 16 ml prewarmed keratinocyte growth medium (KGM; 2 ml inside, 14 ml outside the insert) for 2 hours at 37 °C in a 5% CO₂/95% air environment in a humidified incubator. The medium was then removed from the insert and replaced by 2 ml KGM containing 3× 10^5 keratinocytes per cm² (~ 1.3× 10^6 cells). The submerged culture was allowed to grow overnight, followed by a change to 10 ml serum-free keratinocyte-defined medium (SKDM) outside the insert, and the cultures were kept at the air-liquid interface from that time point onwards. SKDM is a high-Ca²⁺ medium (1.5 mM) consisting of KGM except for the omission of bovine pituitary extract and containing 10 µg/ml transferrin (Clonetics, CellSystems, St. Katharinen, Germany), 0.1% BSA (Sigma, St. Louis, MO, USA) and 50 µg/ml L-ascorbic acid. Culture medium for SE was replaced by fresh prewarmed SKDM every 2–3 days and culturing was continued for up to 6 days. At specific days of culturing samples were taken by punch biopsy, formalin-fixed and processed for either hematoxylin-eosin staining or immuno-fluorescence.

The wound healing model used is a skin organ culture model (“supravital human skin”) of a small biopsy of healthy human skin. Its central portion (diameter ca. 3 mm) was removed, thereby generating a keratinocyte-free area as a wound model. The human skin organ culture model used for wound healing studies has been described (Moll et al., 1998). Briefly, skin samples were trimmed immediately after excision to pieces with a diameter of ca. 6 mm and punch biopsies (diameter ca. 3 mm) including the epidermis and the upper dermis were removed from their centers. Each piece was placed dermis down on gauze in a culture dish ( Falcon, Becton Dickinson, Heidelberg, Germany; diameter 1 cm) filled with Dulbecco’s modified Eagle’s medium supplemented with hydrocortisone, 5% fetal calf serum, penicillin, and streptomycin in such a way that the medium was only in contact with the dermis, and the epidermis remained constantly exposed to the air. These “wound models” were incubated at 37 °C with 10% CO₂ for 7 h, 18 h, 24 h, 2 d, 3 d, 5 d, and 7 d, the medium being changed every other day. The samples were snap-frozen in isopentane pre-cooled in liquid nitrogen and stored at −80 °C until use.

Antibodies, nuclear dyes and primers

Antibodies specific for protein ZO-1 (PAD Z-R1), occludin (PAD Z-T22) and Claudin 1 (PAD MH25) were purchased from Zymed Laboratories (San Francisco, CA, USA). Antibodies specific for filaggrin (clone 576) were purchased from Quartett (Berlin, Germany).
For nuclear staining DAPI (Boehringer Mannheim, Mannheim, Germany) and Hoechst No. 33258 (Sigma-Aldrich, Taufkirchen, Germany) were used.

For PCR (see below) the following primers were used: Claudin 1 sense: 5'GCTCTAGAATTCCAGGGACTCATGCTGCAACG3'; claudin 1 antisense: 5'GCTCTAGAATTCTACACATTGATGCTTCCCGCT3'; claudin 2 sense: 5'GCTCTAGAATTCTGCTTGCGCTTGCGACACG3'; claudin 2 antisense: 5'GCTCTAGAATTCCTCCCTCCTCCGGGTATATGCT3'; claudin 4 sense: 5'GCTCTAGAATTCCTACACATCTTTCCGGTG3'; claudin 4 antisense: 5'GCTCTAGAATTCTTAGACATACTGACTTCTGG3'; claudin 5 sense: 5'GCTCTAGAATTCATGGGCTGTCGGGATGTCCAC; claudin 5 antisense: 5'GCTCTAGAATTCATGCCTGTATATAACCGTC3'; claudin 7 sense: 5'GCTCTAGAATTCGTATCCTACTCCGGCCCGCC; claudin 7 antisense: 5'GCTCTAGAATTCCAGGGTGGCCGGC3'; claudin 8 sense: 5'GCTCTAGAATTCGTATCCTACTCCGGCCCGCC; claudin 8 antisense: 5'GCTCTAGAATTCATGCCTGTATATAACCGTC3'; claudin 10 sense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 10 antisense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 11 sense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 11 antisense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 12 sense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 12 antisense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 13 sense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 13 antisense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 14 sense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 14 antisense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 15 sense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 15 antisense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 17 sense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 17 antisense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 20 sense: 5'GCTCTAGAATTCTACATCTGCTTGCT3'; claudin 20 antisense: 5'GCTCTAGAATTCTACATCTGCTTGCT3';

**Isolation of RNA, reverse transcription and polymerase chain reaction**

Total mRNA was isolated, and reverse transcription was performed using the Invisorb RNA Kit II (Invitec, Berlin, Germany) and sequenced by MWG AG (Ebersberg, Germany). The primers were used in the following reactions:

- 1.5 lM MgCl2, 5 lM dNTPs (final concentration 2mM), 0.5 Taq (5 U/l), reaction buffer (GIBCO BRL, Karlsruhe, Germany), and H2O was added to a final volume of 50 l.
- PCR was carried out in the following steps: Cycle 1: 1 min 95°C, 5 min 80°C, 30 sec 55°C; cycle 2: 1 min 95°C, 30 sec 55°C; cycle 3: 1 min 55°C, 30 sec 72°C; cycle 4: as cycles 2-3, but with an extension of 10 min at 72°C.

The resulting products were separated by electrophoresis on a 2% agarose gel, isolated from the gel using the ™ Gel Extraction Kit ∫ (Qiagen, Hilden, Germany) and sequenced by MWG AG (Ebersberg, Germany).

**Gel electrophoresis and immunoblotting**

Proteins were separated by sodium dodecyl sulfate (12–15%) polyacrylamide gel electrophoresis (SDS-PAGE) as described (Thomas and Kornberg, 1975) and electroblotted on nitrocellulose. Filters were blocked for 10 min with Tris-buffered saline with or without 0.1% Tween (TBST) and for 1 h with TBST containing 0.1% non-fat dry milk, and incubated with antibodies (dilution 1:200–1:1000 in TBST/5% non-fat dry milk) for 1 h. Bound antibodies were detected by enhanced chemiluminescence (ECL; Amersham, Braunschweig, Germany).

**Immunofluorescence microscopy**

Crystall sections (4–7 μm) of frozen tissues were fixed in –20°C acetone for 10 min. Cultured cells were fixed in cold methanol (–20°C, 5 min) and acetone (–20°C, 30 sec). Rabbit primary antibodies were diluted 1:200–1:1000. Incubations with antibodies were performed essentially as described (Brandner et al., 1998).

Confocal laser scanning immunofluorescence microscopy was done on a Zeiss LSM 510 (Carl Zeiss, Göttingen, Germany). For simultaneous double-label fluorescence, an Arion ion laser operating at 488 nm and a Helium neon laser operating at 543 nm were used together with a band-pass filter combination of 510–525 and 590–610 for visualization of Cy-2 and Cy-3 fluorescence.

**Electron microscopy**

For conventional electron microscopy, samples were fixed with glutaraldehyde, extensively washed with buffers, postfixed with osmium tetroxide, dehydrated and embedded in Epon. Immunoelectron microscopy on frozen tissue sections was performed essentially as described (Rose et al., 1995), using the colloidal gold-silver enhancement technique (e.g. (Scopisi et al., 1986)). For the evaluation of both a LEO-EM910 (Zeiss-Leo, Göttingen, Germany) was used.

**Results**

**Identification and localization of TJ proteins in human skin and keratinocytes**

Using RT-PCR techniques we were able to identify mRNAs encoding occludin, protein ZO-1 and claudins 1, 4, 5, 7, 8, 10, 11, and 17 in total human skin (Fig. 1a). In cultured primary keratinocytes, claudins 5 and 10 were absent (Fig. 1b). Claudin 2 was missing in both. All PCR products were confirmed by sequencing.

The existence of claudin 1 (23 kDa), occludin (65 kDa) and protein ZO-1 (220 kDa) in the epidermis was confirmed by immunoblotting experiments. They were identified in protein preparations of human epidermis, of cultured keratinocytes of
Identification of TJ- and TJ-related structures in human epidermis

Using electron microscopy, we were able to identify typical TJ structures (“kissing junctions”) in the uppermost layer of the living human epidermis (stratum granulosum), adjacent to the stratum corneum (Fig. 5a–c presents some examples of fetal skin). In addition, we noticed junctional regions in which the two plasma membranes were in close contact but leaving some sort of a “midline structure” (e.g., as denoted by the bars in Fig. 5). These TJ and probably TJ-related structures were also detected in adult human epidermis and in other squamous stratified epithelia (not shown; (Brandner et al., 2001a) for a detailed study see (Langbein et al., submitted)).

Immunofluorescence microscopy of cryostat sections grazing to the stratum granulosum of such skin tissue allowed the demonstration that the corresponding occludin-positive structures extended over long portions of the cell-cell boundaries (Fig. 6a) suggesting that they encircle these cells in a zonula occludens-like fashion. Using immunoelectron microscopy (Fig. 6b), we could further show that these TJ and compositionally related junctions occupied a large portion of the total interdesmosomal plasma membrane in this layer (arrows in Fig. 6b).

Synthesis and assembly of TJ proteins during the Ca\(^{2+}\)-induced stratification of cultured keratinocytes

At low Ca\(^{2+}\) concentrations, the TJ proteins occludin and claudin 1 were only detected locally and heterogeneously at the plasma membranes of highly confluent and differentiated keratinocytes (Fig. 7a, a’ and b, b’). Interestingly, in the same cultures protein ZO-1 showed a more widespread distribution (Fig. 7c, c’). When stratification of the cells was induced by switching to high-Ca\(^{2+}\) medium, the number of the keratinocyte-positive for claudin 1, occludin and protein ZO-1 at plasma membranes increased (Fig. 8). Groups of cells positive for claudin 1 and protein ZO-1 were now relatively frequent but occludin was still restricted to few highly confluent areas (Fig. 8b, b’).

TJ proteins during organization and regeneration of human epidermis

Using human “skin equivalents” we studied the synthesis of TJ proteins at various time points after the induction of stratification (Fig. 9). Interestingly, claudin 1 and – even more surprisingly – occludin were detected in stratifying skin equivalents already at day 2 (Fig. 9a, a’), when the stratum corneum and partly also the stratum granulosum were still absent, as demonstrated by histological staining and by the absence of filaggrin (Fig. 9a, a’). Claudin 1 and occludin were both localized at the outermost layers of the skin equivalent, but claudin 1 was in addition noticed in all other cell layers, with a somewhat weaker reaction in the stratum basale (Fig. 9a’).

With increasing stratification, characterized by the development of a continuous stratum granulosum and a stratum corneum (day 4 to day 8; Fig. 9b–d), claudin 1 distributed to its typical localization in normal epidermis, but showed a somewhat fainter staining in the stratum granulosum (e.g., arrows in Fig. 9b–d). Occludin was prominent in the stratum granulosum, as in normal epidermis, but a weak staining was sometimes also detected in the upper layers of the stratum spinosum.

Using an experimental wound healing model, we investigated the synthesis and distribution of TJ proteins at various time points after removal of the epidermis, including the epidermal barrier. We were able to identify the TJ transmembrane proteins, claudin-1 and occludin (Fig. 10a, b), as well as the TJ plaque protein ZO-1 (Fig. 10c) already early during wound healing, i.e., in the first cells of the ingrowing epithelial cell group, long before the reconstruction of the stratum corneum. By comparison, desmocollin 1, a differentiation-related desmosomal protein, which is also abundant in the stratum granulosum of healthy epidermis, was not yet detected in the regenerating epidermis (cf. (Moll et al., 1999)).

Discussion

The existence of TJ in squamous stratified epithelia of mammals, in particular in the epidermis, has been controver-
sially discussed for decades (e.g., (Hashimoto, 1971; Elias and Friend, 1975, 1976; Elias et al., 1977; Landmann, 1986; Fleck et al., 1989; Fawcett, 1994; Elias, 1996)). Most authors have concluded that typical TJ (zonulae occludentes) do not exist in mammalian epidermis although they have been clearly demonstrated in lower vertebrates, notably amphibia and reptiles (e.g., (Farquhar and Palade, 1965; Landmann et al., 1981; Fox, 1986; Landmann, 1986)). Recent advances in the elucidation of the molecular composition of TJ (for review see (Tsukita et al., 2001)) and in immunoelectron microscopy have now made it possible to solve this problem. Our finding in this study (see also (Brandner et al., 1999, 2000, 2001a, b)) of extended junctional structures interconnecting the cells of the stratum granulosum and, partly, to the transition cell layer, and protein ZO-1 shows an intermediate distribution with additional localization in the upper layer(s) of the stratum spinosum. Protein ZO-1 also shows a positive reaction of some blood vessels in the dermis. Note that the stratum corneum (sc) is always negative. Bar 50 μm.

Fig. 3. Immunofluorescence microscopy on vertical sections of frozen samples of adult human skin demonstrating the localization of claudin 1 (a, epifluorescence; a’, overlay of epifluorescence and corresponding phase contrast), occludin (b, b’), and protein ZO-1 (c, c’). The level of the basal lamina is indicated by the white line. While claudin-1 is seen in all viable epidermal layers, occludin is restricted to the stratum granulosum and, partly, to the transition cell layer, and protein ZO-1 shows an intermediate distribution with additional localization in the upper layer(s) of the stratum spinosum. Protein ZO-1 also shows a positive reaction of some blood vessels in the dermis. Note that the stratum corneum (sc) is always negative. Bar 50 μm.
the occludin immunolabelling of rodent skin by Morita et al. (1998). Of course, without direct functional data we cannot formally exclude the possibility that this TJ system is somewhat leaky and represents an incomplete TJ system (e.g., (Elias et al., 1977; Elias, 1996)).

Moreover, the zonula occludens-like continuity of the human epidermal TJ system is not confined to the interfollicular regions but integrates the epidermal glands and ducts as well as the Henle layer of hair follicles ((Langbein et al., submitted); for animal species see also (Orwin et al., 1973; Muto et al., 1981; Morita et al., 1998)). Thus we think that the entire surface of the human body is covered at the level of the granular/Henle layer by a continuous zonula occludens. Clearly, this demonstration of a TJ-containing cell layer will now lead to novel-designed physiological experiments examining its possible barrier function for the translocation of molecules across the epidermis.

The occurrence of certain TJ hallmark proteins beyond the TJ structures of the granular layer is especially remarkable. We have identified a number of other members of the claudin

Fig. 4. Laser-scanning confocal microscopy, showing single (a, a′) and double-label (a′′) immunofluorescence localization of occludin (a, a′, red) and protein ZO-1 (a′′, green) on near-horizontal cryostat sections of adult human skin. Over large areas, occludin colocalizes with protein ZO-1. There are, however, some structures mutually exclusive for occludin or protein ZO-1 (e.g., in the lower left area of a′′) that may reflect local differences of channel intensity. Arrows denote examples of cell circumferences completely positive for occludin. Bar 50 μm.

Fig. 5. Electron micrographs of ultrathin sections through fetal plantar epidermis (pregnancy week 21) showing the transition of the stratum corneum (sc) to the first living layer, stratum granulosum, which contains numerous intercellular contact sites (arrows) typical of tight junctions ("kissing points"); the horizontal bar in (c) denotes a close, but not tight contact site), interspersed with desmosomes. Asymmetrically appearing desmosomes connecting sc with the stratum granulosum are denoted by arrowheads (a, b). Bars 0.5 μm (a, b) and 0.2 μm (c).
family in the epidermis (see Results), and the immunolocalizations of claudins in general, notably claudin 1 clearly extend over most layers of the stratum spinosum and the stratum basale. Protein ZO-1 also occurs in upper spinous layers. This indicates the existence of junction structures other than typical occludin-containing TJ in plasma membranes of epidermal keratinocytes, and we have recently indeed identified a number of candidate structures, including the “close but not tight” type of junction shown in Figure 5c. Several examples of TJ-related junctions in diverse stratified epithelia will be presented elsewhere (Langbein et al., submitted).

The well studied TJ in simple epithelial and endothelial cells and in corresponding cell culture model systems such as MDCK cells have revealed a dependence of TJ formation on the Ca\(^{2+}\) concentration in the environment (for review see Denker and Nigam, 1998)). In agreement with this we have demonstrated in cultured human epidermal keratinocytes that the shift from low to high Ca\(^{2+}\) concentrations also results in an increased appearance of TJ proteins at the plasma membrane (see also Kitajima et al., 1983)). Here again, occludin has been noted as the most restricted TJ protein and its local co-distribution with claudin(s) and protein ZO-1 in certain keratinocyte colonies may well reflect TJ-related structures.

As TJ play an important role in the barrier function of the cells of simple epithelia and endothelia and the TJ proteins and structures in the epidermis are found in the differentiated stratum granulosum, we have also examined in experimental keratinocyte models the formation of the barrier function of the epidermis. In both processes, i.e. stratification of skin equivalents and epidermal regeneration during wound healing we have found that the synthesis of TJ proteins clearly precedes
that of other differentiation markers such as desmocollin 1 (Moll et al., 1999) or the cornified envelope marker filaggrin and the formation of any morphological stratum corneum.

Various clinical aspects argue for an – at least partial – breakdown of TJ-mediated barrier functions in the course of some diseases. For example, the binding of Clostridium perfringens toxins to claudins 3 and 4 at the TJ of intestinal epithelia induce severe diarrhea (Katahira et al., 1997), and the interaction of house dust mite allergens with occludin in the lung epithelium has been reported to be an important step in allergic sensitization (Wan et al., 1999). Moreover, Yoshida et al. (2001) have recently shown that in psoriasis the expression of occludin is no longer restricted to the stratum granulosum but extends to parts of the stratum spinosum. The molecular mechanisms involved in specific skin diseases and the possible part of TJ in the barrier function of the skin will now have to be examined on the basis of our findings of an extended TJ system in the upper layers of the epidermis.

**Fig. 9.** Localization of TJ proteins in developing “skin equivalents”. Immunofluorescence and hematoxylin-eosin stainings of skin equivalents were performed at day 2 (a–a′), day 4 (b–b′), day 6 (c–c′) and day 8 (d–d′) after the induction of stratification and differentiation. (a–d) Hematoxylin-eosin staining. (a′–d′) Triple immunofluorescence staining demonstrating the localization of claudin-1 (red), filagrin (green) and nuclear chromatin (Hoechst, blue). (a′–d′) Immunofluorescence staining for occludin showing a restriction to the upper layers of the developing epidermis. The dotted lines indicate the upper and lower borders of the epidermis. Arrows indicate claudin 1 staining in the stratum granulosum. Bar 50 µm.
Acknowledgements. We thank Ewa Wladykowski and Birgit Hüsing (Hamburg) for excellent technical assistance and Jutta Müller-Osterholt (Heidelberg) for competent photographic work. We also gratefully acknowledge Drs. Shoichiro Tsukita (Kyoto), Roland Moll (Marburg), Lutz Langbein (Heidelberg) and Nikolas K. Haass (Hamburg) for stimulating discussions. We are indebted to Dr. Herbert Spring (Heidelberg) for his expert cooperation in the laser scanning confocal microscopy as well as Guido Bruning and Dr. Andrea Diederich for clinical support and Dr. Peter von den Driesch (all Hamburg) for expert histopathological diagnosis. This work has been supported by the Deutsche Forschungsgemeinschaft (grant Mo 644/4–1 to Drs. J.M. Brandner, W.W. Franke and I. Moll).

Note added. After completion of this manuscript we learnt of a study by Pummi et al. (2001) on normal, diseased and fetal human skin, using antibodies to occludin and protein ZO-1, which is essentially in agreement with the present report.

Fig. 10. Immunofluorescence microscopy of TJ proteins on frozen sections of a human skin organ culture model during wound healing 18 h (left column), 24 h (middle column) and 5 d (right column) after “wounding” (total epidermis has been removed; for details see Materials and methods). Uppermost panel: schematic illustration of the wound-healing skin organ culture model (green, D, dermis; yellow, E, epidermis). The “magnifying glass” shows the location of the tissue region shown in the epifluorescence-phase contrast combination picture below. a. Localization of claudin 1 (red) and nuclei (DAPI, blue). b. Localization of occludin (red) and nuclei (blue). c. Localization of protein ZO-1 (red) and nuclei (blue). The arrows denote the first cells of the margin of the regenerating epithelial tissue. The TJ proteins occur in the first cells of the wound-tongue. Bar 50 μm.

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