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Short Communication

Human Keratinocytes Express Cellular Prion-Related Protein *in Vitro* and during Inflammatory Skin Diseases

Johannes Pammer,* Wolfgang Weninger,† and Erwin Tschachler†

From the Institute of Clinical Pathology and Division of Immunology, Allergy, and Infectious Diseases,† Department of Dermatology, University of Vienna Medical School, Vienna, Austria*

Prion diseases are transmissible spongiform encephalopathies of humans and animals characterized by the accumulation of a proteinase-resistant isoform of the cellular prion-related protein (PrP^c) within the central nervous system. In the present report we demonstrate for the first time the presence of PrP^c on squamous epithelia of normal and diseased human skin and show that inflammatory cytokines regulate PrP^c expression in cultured human keratinocytes (KCs). By immunohistochemistry, only little expression of PrP^c, which was mainly confined to KCs, was detected in normal skin. In contrast, in inflammatory skin diseases including psoriasis and contact dermatitis, PrP^c was strongly present on both KCs and infiltrating mononuclear cells. Strong PrP^c expression was also observed in squamous cell carcinomas and viral warts whereas basal cell carcinomas were mostly negative. In mucous membranes of the upper digestive tract and the genital region, distinct PrP^c expression by basal squamous epithelial cells was a constant feature. In tissue culture, primary KCs constitutively expressed PrP^c mRNA and protein. Exposure of these cells to transforming growth factor (TGF)-α or interferon (IFN)-γ led to an increase of PrP^c protein expression. The presence of PrP^c on epithelial cells of skin and mucous membranes suggests that these cells represent possible first targets for peripheral infection with prions. ***(Am J Pathol 1998, 153:1353–1358)***

Cellular prion-related protein (PrP^c) is a cell surface glycoprotein predominantly expressed within the central nervous system (CNS).[1,2] Accumulation of a modified proteinase-resistant isoform, ie, scrapie-associated PrP (PrP^{Sc}), in the CNS is the common pathogenic denominator for prion diseases, a group of neurodegenerative disorders of humans and animals.[1,2] The inoculation of prions is thought to trigger the continuous conversion of PrP^c to PrP^{Sc}.[1–3] In the laboratory setting, prion diseases are transmitted by direct inoculation of infected tissue.[3] In humans, infection has primarily been related to dura grafts and the parenteral administration of pituitary extracts.[2] In contrast, the routes of transmission in the field are still not completely elucidated, and several different modes, such as the ingestion of contaminated food and peripheral inoculation, have been suggested.[4,5]

Recently, the occurrence of a variant form of Creutzfeldt-Jakob disease (CJD) has raised the question of interspecies transmission of BSE from cows to humans. Whereas evidence suggests that BSE has spread to man[6], no clear relationship between risk factors and disease occurrence has been established.[7] Scrapie of sheep is one of the longest known prion-associated disorders, but the transmission routes have not been clarified for this disease either.[2,8] Successful transmission of prion diseases via inoculation into the skin has been recently reported in rodents.[5] Several cell types found in the skin, including fibroblasts[9] and certain hemopoietic cells,[10,11] have been found to express PrP^c; however, to the best of our knowledge, the skin compartment itself has otherwise not yet been studied for its possible involvement in prion diseases. Therefore, we set out to investigate the distribution of PrP^c in human skin and mucous membranes.

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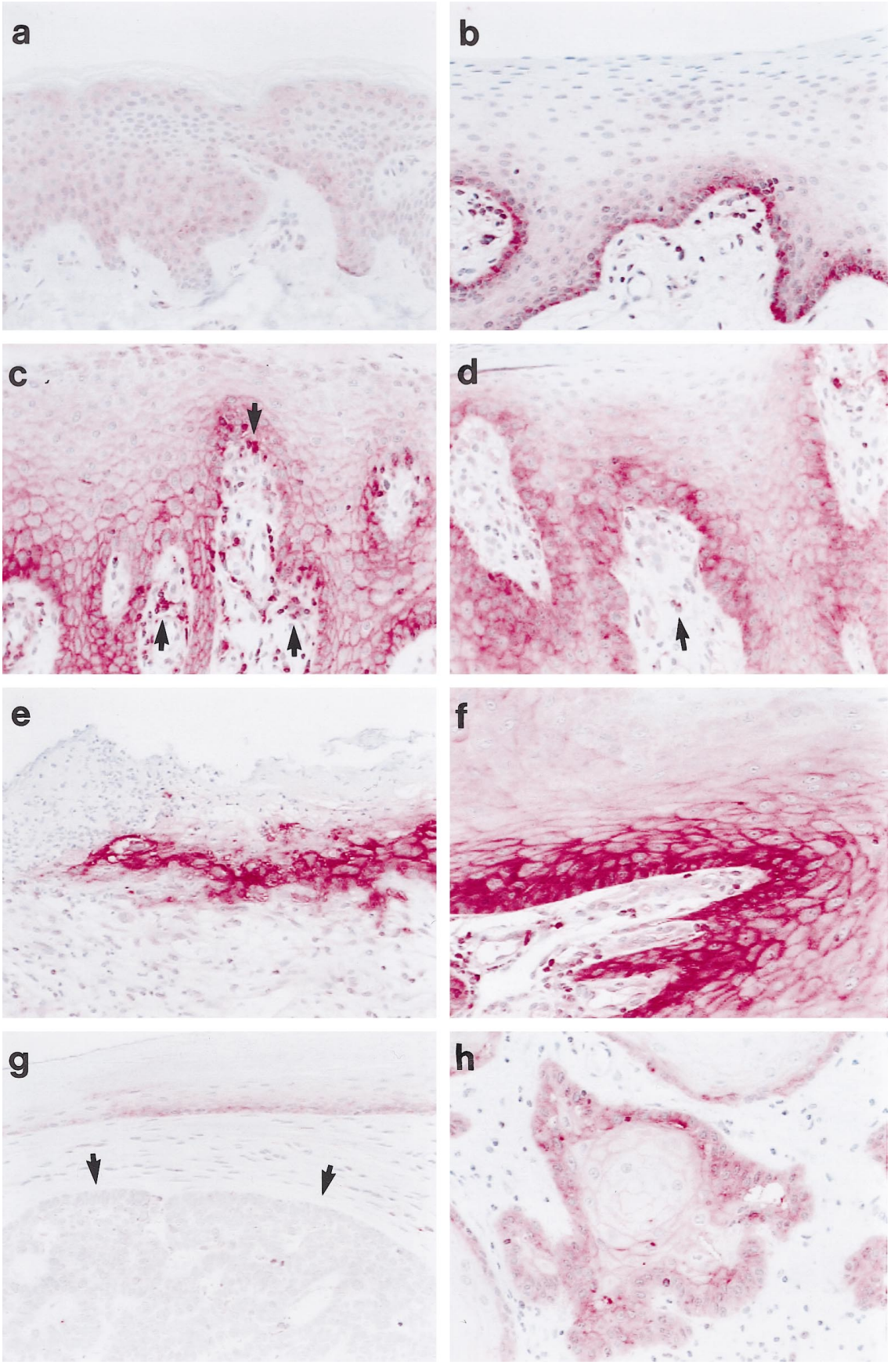
Address reprint requests to Dr. Erwin Tschachler, Division of Immunology, Allergy, and Infectious Diseases, Department of Dermatology, University of Vienna Medical School, Währinger Gürtel 18–20, A-1090 Vienna, Austria. E-mail: erwin.tschachler@akh-wien.ac.at.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Materials and Methods

Immunohistochemistry

Expression of PrP^c was analyzed on sections derived from paraffin-embedded specimens collected for routine histopathology. After microwave treatment, immunostaining was performed using either anti-PrP monoclonal antibody (MAb) 3F4 (1:400; Chemicon, Temecula, CA) or 6H4 (1:300; Prionics AG, University of Zurich, Zurich, Switzerland[12]) following a standard protocol.[13] Control stainings with irrelevant isotype-matched control MAbs, ie, IgG1 (Coulter, Hialeah, FL) for MAb 6H4 and IgG2a (Coulter) for MAb 3F4 in adequate concentrations were regularly included in our experimental procedures.

Western Blot Analysis

Cells were lysed in a buffer containing 1% Nonidet P-40 and 1% SDS buffer. SDS-polyacrylamide gel electrophoresis and Western transfer to nitrocellulose membranes were performed under standard conditions. For some experiments, gels were electrophoresed in duplicate under identical conditions; one was stained with Coomassie blue and the other was subjected to Western blot analysis to assure equal loading of protein. Membranes were reacted to MAb 3F4 or to an isotype-matched control MAb followed by horseradish-peroxidase-labeled sheep anti-mouse IgG (Amersham Life Science, Little Chalfont, UK). After rinsing in enhanced chemiluminescence reagent (Amersham), membranes were exposed to X-OMAT-AR film (Eastman Kodak, Rochester, NY). For deglycosylation experiments, PNGase F (New England BioLabs, Schwalbach, Germany) was used according to the manufacturer's instructions. For some experiments, protein glycosylation was inhibited with tunicamycin (5 μg/ml; Calbiochem, San Diego, CA) for 24 hours.

Cell Cultures

Human neonatal foreskin KCs (Clonetics, San Diego, CA) were cultured under low-Ca^{2+} conditions (0.15 mmol/L) in serum-free KC growth medium (KGM) consisting of KC basal medium (KBM) supplemented with human recombinant epidermal growth factor (0.1 ng/ml), bovine pituitary extract, insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), and gentamicin/amphotericin B (50 μg/ml/50 ng/ml; all Clonetics) at 37°C in 5% CO_2. Cells were routinely passaged at a confluence of 60% to 80%. All experiments were carried out between passages 2 and 5. For stimulation experiments, KCs were grown either in KBM alone or in KBM supplemented with interferon (IFN)-γ (1000 U/ml; Imukin, Bender-Med, Vienna, Austria), transforming growth factor (TGF)-α (100 ng/ml; Eubio, Vienna, Austria), interleukin (IL)-1β (4 pg/ml; Eubio), tumor necrosis factor (TNF)-α (50 U/ml; Biomol, Hamburg, Germany) or phorbol myristate acetate (100 nmol/L; Sigma Aldrich, Vienna, Austria). The epidermoid carcinoma cell line A431, the immortalized KC cell line HaCaT, and the melanoma cell line SkMEL28 were grown in RPMI-1640 supplemented with 10% fetal bovine serum and 1% L-glutamine (all GIBCO BRL, Gaithersburg, MD).

Northern Hybridization

For Northern blot analysis, total RNA was size fractionated in 1% agarose gels containing 1.48% formaldehyde and transferred to nylon membranes (Nytran, Schleicher and Schüll, Dassel, Germany) as described previously.[14] For hybridization, a mouse PrP^c cDNA probe (kindly provided by A. Aguzzi[15]) was used under high-stringency conditions.

Results

In normal skin, only little expression of PrP^c was detectable by immunohistochemistry (Figure 1a). Staining was confined mainly to epithelial cells (Figure 1a) and to sporadic mononuclear cells within the dermis. In squamous epithelium of mucous membranes of different sites, distinct constitutive PrP^c expression was detected in basal but not suprabasal KCs (Figure 1b). This pattern of expression was consistently observed in pharynx (n = 5, Figure 1b), larynx (n = 3), esophagus (n = 2), vulva (n = 3), and the ectocervix (n = 3). In contrast to low PrP^c expression in normal epidermis, this protein was strongly up-regulated in basal and suprabasal KCs up to the granular layer in eczema (n = 5, Figure 1c) and psoriasis (n = 5, Figure 1d) and adjacent to skin ulcerations (n = 4, Figure 1e). In addition, dermal mononuclear cells were also distinctly positive for PrP^c in these diseases (Figure 1, c and d, arrows). These infiltrating cells essentially comprise T lymphocytes and macrophages,[16] both of which are able to express PrP^c.[10,11,17] In epithelial tumors, PrP^c expression was most pronounced in common warts (n = 7, Figure 1f) and squamous cell carcinomas (n = 6, Figure 1h), whereas basal cell carcinomas (n = 4, Figure 1g) were mostly negative. In all experiments, the replacement of anti-PrP^c MAbs with the appropriate isotype-matched control MAbs resulted in no specific staining (not shown).

KCs in primary culture were positive for PrP^c by both Western blotting (Figure 2a) and FACS analysis (data not shown). PrP^c protein moieties ranged between 30 and 43 kd, corresponding to reports for other tissues.[11,18] In contrast to KCs in primary culture and to the melanoma

Figure 1. Human KCs *in situ* express PrP^c. Immunohistochemical staining was performed on paraffin sections after microwave treatment using the anti-PrP MAb 3F4 (1:400). PrP^c is weakly present in the cytoplasm of KCs of normal skin (a) but distinctly in basal cells of squamous epithelium of mucous membranes (pharynx; b). Besides a weak cytoplasmic staining, basal and suprabasal KCs up to the granular layer stain strongly for PrP^c in a chicken-wire pattern characteristic for membrane proteins in eczema (c) and psoriasis (d), adjacent to ulcerations (e), and in common warts (f). In addition, mononuclear cells within the dermis and epidermis (c and d, arrows) express PrP^c. Whereas basal cell carcinomas (g, arrows) showed weak to no PrP^c expression, squamous cell carcinomas (h) stained distinctly for PrP^c. Original magnification, ×200.

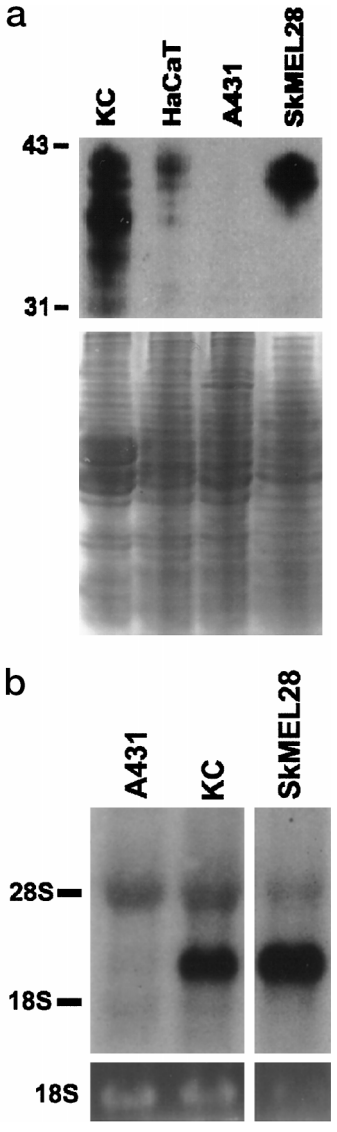

Figure 2. KCs in primary culture express PrP^c mRNA and proteins. a: Western blot analysis was performed with the 3F4 anti-PrP MAb. Lysates of KCs in primary culture (lane 1) were strongly positive for PrP^c whereas in lysates of HaCaT (lane 2) and A431 cells (lane 3) little or no reactivity was detectable. The SkMEL28 melanoma cell line (lane 4) also strongly expressed PrP^c. Equal protein loading was confirmed by Coomassie blue staining of a gel run in parallel under identical conditions (lower part of a). b: When total RNA was hybridized to a cDNA of mouse PrP^c, a specific hybridization signal was detected at approximately 2.3 kb in primary cultured KCs (lane 2) and SkMEL28 melanoma cells (lane 3) but not in A431 cells (lane 1). The lower panel depicts the ethidium bromide staining of 18 S RNA.

cell line SkMEL28, little to no expression was detected in HaCaT and A431 epithelial cell lines (Figure 2a). In keeping with the protein data were the data obtained by Northern hybridization with a PrP^c cDNA probe.[15] A specific band of approximately 2.3 kb was present in RNA from epithelial cells in primary culture and in SkMEL28 cells but not in A431 cells (Figure 2b). The expression of PrP^c by KC in tissue culture was up-regulated by IFN-γ and transforming TGF-α but not by IL-1β or TNF- α (Figure 3a). TGF-α also strongly induced the expression of PrP^c in HaCaT (Figure 3a) but not in A431 cells (data not shown). Interestingly, stimulation with TGF-α resulted in

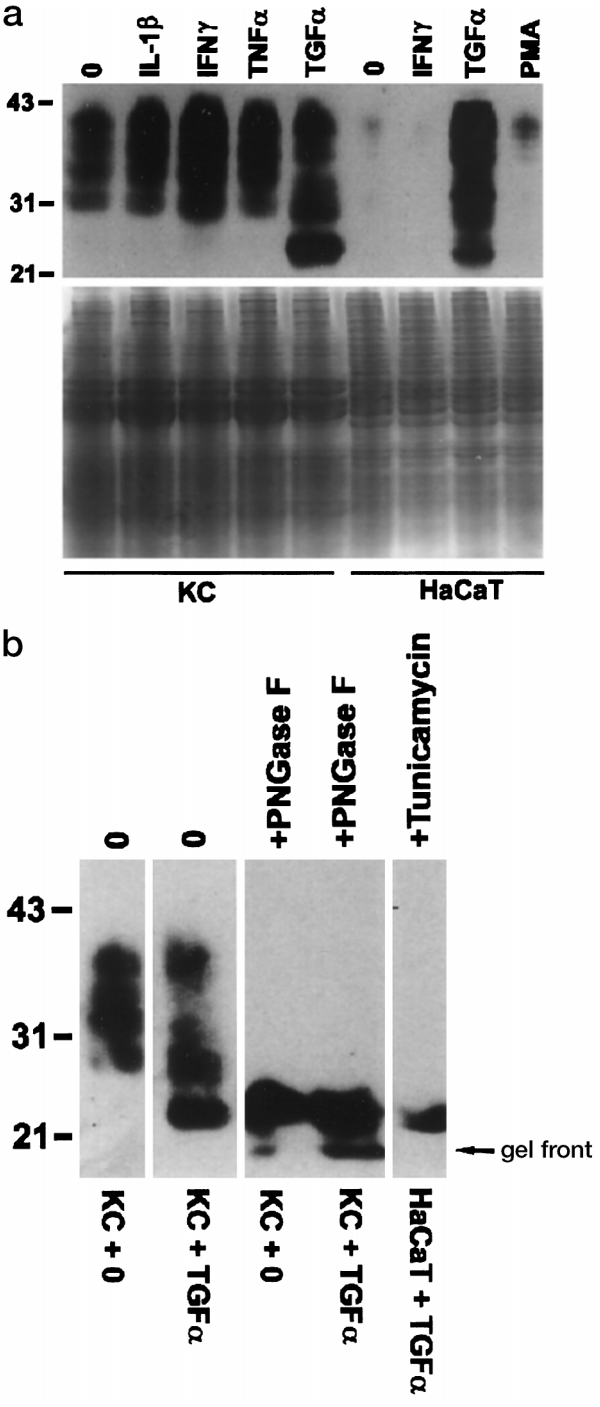

Figure 3. PrP^c expression in human KCs is up-regulated by TGF-α and IFN-γ. a: In KCs in primary culture (left panel), PrP^c was up-regulated after a 48-hour exposure to TGF-α (lane 5) and IFN-γ (lane 3) but not to IL-1β (lane 2) or TNF- α (lane 4). Lane 1 contains lysates of unstimulated KCs. In HaCaT cells (right panel), strong induction of PrP^c was found 24 hours after exposure to TGF-α (lane 3), whereas incubation with IFN-γ (lane 2) and phorbol myristate acetate (lane 4) had only little or no effect as compared with unstimulated cells (lane 1). Equal protein loading was confirmed by Coomassie blue staining of a gel run in parallel under identical conditions (lower part of a). b: Deglycosylation of lysates of unstimulated (lanes 1 and 3) and TGF-α-stimulated KCs (lanes 2 and 4) with PNGase F (lanes 3 and 4) resulted in a band of approximately 23 kd. PrP^c of the same size was detected after inhibition of protein glycosylation with tunicamycin in HaCaT cells (lane 5).

strong expression of an additional band of approximately 23 kd, corresponding to nonglycosylated PrP^c.[19] Deglycosylation experiments with either PNGase F or incubation of cells with tunicamycin confirmed that the molecular weight of the KC-derived PrP^c protein backbone was indeed approximately 23 kd (Figure 3b). Shorter exposure of the blot revealed that in lysates of tunicamycin-treated HaCaT cells this moiety consisted of two closely migrating bands (not shown).

Discussion

In the present report we demonstrate for the first time that KCs *in situ* and in tissue culture produce PrP^c mRNA and protein. KC- PrP^c is up-regulated in inflammatory skin diseases and can be induced in cultured KCs by TGF-α and IFN-γ. As TGF-α is strongly expressed in psoriatic epidermis[20] and IFN-γ is produced at inflammatory sites by activated T lymphocytes, these cytokines may also *in vivo* be involved in the regulation of PrP^c expression by KCs in an autocrine and paracrine manner.

In recent years, it has been found that PrP^c is expressed not only by neurons but also by several non-neuronal tissues.[9–11,21] Functionally, PrP^c in the CNS is involved in synaptic transmission in nerve cells,[22] in survival of Purkinje cells,[23] and in sleep regulation.[24] In both human and murine lymphocytes it plays a role in mitogen-induced lymphocyte activation.[10,17] Despite intensive research, the function of PrP^c in other tissues, including the epidermis, is unknown. The fact that PrP^c is a glycolipid-anchored cell surface glycoprotein[3] is suggestive for a role in signaling and/or adhesion. Recent data indeed indicate that at least two ligands, ie, a 37-kd laminin receptor precursor[25] and a 66-kd membrane protein,[26] exist that are able to bind to PrP^c. However, the *in vivo* significance and possible functional implications of these findings remain to be determined.

As to the involvement of PrP^c in the pathogenesis of prion diseases, it has been amply documented that its presence is the prerequisite for the development of transmissible spongiform encephalopathies.[1–3] Therefore, the clinically most relevant question is whether or not epithelia-associated PrP^c plays a role in the acquisition of prion diseases. It is conceivable or even likely that if prions penetrate into the epidermis they would be able to start the conversion of KC-derived PrP^c to PrP^{Sc}. Penetration is certainly possible via the broken skin barrier in eczema and ulcerations. As in these disorders KCs do strongly express PrP^c, the scenario would be particularly favorable for a transmission to occur. After inoculation, the skin-associated lymphoid tissue could play a role in the further propagation of prions to lymphoid organs and to the CNS.[5] Infiltrating mononuclear cells in eczema and psoriasis, mainly composed of T lymphocytes and macrophages,[16] are PrP^c positive (Figure 1c). They thus represent appropriate targets for conversion of PrP^c to PrP^{Sc} in the skin, in the course of primary infection, and could account for the spread of the altered protein to the lymphoid organs. Conversely, in infected individuals, skin-homing lymphocytes might encounter PrP^{Sc} in the lymphoid organs and transport it back to the skin at sites of inflammation. Lymphocyte-associated PrP^{Sc} could thus gain access to the epidermis causing the conversion of KC-associated PrP^c. However, in contrast to other organs where PrP^{Sc} accumulates within cells and in intercellular spaces,[1–3] the epidermis is constantly self-renewing and sheds terminally differentiated cells. This mechanism would probably not allow that PrP^{Sc} accumulates intraepidermally and induces epidermal pathologies. On the other hand, such a mechanism might lead to the shedding of contaminated material from the body surface. Sheep affected by scrapie suffer from severe pruritus, prompting them to rub their hindquarters on any objects.[2] If PrP^{Sc} were present in the skin as argued above, it could readily be deposited on these objects and passed on to noninfected animals that use the same objects for rubbing.

Although there are only few hard data available on the route of transmission of prion diseases, it appears that the intimate contact with contaminated material, by direct inoculation or ingestion, is necessary. CJD is transmitted in the course of dura grafting or after parenteral administration of natural human growth hormone.[2] Infection by ingestion of prion-contaminated material has recently aroused great interest as a result of the BSE crisis where cows have been infected most likely after being fed meat and bone meals derived from scrapie-infected sheep. Kuru, a prion-mediated disease of the Fore people, was acquired during cannibalistic rites and the ingestion of organs of relatives.[3] In the light of our finding it is conceivable that, in addition to the gut, PrP^c-expressing squamous epithelium of the upper gastrointestinal tract might constitute a target for primary generation of PrP^{Sc} in both BSE and kuru. Interestingly, in transmission of kuru, also contamination during the preparation of the ritual meals and peripheral inoculation has been suggested to be involved.[27] As inflammatory skin diseases were frequent among the Fore people,[27] epidermal PrP^c might have indeed represented a relevant first target for prions in kuru. As to other human prion diseases, ie, CJD and its new variant,[7] there exists no epidemiological indication that acquisition of prions via the skin plays a role in their spread. Nevertheless, we believe that our data on the expression of PrP^c in epithelial cells of skin and mucous membranes justify the inclusion of these organs in future studies on prion disease transmission and pathogenesis.

A potential further implication of our findings concerns the current practice of grafting of skin substitutes. Skin substitutes contain *in vitro* expanded keratinocytes and are routinely used for autologous and allogeneic grafting on surgical and burn wounds and chronic leg ulcers.[28] Growth of these cells mostly is carried out in medium supplemented with either fetal bovine serum or with bovine pituitary extracts.[29] If epithelial PrP^c indeed constituted a possible target for prions, skin substitutes may represent a possible route for inoculation of bovine prions into humans. As currently there are no routine tests available to detect prion-contaminated material, our data emphasize the necessity to develop cell culture systems devoid of undefined animal proteins.

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