Creatine Kinase and Creatine Transporter in Normal, Wounded, and Diseased Skin

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Skin comprises many cell types that are characterized by high biosynthetic activity and increased energy turnover. The creatine kinase system, consisting of creatine kinase isoenzymes and creatine transporter, is known to be important to support the high energy demands in such cells. We analyzed the presence and the localization of these proteins in murine and human skin under healthy and pathologic conditions, using immunoblotting and confocal immunohistochemistry with our recently developed specific antibodies. In murine skin, we found high amounts of brain-type cytosolic creatine kinase coexpressed with lower amounts of ubiquitous mitochondrial creatine kinase, both mainly localized in suprabasal layers of the epidermis, different cell types of hair follicles, sebaceous glands, and the subcutaneous panniculus carnosus muscle. With exception of sebaceous glands, these cells were also expressing creatine transporter. Muscle-type cytosolic creatine kinase and sarcomeric mitochondrial creatine kinase were restricted to panniculus carnosus. Immediately after wounding of murine skin, brain-type cytosolic creatine kinase and a creatine transporter-subspecies were transiently upregulated about 3-fold as seen in immunoblots, whereas the amount of ubiquitous mitochondrial creatine kinase increased during days 10–15 after wounding. Healthy and psoriatic human skin showed a similar coexpression pattern of brain-type cytosolic creatine kinase, ubiquitous mitochondrial creatine kinase, and creatine transporter in this pilot study, with creatine transporter species being upregulated in psoriasis. ***Key words: creatine kinase/creatine transporter/cutaneous wound repair/IgY/psoriasis.***
J Invest Dermatol 118:416–423, 2002

Many cells and tissues such as sperm cells, muscle, or brain use metabolically inert creatine and phosphocreatine together with isoenzymes of creatine kinase (CK) to cope efficiently with high and/or alternating adenosine triphosphate (ATP) requirements (reviewed in Wallimann *et al*, 1992; Schlattner *et al*, 1998). CK catalyzes the reversible transphosphorylation between ATP and phosphocreatine. The enzyme is therefore able to stock the free energy of ATP in the form of phosphocreatine and, vice versa, to use phosphocreatine to replenish cellular ATP pools. Expression of CK isoenzymes in vertebrates is tissue and compartment specific. Two dimeric cytosolic isoenzymes, MM-CK (*m*uscle-type) and BB-CK (*b*rain-type) and two octameric mitochondrial isoenzymes, sMtCK (*s*arcomeric) and uMtCK (*u*biquitous) are encoded by separate nuclear genes. Cytosolic and mitochondrial isoenzymes are usually coexpressed, with MM-CK and sMtCK found in skeletal muscle, and BB-CK and uMtCK in brain, smooth muscle, and many other organs and tissues. The interplay between cytosolic and mitochondrial CK isoenzymes, the CK/phosphocreatine circuit, provides an efficient energy buffer and energy shuttle system that plays a pivotal role in cellular energy supply and energy homeostasis (Bessman and Carpenter, 1985; Wallimann *et al*, 1992; Schlattner *et al*, 1998). The creatine transporter (CRT) is an important component of the CK/phosphocreatine system, responsible for the Na^+/CL^-dependent creatine uptake into the cell (Guimbal and Kilimann, 1993; Sora *et al*, 1994; Saltarelli *et al*, 1996; Dai *et al*, 1999), leading to intracellular total creatine (creatine + phosphocreatine) concentrations of up to 40 mm.

CK has been detected in many different epithelia. This may be due to manifold energy requirements of these cells, including high proliferation rates, active ion pumps, and transport processes. Where examined, a coexpression of BB-CK and uMtCK was observed (Keller and Gordon, 1991), with BB-CK being the predominant isoenzyme, by far; however, the abundance of CK in epithelia varies considerably. Certain organs contain epithelia that are highly enriched in CK, e.g., the electric organ of Torpedo (Barrante *et al*, 1983), where also CRT was detected (Guimbal and Kilimann, 1994), the choroid plexus (Kaldis *et al*, 1996), kidney (Ikeda, 1988), the adult eye lens (Friedman *et al*, 1989), or prostate (Silverman *et al*, 1979). Lower amounts of BB-CK were found in many epithelia of the urogenital system and the digestive tract (Wold *et al*, 1981; Gordon and Keller, 1992; Sistermans *et al*, 1995). In epithelia of the respiratory tract, only traces of CK were observed (Braegger, Schlattner, Utiger, Wallimann, and Sennhauser, unpublished data).

Although representing one of the largest epithelia, skin has not yet been analyzed in detail for CK isoenzymes and their tissue localization. Furthermore, no information at all has been published on the presence and localization of CRT in this tissue. CK activity was reported in extracts of normal and psoriatic human skin, skin tumors, and keratinocyte cell cultures (Zemtsov *et al*, 1994a) and

Manuscript received July 19, 2001; revised November 19, 2001; accepted for publication November 23, 2001.

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Abbreviations: CK, creatine kinase, CRT, creatine transporter; MM-CK, BB-CK, muscle-type and brain-type cytosolic CK; uMtCK, sMtCK, ubiquitous and sarcomeric mitochondrial CK.

BB-CK was localized in human skin epidermis and blister fluid (Kiistala *et al*, 1989), as well as in murine skin (Chida *et al*, 1990; Sistermanns *et al*, 1995). Phosphocreatine in skin has been detected by high-performance liquid chromatography (Zemtsov *et al*, 1993) and more unambiguously by high-resolution ^{31}P-NMR imaging (Bohning *et al*, 1996), indicating a functional phosphocreatine/CK system in this tissue. In addition to basic cellular functions of skin, the CK/phosphocreatine system may also be involved in repair processes like wound healing or in hyperproliferative skin disease, such as psoriasis. During wound healing, enzymes of the *de novo* nucleotide biosynthetic pathway (Gassmann *et al*, 1999) are highly upregulated in the wounded epidermis, reflecting the elevated need for nucleoside triphosphates in cellular energy transduction, nucleic acid synthesis, and biosynthetic pathways in general. Similar conclusions may be true for psoriasis, where highly elevated phosphocreatine levels point to an especially active CK/phosphocreatine system (Zemtsov *et al*, 1994b). Psoriasis is an inflammatory skin disease that is characterized by increased angiogenesis and hyperthickening of the epidermis due to an imbalance in keratinocyte proliferation and differentiation (Kadunce and Krueger, 1995).

In this study, we set out to identify and localize CK and CRT species in healthy and diseased skin, as well as during the wound healing process. Using highly specific antibodies for immunoblotting and immunohistochemistry with fluorescence and confocal microscopy, we could show coexpression of BB-CK, uMtCK, and CRT in several specific cell types and changes in protein levels during wound healing and in psoriatic skin disease.

MATERIALS AND METHODS

Proteins and antibodies against CK isoenzymes CK isoenzymes were expressed in *Escherichia coli* and purified as described earlier (Eder *et al*, 1999; Schlattner *et al*, 2000). Final gel filtration chromatography on Superose 12 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to obtain highly pure protein. Polyclonal antibodies against human sMtCK and uMtCK were produced in rabbits by standard procedures. Rabbits were injected subcutaneously with 200 μg aliquots of protein in complete Freund's adjuvant, followed by three boosts, every 3 wk, in incomplete Freund's adjuvant. Immune sera were collected 3, 5, and 7 wk after the last immunization. Polyclonal antibodies against human BB-CK were obtained from egg yolk of immunized SHAVER outdoor 579 hens. Forty micrograms of BB-CK in complete Freund's adjuvant were injected into the pectoral muscle of each hen, followed after 8 wk by one boost of 40 μg BB-CK in incomplete Freund's adjuvant. Eggs were collected after one further week and stored at 4°C. A simple water dilution method was used to separate yolk plasma proteins from the granules and lipids (Akita and Nakai, 1993; Kokko and Kärenlampi, 1998). The egg yolk was separated, washed and diluted with 9 vol. of distilled water. The emulsion was adjusted to pH 5.0, incubated overnight at 4°C, and centrifuged at 8000 × **g** for 25 min at 4°C. Proteins containing egg immunoglobulins (IgY) were precipitated with sodium acetate (200 g per l) at room temperature and resuspended in 5–8 ml Tris-buffered saline (TBS) per egg. BB-CK-specific IgY were further purified by affinity chromatography, using human BB-CK coupled to *N*-hydroxysuccinimide-ester activated methacrylate copolymer (Affi-Prep 10, Bio-Rad, Reinach, Switzerland). Bound IgY was eluted by 0.2 m glycine, 0.15 m NaCl, pH 2.3, neutralized in 1 m Tris–HCl buffer, pH 8.0, and concentrated by ultrafiltration (Centriplus YM-100, Millipore, Bedford, MA). For extended storage, IgY preparations were adjusted to 40% glycerol and stored at −20°C. Purity and recovery of IgY were monitored by 8% nonreducing standard sodium dodecyl sulfate (SDS)–polyacrylamide gels and Coomassie staining.

Wounding of mice and preparation of wound tissue BALB/C mice were obtained from RCC (Füllinsdorf, Switzerland). They were housed and fed according to Swiss federal guidelines, and all procedures were approved by the local authorities. Full thickness excisional wounds were generated as described earlier (Werner *et al*, 1994). Briefly, mice (8–12 wk of age) were anesthetized with a single intraperitoneal injection of ketamine/xylazine. The hair on the animals' back was shaved and the skin was wiped with 70% ethanol. Four full-thickness excisional wounds (5 mm diameter, 3–4 mm apart, two wounds on each side of the spinal cord) were generated on the back of each animal by excising skin and panniculus carnosus. The wounds were allowed to dry to form a scab. The back skin that was excised upon generation of the wounds served as control (0 d, healthy skin). Animals were killed at different time points after injury (1–14 d). For tissue extracts, the wounds on the left side, including 2 mm of the epithelial margins were isolated and immediately frozen in liquid nitrogen. Wound tissue of several animals was pooled and soluble proteins were extracted in lysis buffer containing NP-40 and urea as described elsewhere (Werner *et al*, 1993; Wakita and Takigawa, 1999). For immunohistochemistry, the complete wounds on the right side were isolated, bisected, fixed in 95% ethanol/1% acetic acid, and embedded in paraffin.

Human tissue samples Human tissue samples (brain, kidney, heart, and skeletal muscle) were obtained from Dr V. Adams (University of Leipzig, Germany), and soluble protein and mitochondria-enriched pellets were extracted according to Schlegel *et al* (1988). Human skin biopsies of three healthy and three psoriasis patients containing epidermis and dermis of upper or lower extremities were obtained from Dr. C. Mauch (University of Cologne, Germany; see Hanselmann *et al*, 2001). They were immediately frozen without fixing procedure. Protein extracts were prepared as described previously (Werner *et al*, 1993).

SDS–polyacrylamide gel electrophoresis and immunoblots Samples were separated by standard 12% SDS–polyacrylamide gel electrophoresis and electrotransferred by semidry blotting (Trans Blot SD, Bio-Rad) on to nitrocellulose (0.45 μm pore size; Schleicher & Schüll, Dassel, Germany) according to the manufacturer's instructions. Loading of the blots was verified by a reversible staining with Ponceau S (0.2% in 0.3% trichloroacetic acid (TCA); Serva, Heidelberg, Germany). Membranes were blocked with 4% fat-free milk powder in TBS (20 mm Tris–HCl, pH 7.5, 150 mm NaCl), incubated for 1 h with CK or CRT immune sera (see below, 1:1000 dilution in blocking buffer) or affinity-purified purified anti-BB-CK IgY (1:500 dilution in blocking buffer), washed three times in TBS, incubated 1 h with peroxidase-coupled secondary antibodies (1:3000 dilution in blocking buffer), either goat anti-rabbit IgG (Nordic, Lausanne, Switzerland) or rabbit anti-chicken IgY (Jackson ImmunoResearch, West Grove, PA) and finally washed three times with TBS. Blots were developed with enhanced chemiluminescence substrate (NEN, Zaventem, Belgium) and exposed to X-ray film (1–60 s) or analyzed with a digital imager (Kodak Image Station; Kodak, Rochester, NY).

Immunostaining Ethanol/acetic-acid fixed and paraffin wax embedded samples of mouse skin and nonfixed samples of human skin were cut with a microtome into 5–7 μm thin sections. Paraffin-embedded sections were deparaffinized, rehydrated, and incubated in 0.05% sodium borohydride ($NaBH_4$) in phosphate-buffered saline (PBS) to quench autofluorescence. After rinsing with PBS, sections were permeabilized with 0.2% Triton-X-100 for 10 min, followed by a wash in PBS. Frozen sections stored at –80°C were directly fixed in acetone at –20°C for 10 min, washed three times in PBS, and blocked for 30 min with 5% goat serum albumin/1% bovine serum albumin (BSA). Sections were then incubated overnight at 4°C in a moist chamber with different primary antibodies, diluted in PBS, containing either 0.2% Tween-20 (polyoxyethylenesorbitan monolaureate) and 1% BSA (paraffin-embedded) or 5% goat serum albumin/1% BSA (frozen sections). Polyclonal primary antibodies were (dilution in brackets): affinity-purified chicken IgY against human BB-CK (lot 23–1; 1:100), preimmune IgY (1:100), and rabbit anti-sera against human sMtCK (lot r3i3; 1:200), human uMtCK (lot r7i3; 1:200), chicken MM-CK (lot 1985; 1:100), and rat CRT C-terminus (lot 75898; 1:100), as well as the corresponding rabbit preimmune sera (same dilutions). Monoclonal primary antibodies were (dilution in brackets): anti-keratin 10 and keratin 14 (Dako Diagnostics AG, Zug, Switzerland; 1:100); anti-CD31 (Dako; prediluted), anti-F4/80 (Serotec, Oxford, GB; 1:100), and anti-wheat germ agglutinin (Dako; 1:200). After washing four times in PBS/0.1% Tween-20, sections were incubated for 1 h at room temperature in a moist chamber with secondary antibody diluted in PBS, containing either 0.1% Tween-20 and 12% BSA (paraffin embedded) or only 1% BSA (frozen sections). Secondary antibodies were Cy3-conjugated rabbit or donkey anti-chicken IgY (Jackson ImmunoResearch; 1:500), Texas Red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch; 1:100), Cy3- or fluorescein isothiocyanate-conjugated goat anti-rabbit or anti-mouse IgG (Pierce, Rockford, IL; 1:200–1:500). Sections were again washed four times in PBS, including 0.1% Tween-20 and 0.02% sodium azide, and finally mounted in an anti-fading solution (30 mm Tris-HCl, pH 9.5, 70% (vol/vol) glycerol, and 240 mm *N*-propyl-gallate).

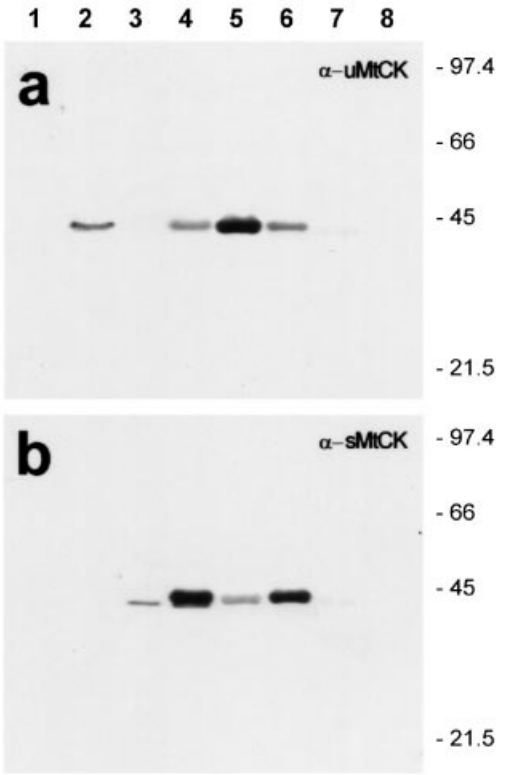

Figure 1. Immunoreactivity of anti-MtCK rabbit serum antibodies. Immunoblots probed with polyclonal rabbit antibodies against (*a*) human uMtCK and (*b*) human sMtCK. *Lanes*: (1) human brain, soluble proteins (24 μg protein); (2) human brain, mitochondria-enriched pellet (24 μg); (3) human muscle, soluble protein (24 μg); (4) human muscle, mitochondria-enriched pellet (24 μg); (5) human uMtCK (1 μg); (6) human sMtCK (1 μg); (7) human BB-CK (3 μg); and (8) chicken MM-CK (3 μg).

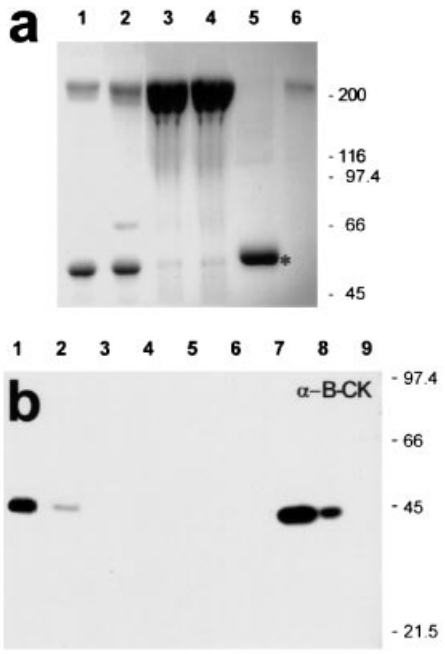

Figure 2. Purification and immunoreactivity of anti BB-CK IgY antibodies. (*a*) Nonreducing SDS–polyacrylamide gel electrophoresis of IgY preparations from chicken egg yolk (for details see *Materials and Methods*). *Lanes*: (1) supernatant of diluted preimmune egg yolk; (2) supernatant of diluted immune egg yolk; further processing of immune egg IgY: (3) dissolved pellet of sodium sulfate precipitation; (4) sterile filtered IgY preparation; (5) IgY bound to BB-CK affinity column, stabilized with 0.5 mg per ml BSA; (6) IgY in flow-through of BB-CK affinity column. Note: *lane 5* only shows the BSA band (*), the low amounts of IgY do not stain. (*b*) Reactivity of affinity-purified IgY with human tissue extracts and pure CK. *Lanes:* (1) brain (20 μg); (2) kidney (20 μg); (3) heart muscle (20 μg); (4) skeletal muscle (20 μg); (5) human uMtCK (3 μg); (6) human sMtCK (3 μg); (7) human BB-CK (3 μg); (8) human BB-CK (0.3 μg); (9) rabbit MM-CK (3 μg). Note the complete absence of cross-reactivity with any other protein or CK isoenzyme.

Microscopy Fluorescence images of sections were recorded on a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with an attached CCD camera (Hamamatsu 3CCD, Hamamatsu, Herrsching, Germany) using Achrostigmat 5×/0.12 or Plan-Neofluoar 10×/0.3 objectives. Confocal images were recorded with a Leica inverted microscope DM IRB/E connected to a Leica true confocal scanner TCS NT and Leica PL APO 100×/1.4 oil or PL APO 63×/1.4 oil immersion objectives. The system was equipped with an argon/krypton mixed gas laser. Image processing was done on a Silicon Graphics workstation using Imaris software (Bitplane AG, Zurich, Switzerland) and Corel Photo-Paint (Corel, Ottawa, Canada).

RESULTS

Specificity of polyclonal antibodies For immunohistochemical studies, we have raised polyclonal antibodies against heterologously expressed and highly purified human CK isoenzymes in rabbit and chicken. Rabbit anti-sera against human sMtCK and uMtCK specifically detected mitochondrial isoenzymes, with relatively high selectivity for the corresponding isoenzyme. Anti-uMtCK anti-sera (**Fig 1*a***) visualized human uMtCK in mitochondria-enriched brain extracts (**Fig 1*a***, *lane 2*) and purified uMtCK (**Fig 1*a***, *lane 5*), with some cross-reactivity with abundant sMtCK in muscle (**Fig 1*a***, *lane 4*) and pure sMtCK (**Fig 1*a***, *lane 6*). Anti-sMtCK anti-sera (**Fig 1*b***) reacted mainly with human sMtCK in mitochondria-enriched muscle extracts (**Fig 1*b***, *lane 4*) or purified sMtCK (**Fig 1*b***, *lane 6*), with minor cross-reaction with pure uMtCK (**Fig 1*b***, *lane 5*). Antibodies did neither cross-react with other proteins in several human tissue extracts nor with cytosolic CK (**Fig 1**, *lanes 1–4*), except for a faint cross-reactivity of sMtCK antibodies with cytosolic MM-CK in human muscle extracts, running just below the sMtCK band (**Fig 1*b***, *lane 3*); however, no signal was observed with chicken MM-CK (**Fig 1*b***, *lane 8*).

Chicken IgY against heterologously expressed and purified human BB-CK were isolated from egg yolk and affinity-purified to eliminate any cross-reactivity with other proteins (**Fig 2*a***). The final IgY preparation only reacted with tissue containing BB-CK, such as brain (**Fig 2*b***, *lane 1*) and kidney (**Fig 2*b***, *lane 2*), as well as with purified human BB-CK (**Fig 2*b***, *lanes 7, 8*). Rabbit polyclonal antibodies against the C-terminal part of rat CRT have already been described elsewhere (Guerrero-Ontiveros and Wallimann, 1998). All antibodies reacted in a similar way with CK and CRT from rat and mouse tissue extracts (data not shown).

CK isoenzymes and CRT in murine skin Sections of paraffin-embedded murine skin samples were analyzed by immunohistochemistry for the presence of different CK isoenzymes and CRT (**Fig 3**, *left panel*). Fluorescence microscopy revealed strong and cell-type specific expression of BB-CK, uMtCK, and CRT (**Figs 3*c***, ***f***, ***l***) in epidermis, hair follicles, and to a lesser extent in panniculus carnosus, the subcutaneous striated muscle. Dermis and subcutis (adipose tissue) stained much weaker for BB-CK, uMtCK, and CRT. Signals for sMtCK (**Fig 3*i***) and MM-CK (data not shown) were restricted to panniculus carnosus and therefore not due to cross-reactivity with uMtCK or BB-CK. In 3 d old murine skin wounds, the hyperproliferative epithelium at the wound edge stained especially strong for BB-CK, uMtCK, and CRT (**Fig 3**, *central panel*). Control incubation with preimmune IgY and

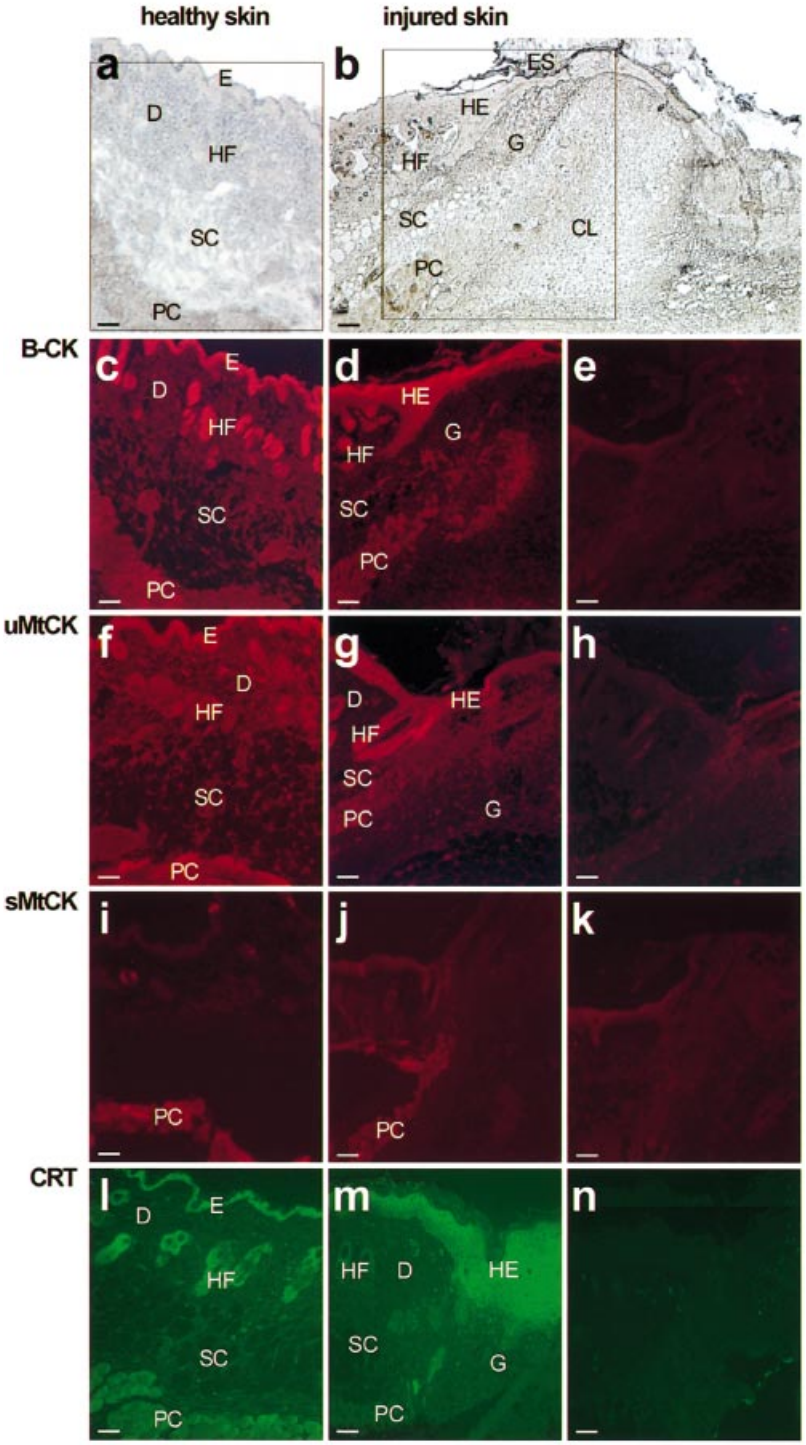

Figure 3. Distribution of CK isoenzymes in healthy and wounded murine back skin. (*a*, *b*) Phase contrast and (*c–n*) immunofluorescence microscopy images of 7 μm sections of healthy skin (left panel) and 3 d full thickness excisional wounds (middle and right panel; overviews of the left half of a wound are shown). Sections were stained for (*c*, *d*) BB-CK (*f*, *g*) uMtCK (*i*, *j*) sMtCK, and (*l*, *m*) CRT. The specificity of the applied antibodies was verified with (*e*) preimmune IgY or (*h–n*) the corresponding preimmune sera. Secondary antibodies were Cy3-conjugated for BB-CK (*c–k*; red channel) and fluorescein isothiocyanate-conjugated for CRT (*l–n*, green channel). Sections indicated in (*a*, *b*) are given as immunofluorescence image in (*c*, *d*). For details see *Materials and Methods*. D, dermis; E, epidermis; ES, eschar; G, granulation tissue; HF, hair follicle; HE, hyperproliferative epidermis; PC, panniculus carnosus; SC, subcutis. *Scale bars*: 50 μm.

preimmune rabbit sera (**Fig 3**, *right panel*) showed only very faint background staining, confirming the low cross-reactivity of the applied antibodies in mouse tissue.

Confocal microscopy of murine skin sections revealed expression of BB-CK in specific cell types (**Fig 4**, *left panels*). In murine epidermis, the suprabasal layers costained with keratin 10 (**Fig 4*b***) rather than the basal layers costained with keratin 14 (**Fig 4*a***) were strongly positive for BB-CK. As evident in **Fig 4*b***, BB-CK was mainly located in the cell periphery near or at the plasma membrane, showing a particulate pattern. In the dermis, the majority of cells in the pilosebaceous apparatus were positive for BB-CK (**Fig 4*e*, *f*, *i***). The enzyme was especially abundant in the sebaceous glands and specific parts of the hair follicle, including the dermal hair papilla (**Fig 4*e***) and the inner root sheath, which is the cell layer surrounding the growing hair (**Fig 4*e*, *f*, *i***). By contrast, the outer root sheath costained with keratin 14 is nearly devoid of BB-CK (**Fig 4*f***). Strong staining was also detectable in endothelial cells of blood vessels in the subcutis and in the panniculus carnosus (**Fig 4*j***). Among the CK-positive cells in the granulation tissue of wounds we identified endothelial cells and macrophages, using costaining with CD-31 and F4/80, respectively (data not shown; Austyn and Gordon, 1981). Staining for uMtCK in all these sections was nearly identical to BB-CK (data not shown, compare to **Fig 3*f*, *g***).

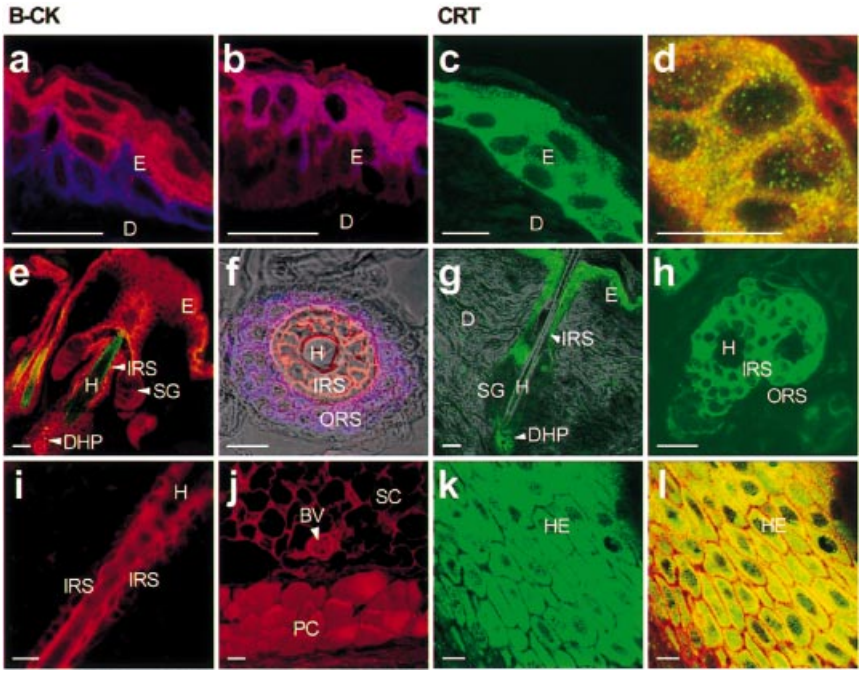

Figure 4. Details of BB-CK and CRT localization in murine back skin. Immunofluorescence confocal microscopy images of 7 μm sections either stained for BB-CK (affinity-purified IgY) using Cy3 conjugated secondary antibody (red channel, left panels), or stained for CRT (rabbit antibodies raised against the CRT C-terminus) using fluorescein isothiocyanate-conjugated secondary antibody (green channel, right panels). (*a–d*) epidermis and dermis, double stained (*a*)with keratin-14 (blue channel, basal epidermal layer), (*b*) keratin-10 (blue channel, suprabasal epidermal layers), or (*d*) wheat germ agglutinin (red channel, extracellular matrix); (*e–i*) hair follicle and connected glands (pilosebaceous apparatus) (*e*, *g*, *i*) longitudinal sections (*f*, *h*) transverse sections just above the hair bulb, in (*f*) double stained with keratin-14 (blue channel); (*j*) subcutis with panniculus carnosus; (*k*, *l*) hyperproliferative epithelium at the wound edge, in (*l*) double stained with wheat germ agglutinin (red channel, extracellular matrix). Note the green autofluorescence of the hair in (*e*) and the BB-CK staining of suprabasal epidermal layers and the inner root sheath in (*b*) and (*f*), respectively. BV, blood vessel; D, dermis; DHP, dermal hair papilla, E, epidermis; ORS, outer root sheath; H, hair; HF, hair follicle; HE, hyperproliferative epithelium, IRS, inner root sheath; PC, panniculus carnosus; SC, subcutis; SG, sebaceous gland. *Scale bars*: 10 μm.

Most cells strongly expressing BB-CK and uMtCK were also positive for CRT (**Fig 4**, right panels). A strong signal was observed in different layers of the epidermis (**Fig 4*c*, *d***), the dermal hair papilla (**Fig 4*g***), the inner root sheath of the hair follicle (**Figs 4*g*, *h***), and the panniculus carnosus. In contrast to CK isoenzymes, CRT was less abundant in sebaceous glands (**Fig 4*g***). CRT showed only faint staining at the cell periphery as indicated by a slight overlapping with the extracellular marker wheat germ agglutinin (yellow in **Fig 4*d*, *l***). Most CRT was not related to plasma membrane, but showed a particulate pattern inside the cell as observed in epidermis (**Fig 4*d***) or was abundantly present in the whole cell body except the nucleus as seen in hyperproliferative epithelium (**Figs 4*k*, *l***).

CK isoenzymes and CRT protein during cutaneous wound repair As CK and CRT were detected in newly regenerating tissue after wounding, we examined the time course of these proteins in wound tissue extracts by immunoblotting (**Fig 5**). The intensity of the signals suggested that at least 10 times more BB-CK is present in skin tissue as compared with the other proteins examined (**Fig 5*a***). After wounding, the amount of BB-CK increased rapidly in an early response as soon as 12 h after injury (data not shown). After 24 h, at least three times more BB-CK protein was present compared with control, but then declined rapidly to reach control levels after 7 d. The amount of uMtCK remained roughly constant during this time period, but increased transiently to a variable degree during days 10–15 after wounding. MM-CK and sMtCK were present, but disappeared during the initial phase of wound healing most likely due to excision of the panniculus carnosus.

Polyclonal antibodies against N- and C-terminal peptides of CRT recognized up to three different protein bands in mouse tissue extracts separated by SDS–polyacrylamide gel electrophoresis (**Fig 5** and unpublished data; see also Guerrero-Ontiveros and Wallimann, 1998; Neubauer *et al*, 1999; Tran *et al*, 2000; Murphy *et al*, 2001). These bands migrate at an apparent molecular weight of about 55, 65, and 70 kDa (CRT55, CRT65, and CRT70; **Fig 5**). After wounding, CRT55 and CRT70 did not change, but CRT65 showed a transient increase during days 1–3. This correlated very well with the above-described upregulation of BB-CK. Later, during days 5–10, all CRT bands showed a temporal downregulation before returning to control levels at day 13.

CK isoenzymes and CRT in healthy and psoriatic human skin Immunohistochemistry of human skin revealed an overall expression pattern of the CK system that is similar to mice (**Fig 6**); however, BB-CK intensely stained the entire epidermis, including apparently also the more basal layers. In addition, the particulate pattern at or near the plasma membrane was more pronounced than in murine skin (**Fig 6*a–c***). uMtCK colocalized with BB-CK, but the staining was weaker compared with murine skin (data not shown). As psoriasis, a hyperproliferative inflammatory skin disease, often shows similarities to wounded skin, we analyzed the expression of the CK system in a pilot study involving three patients and three healthy volunteers. BB-CK (**Fig 6*d–f***), uMtCK and CRT (data not shown) were coexpressed across the entire, much thicker epidermal layers, suggesting a strong expression of these proteins in psoriatic skin. Immunoblotting results normalized to total protein concentration confirmed the upregulation of

CRT55 and CRT65 subspecies in psoriatic skin samples of all three patients as compared with healthy controls (**Fig 7**). In two individuals, antibodies against CRT N- and C-termini recognized an additional band at 115 kDa, which is occasionally also observed in mouse tissues and may represent a nondissociated dimeric CRT species. In case of CK isoenzymes, a very strong variability among individuals made it impossible to detect statistically significant changes in expression levels with the given number of samples.

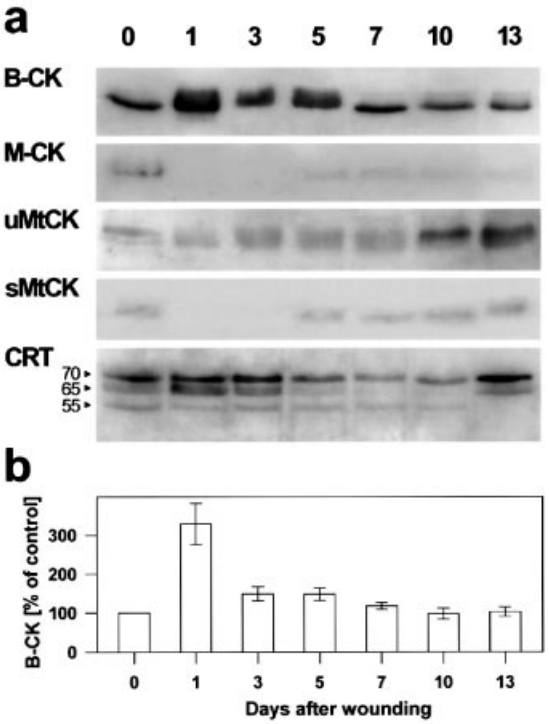

Figure 5. CK isoenzymes and creatine transporter in murine skin during wound healing. (*a*) Immunoblots of skin extracts before (day 0) and at different time points after injury (days 1–13). Samples contained epidermis, dermis, adipose tissue, and, where present, panniculus carnosus. 40 μg of total protein were applied to each lane and equal loading was verified by Ponceau S staining and quantitation on a Kodak image station. Blots were developed as described in detail in *Materials and Methods*. For immunoluminescence detection, blots were exposed to X-ray film for 1 s (BB-CK) or 60 s (MM-CK, uMtCK, sMtCK, CRT). M_r for bands revealed with antibodies against the CRT C-terminus is indicated. (*b*) Relative amount of BB-CK ± SD (n = 4) in skin samples (shown in *a*), quantified by exposure of the immunoluminescent blots in a Kodak image station. BB-CK before injury (day 0) was taken as 100%.

DISCUSSION

In this study we have analyzed the CK/phosphocreatine system in murine and human skin. Using largely isoenzyme-specific antibodies, we could show that cytosolic and mitochondrial CK, as well as CRT, are specifically coexpressed in many cell types of murine and human skin. In addition, we found upregulation of BB-CK and a CRT-subspecies after wounding of murine skin and increased abundance of CRT species in psoriatic human skin.

Suitable antibodies against the ubiquitous CK isoenzymes (BB-CK and uMtCK) for histologic studies with mouse tissues were not available so far. Our new polyclonal antibodies generated in rabbits against recombinant human uMtCK and sMtCK, however, are MtCK-specific, applicable to different species, and show only very low cross-reactivity with the corresponding other MtCK isoenzyme, as also demonstrated in two recent studies (Tarnopolsky *et al*, 2001; Kornacker *et al*, 2001). Affinity-purified anti-BB-CK IgY from chicken egg yolk specifically recognized BB-CK in several human tissues, without cross-reactivity to other proteins than BB-CK in human skeletal muscle, brain, and kidney. This compares very well with a monoclonal anti-BB-CK antibody such as 21E10 (Sistermanns *et al*, 1995). Although these monoclonals are very useful, they present some important limitations for histologic studies in mice. Many tissues give a background signal in immunofluorescence applications due to reaction of secondary antibodies with endogenous mouse IgG. Furthermore, some tissues known to contain BB-CK showed a very low signal, indicating that the epitope recognized by the monoclonals is possibly hidden (Sistermans *et al*, 1995). As we can demonstrate in murine skin, polyclonal IgY can overcome these drawbacks. Chicken IgY can be used on mouse tissue for detection at a high level of sensitivity without problems of background staining. In addition, IgY recognize a variety of different epitopes due to their polyclonal origin and the phylogenetic distance between chicken and human, mouse or rat. A further advantage of IgY is their cheap and harmless production in egg yolk, giving a high titer of antibodies in a rather short time span.

Using these antibodies to analyze murine and human skin by immunoblotting and immunofluorescence confocal microscopy,

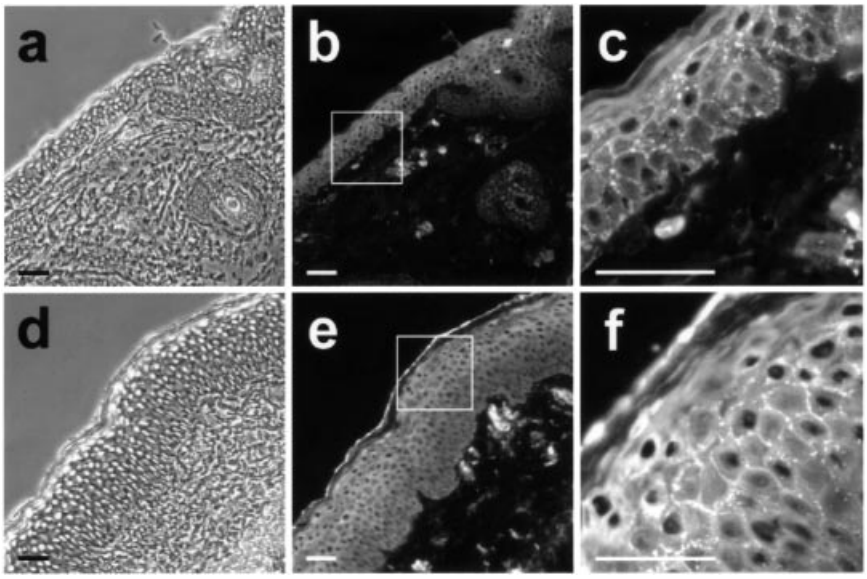

Figure 6. Distribution of BB-CK in healthy and psoriatic human skin. Phase contrast (left panel) and immunofluorescence microscopy images (central and right panels) of 5 μm sections of healthy (*a–c*) and psoriatic skin (*d–f*) stained for for BB-CK using Cy3 conjugated secondary antibodies. The sections indicated in (*b*, *e*) are enlarged in (*c*, *f*). *Scale bars*: 50 μm.

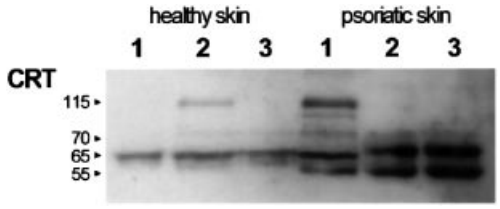

Figure 7. Creatine transporter in healthy and psoriatic human skin. Immunoblots of protein extracts from human skin biopsies containing epidermis and dermis. Three samples each of healthy and psoriatic skin are shown. Forty micrograms of total protein were applied to each lane and equal loading was verified by Ponceau S staining. Blots were developed as described in detail in Materials and Methods. M_r for specific bands is indicated.

we could mostly detect coexpression of BB-CK and uMtCK together with CRT. High expression of these proteins was confined to specific cell types that are characterized by especially high metabolic rates and energy turnover. The suprabasal layers of the epidermis and cells of the inner root sheath in hair follicles have high biosynthetic activity, characterized by synthesis of keratins and of the cornified envelope. Hyperproliferative epithelium and the hair papilla are actively dividing tissues involved in wound healing and hair growth, respectively. Only CK expression in sebaceous glands was not paralleled by a high amount of CRT protein. Suprabasal epithelial layers of the hyperproliferative epithelium not only contained most of the CK and CRT, but are also highly expressing key enzymes involved in nucleotide biosynthesis (Gassmann *et al*, 1999), thus emphasizing the highly active nucleic acid synthesis and energy metabolism in these cells. MM-CK and sMtCK were restricted to the subcutaneous striated muscle panniculus carnosus and absent in skin, thus not confirming earlier detection of MM-CK in epidermis, which was rather due to contamination by muscle fibers (Zemtsov *et al*, 1994a). In addition, immunohistochemical signals for BB-CK and uMtCK were observed in this muscle. Although a minor part of the uMtCK signal may be due to cross-reactivity with sMtCK (see **Fig 1*a***), the data suggest a coexpression of all four CK isoenzymes in panniculus carnosus.

Co-localization of creatine uptake together with mitochondrial and cytosolic CK activity in metabolically highly active cells and the presence of an intracellular phosphocreatine pool (Zemtsov *et al*, 1993; Bohning *et al*, 1996) clearly indicate a functional CK/phosphocreatine shuttle (Wallimann *et al*, 1992) in these cells; however, as judged from immunoblots, CK-positive skin cells contain much more cytosolic BB-CK than mitochondrial CK. This is certainly related to the predominantly glycolytic metabolism of normal and particularly wounded skin (Hunt *et al*, 1978), with only a minor contribution of oxidative phosphorylation. The CK/phosphocreatine-system will therefore predominantly provide a cytosolic energy buffer, bridging sites of glycolytic activity with sites of energy consumption. Some of the major energy consuming sites may be localized at the plasma membrane (e.g., ion pump ATPases), as indicated by accumulation of BB-CK in the vicinity of plasma membrane in murine and human skin sections.

Of further importance for the above described CK/phosphocreatine shuttle are the multiple CRT species detected in immunoblots (Guerrero-Ontiveros and Wallimann, 1998; Neubauer *et al*, 1999; Tran *et al*, 2000; Murphy *et al*, 2001). The three to four proteins recognized by polyclonal antibodies against CRT C- and N-termini are probably generated by alternative splicing, secondary modification due to glycosylation and possibly also dimerization (unpublished data); however, apart from the origin of these proteins, there is growing evidence for a different localization of these CRT species. Very recent data suggest that CRT55 and CRT70 may be at least in part located in mitochondria (unpublished data). This corresponds very well to the particulate pattern of CRT in most skin cell types examined. *In vivo* biotinylation labeling and creatine uptake experiments indicate that the third protein, CRT65, is likely representing the intrinsic plasma membrane CRT, which labels more poorly in immunohistochemistry (Murphy *et al*, 2001). Thus, only part of the histologically detected CRT would be directly involved in creatine uptake from the extracellular space, whereas the role of mitochondrial CRT still remains enigmatic.

Wound healing is a complex process involving inflammation, new tissue formation, and finally tissue remodeling (reviewed in Clark, 1996 and Martin, 1997). Interestingly, both the inflammatory and the proliferative phase of repair were characterized by increased levels of BB-CK and CRT65 protein. We could observe a significant increase of BB-CK and the CRT65 protein during the very first phase following injury, already after 12 h. This correlates with the upregulation of key regulatory enzymes in purine and pyrimidine metabolism such as adenylosuccinate kinase, PRPP synthetase, and others (Gassmann *et al*, 1999), most likely triggered as an early response by keratinocyte growth factor and other growth factors present in the wound. Thus, the initial phase of cutaneous wound repair is characterized by nucleotide biosynthesis and maintenance of high [ATP]/[adenosine diphosphate] ratios by CK. This also makes BB-CK and CRT65 likely candidates for regulation by growth factors. Later, during days 10–15 of wound healing, there is a suspicious rise of uMtCK, correlated with apoptotic cell elimination and matrix remodeling, including the redifferentiation into subcutis and dermis in the granulation layer of the wound. Possibly, these processes rely much more on an oxidative metabolism, with uMtCK using mitochondrially produced ATP. The opposite time course of muscle-specific cytosolic MM-CK and mitochondrial sMtCK, disappearing during the initial wound healing, is most likely due to excision of panniculus carnosus muscle during the application of wounds and following regeneration.

Like injured skin, psoriatic skin is characterized by upregulation of epithelial mitogens, cell proliferation, angiogenesis, and an inflammatory process, which finally leads to a greatly thickened epidermal mass (Kadunce and Krueger, 1995). Earlier publications reported highly elevated phosphocreatine levels and increased CK activity in psoriatic skin as compared with normal skin (Zemtsov *et al*, 1994a, b). In this pilot study, all psoriasis biopsies consistently showed an upregulation of CRT species, including CRT65, the likely candidate for the plasma membrane CRT. This could indeed lead to accumulation of intracellular creatine and finally to higher phosphocreatine concentrations. Our results could not give a definitive answer on changes in CK isoenzyme expression due to high variability among individuals and a small sample size. uMtCK would be a good candidate for upregulation, as psoriatic skin partially switches from glycolytic to oxidative metabolism (Zemtsov *et al*, 1994b) and uMtCK is often overexpressed as a compensatory measure under energy stress, e.g., in aged *vs* adult animals (Lee *et al*, 1999) or patients with mitochondrial disorders (Heddi *et al*, 1999). Definitively, a much broader study is needed to address this hypothesis.

Taken together, we have obtained good evidence for the presence of a functional CK/phosphocreatine system in many metabolically very active cell types in skin, especially in epidermis and hair follicles. In addition, BB-CK and the CRT65-species most probably localized in the plasma membrane are upregulated after injury during cutaneous wound repair and CRT species are also more abundant in psoriatic skin. It will be important to analyze the molecular basis of CK and CRT regulation, as well as the contribution of the CK/phosphocreatine system to epidermal and hair growth in healthy and injured skin. The fact that postoperative perfusion of ischemically compromised skin flaps with solution containing phosphocreatine leads to improved survival of such flaps (Cuono *et al*, 1987) points to important functions of the CK/phosphocreatine system in skin.

We would like to thank Dr. Be Wieringa for sending us anti-BB-CK monoclonal antibodies, Dr. Cornelia Mauch for human skin samples, and Else Zanolla for immunohistochemical work. This study was in part supported by the Swiss Cancer League (grant to U.S. and T.W) and the Zentralschweizer Krebsstiftung (grant to U.S. and T.W).

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