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SHORT COMMUNICATION

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Immunohistochemical localization of sulfhydryl oxidase correlates with disulfide crosslinking in the upper epidermis of rat skin

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Striking morphological changes occur during the terminal stages of epidermal differentiation, such as the loss of major cellular organelles, the aggregation of keratin filaments and the formation of the cornified cell envelope (CCE). The CCE is a highly specialized structure in the cell periphery [1–3], and serves as a physical barrier for the organism and helps to maintain the structural integrity of the upper epidermis. The CCE is rendered insoluble by various post-translational modifications including isopeptide crosslinking and disulfide (S-S) bonding. Isopeptide crosslinking is mainly catalyzed by a membrane-associated transglutaminase specific for the epidermis. During the formation of the CCE this enzyme crosslinks different precursor proteins via *N*-(gamma-glutamyl)-lysine isopeptide bonds [4–6].

On the other hand, S-S bonding is thought to cause conformational changes of the membrane-associated proteins [7]. We have been interested in S-S crosslinking reactions in the epidermis and have reported in this journal [8, 9] on the clear presence of S-S bonds in human skin as determined using a new fluorescent thiol reagent, *N*-(7-dimethylamino-4-methyl coumarinyl)-maleimide (DACM; Wako, Tokyo), which specifically binds to thiol groups (-SH) causing fluorescence [8, 9]. Using this method we have shown that free -SH groups are distributed in the cytoplasm of all living layers of the epidermis. However, S-S bridges are only barely detectable in the living layer, but appear suddenly in the periphery of epidermal cells localized in the transition zone of the living and cornified layers of human skin. Based on this fact, we have suggested that -SH groups suddenly become oxidized to S-S bonds at the junction of the granular and cornified layers [8, 9].

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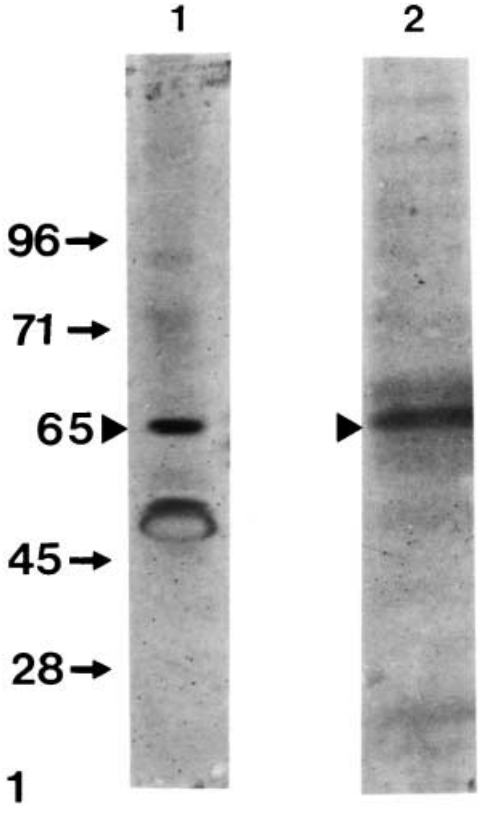
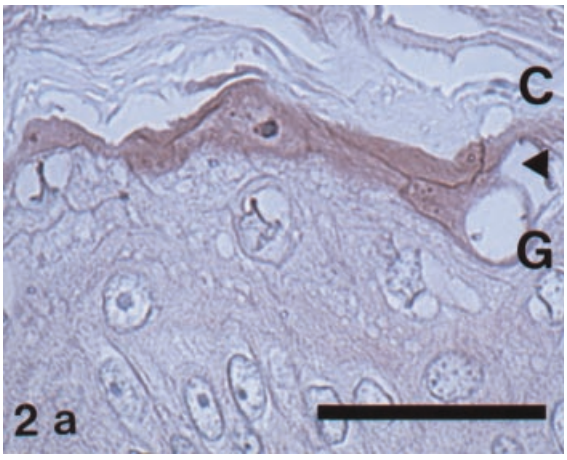


Fig. 1 Detection of SOx in crude extracts from rat seminal vesicle *(lane 1)* and epidermis *(lane 2)* by Western blot analysis using polyclonal antiserum to rat secretory SOx. The molecular weight of SOx was estimated to be 65 kDa in both organs *(arrowheads)*



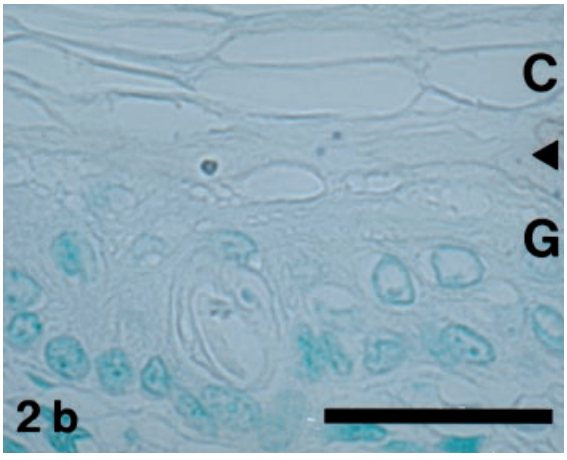


Fig. 2 a, b Immunohistochemical localization of SOx in a cryosection of rat skin. Note the homogeneous distribution of the enzyme in the transitional layer between the granular and the cornified keratinocytes (**a**). There was no positive staining in the control sections using preimmunized rabbit serum as the primary antibody (**b**) (*C* cornified layer, *G* granular layer; *arrowheads* transitional cell layer; *bars* 50 μm)

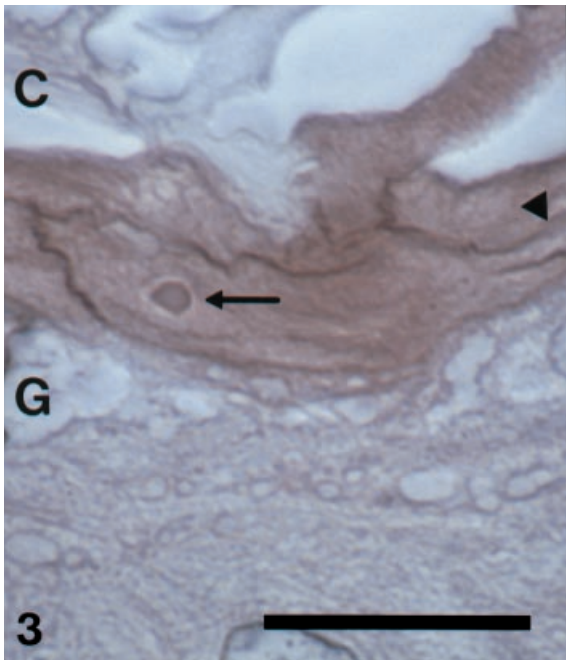
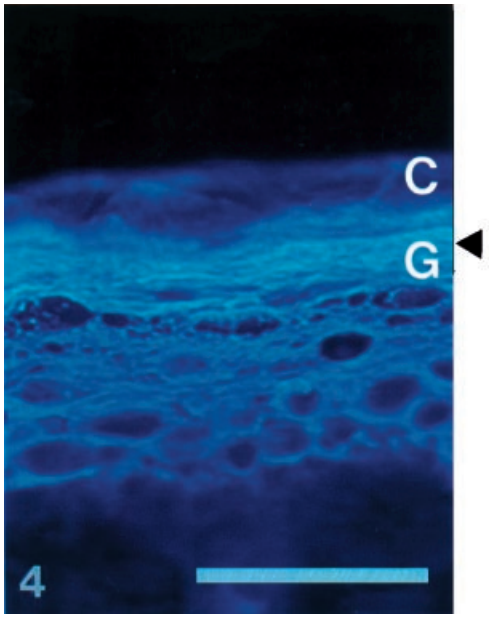
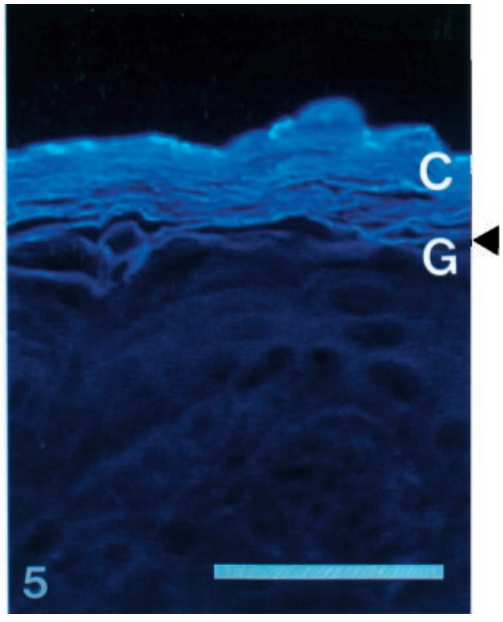


Fig. 3 Higher magnification of immunohistochemical localization of SOx in a cryosection of rat skin. Note the immunopositive layers including some apoptotic nuclei *(arrow)* (*C* cornified layer, *G* granular layer; *arrowhead* transitional cell layer; *bar* 10 μm)

Fig. 4 Identification of thiol groups (-SH) in rat epidermis. First soluble -SH compounds were washed out, then protein-bound -SH groups were labeled with DACM. Strong fluorescence is evident in the cytoplasm of the living layers of the epidermis (*C* cornified layer, *G* granular layer; *arrowhead* transitional cell layer; *bar* 100 μm)

Fig. 5 Distribution of disulfide (S-S) bonds in rat epidermis. First, SH groups were blocked by *N*-ethylmaleimide, then S-S bonds were reduced by dithiothreitol followed by labeling with DACM. S-S bridges are apparent only in the periphery of cells in the cornified layers (*C* cornified layer, *G* granular layer; *arrowhead* transitional cell layer; *bar* 100 μm)





We have also purified the specific enzyme catalyzing S-S formation from rat skin and have characterized it [10] and named it skin sulfhydryl oxidase (SOx). The estimated molecular weight of the enzyme is approximately 66 kDa. We have obtained the highest sulfhydryl oxidase activity in extracts of granular cell-rich fractions from cow snout. However, the localization of skin SOx was unknown until recently, when we succeeded in demonstrating the precise distribution of this enzyme using a polyclonal antiserum against rat seminal vesicle SOx.

For antibody production, SOx was purified from the seminal vesicle secretions of sexually active rats using methods described previously and injected into rabbits [11, 12]. The antiserum raised was characterized as previously described [12, 13]. For gel electrophoresis and immunoblotting, skin from the back of newborn rats and seminal vesicles from sexually active male rats were homogenized in a buffer solution [14]. The SOx antibody showed the same immunoreaction pattern as previously described [14]. For immunohistochemical analysis, cryosections of newborn rat skin were first incubated with the primary antibody, then further incubated with biotinylated goat anti-rabbit IgG antibody (DAKO). For the control study, preimmunized rabbit serum was used as the primary antibody. The sections were then incubated with a solution containing 0.5% 3,3-diaminobenzidine (DAB, Sigma-Aldrich), 1% (w/v) $NiCl_2$, and 0.006% H_2O_2 in 0.05 *M* Tris-HCl buffer, pH 7.6. Nuclear counterstaining was performed using methyl green (Wako, Tokyo).

Western blot analysis of protein extracts from rat seminal vesicle and epidermis showed an immunoreaction of the antibody with a band at 65 kDa (Fig. 1) in both cases. This result indicates that the polyclonal antiserum is not tissue-specific, and SOx from rat skin is similar in size to SOx extracted from rat seminal vesicles. Immunohistochemical analysis using the polyclonal antiserum proved that SOx was localized specifically at the junction of the living and cornified layers in rat skin (Fig. 2 a). No positive reactions were seen in the papillary dermis of the skin. Higher magnification showed that the immunopositive layers included some apoptotic nuclei (Fig. 3, arrow). Thus the layers seem to be the transitional layers (Fig. 2 a and Fig. 3, arrowheads).

DACM staining analysis showed that the distribution of S-S crosslinks in rat skin was almost similar to that in human skin. The oxidization of free -SH groups in rat epidermis (Fig. 4) to S-S bonds (Fig. 5) seemed to occur around transitional layers (arrowheads in Figs. 4 and 5). S-S crosslinking was detectable on the cell membrane and/or in the intracellular spaces (Fig. 5), although the antibody showed homogeneous SOx distribution in the transitional cells (Figs. 2 and 3). CCE has been reported to contain SH-rich components [1–3], and therefore SOx may be one of the thiol oxidases that catalyze the structural stabilization of precursor proteins, such as loricrin, involucrin, and the so-called “cysteine-rich envelope protein”. These findings suggest that this enzyme induces S-S crosslinking of CCE proteins already crosslinked by transglutaminase from the intracellular side of the cornified cells. Moreover, it has also been reported that additional S-S bonds form in the remnants of nuclei of apoptotic cells in the transitional layers [15]. The prominent SOx localization in these transitional epidermal cells suggests that the enzyme is not only a new marker for terminal rat skin differentiation, but also that the structural changes in the transitional layers may partly account for the oxidase-catalyzed conversion of -SH groups to S-S bridges. Therefore, the aim of our current research is to obtain more detailed information on the physiological role of skin SOx.

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