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ORIGINAL ARTICLE

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Ubiquitin expression in skin wounds and its application to forensic wound age determination

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Abstract The time-dependent expression of ubiquitin (Ub) was examined in murine skin wounds and 55 human skin wounds with different wound ages (groups I: 0–12 h, II: 1–5 days, III: 7–14 days and IV: 17–21 days). In murine skin wound specimens, neutrophils, macrophages and fibroblasts showed intensive Ub-positive reactions in the nuclei. In the human wound specimens with wound ages between 4 h and 1 day, neutrophils with strong intranuclear positive reactions for Ub were observed. With increasing wound ages, the nuclei of macrophages and fibroblasts were more intensively stained with anti-Ub antibody. Morphometrically, the intranuclear Ub-positive ratios were very low in group I. The skin wound specimens in groups II and IV showed Ub-positive ratios of >10%, and all samples in group III had Ub-positive ratios of >20%. These results suggest that a ratio of >10% for Ub indicates a wound age of at least 1 day. In contrast, Ub-positive ratios of less than 10% indicate a wound age of <1 day. Moreover, there was a significant difference in the Ub-positive ratio between group III and the other three groups. Thus, Ub-positive ratios considerably exceeding 30%, possibly indicate a wound age of 7–14 days. From the viewpoint of a forensic pathology application, the present study showed that Ub is suitable as a marker of wound age determination.

Keywords Forensic pathology · Wound age determination · Immunohistochemistry · Stress protein · Ubiquitin

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Introduction

Wound examination is one of the most important aspects of the forensic practice and forensic pathologists are often required to estimate wound age. Therefore, many studies on forensic wound age estimation have been performed [1, 2.3, 4, 5, 6, 7, 8]. At present, various kinds of biological substances such as growth factors, cytokines and adhesion molecules are known to be closely involved in various phases of the wound healing process [9, 10]. In forensic pathology, the expression of these biological substances in skin wounds has been examined for wound age determination [11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27].

Ubiquitin (Ub) is a highly conserved protein with a molecular weight of 8,500 [28] and is present in all eukaryotes [29]. This protein mediates non-lysosomal protein degradation in eukaryotic cells by covalently attaching to various proteins by the Ub-protein ligase system [30]. Ub is a member of the heat shock protein family, which is rapidly induced by various types of stimuli such as hyperthermia, chemical or mechanical stress [31]. Ub is presumed to be involved in chronic neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis [32]. Furthermore, Ub gene expression following ischemia/reperfusion has been studied in rat brain and is significantly up-regulated, suggesting that this might be useful as an indicator of ischemic stress [33]. The Ub system appears to be part of the mechanism of neuronal cell death following cerebral ischemia [34, 35]. Recently, Ub expression in various human organs was examined from the forensic aspect [36, 37, 38, 39]. We performed experimental studies on the temporal dynamics of Ub in the healing process of mouse skin wounds. Furthermore, practical availability of Ub as a marker for wound age determination was also examined using human skin wounds with different wound ages.

Materials and methods

Animal experiments

For these experiments 8-week-old Crj-CD1 (ICR) mice weighing 30–37 g (Charles River Breeding Laboratories, Japan), were anesthetised with a single intraperitoneal administration of sodium pentobarbital (50 μg/g). Skin wound preparation was performed according to a previous study [20]. Briefly, after shaving the dorsal region, a 1 -cm full-thickness incision was made on the dorsal skin using a scalpel. After wounding, the mice were individually housed and given sterilised food as well as redistilled water to prevent bacterial infection. Mice were killed by an overdose of sodium pentobarbital at 12 h, 1, 3, 6 and 10 days after wounding, and the wounded skin including an intact 1-cm margin was excised. As the control, unwounded skin specimens were also obtained. All of the animal experiments in the present study were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of Takara-machi Campus of Kanazawa University.

Human skin wound specimens

A total of 55 human skin wounds with different post-infliction intervals between a few minutes and 21 days (15 stab wounds, 8 incised wounds, 25 surgical wounds and 7 lacerations) were removed at forensic autopsy (Department of Legal Medicine, University of Munich). The individual ages ranged from 8 to 75 years (mean age: 40.6 years), and the post-mortem interval was less than 3 days in each case. None of the individuals had suffered from severe malnutrition, malignant diseases or metabolic disorders, and no substances such as cytostatic agents or glucocorticoids, which can possibly influence wound healing, were administered during medical treatment. According to the wound ages, wound specimens were classified into the following 4 groups: I 0–12 h (n=13), II 1–5 days (n=12), III 7–14 days (n=19) and IV 17–21 days (n=11). Uninjured skin from the same individuals was also taken as a control.

Immunohistochemistry

The wound specimens were fixed in 4% formaldehyde solution with phosphate-buffered saline (PBS, pH 7.2), embedded in paraffin and sectioned at thickness of 4 μm. After deparaffinisation, the sections were immersed in 0.3% H_2O_2-methanol for 30 min and incubated overnight with rabbit anti-ubiquitin polyclonal antibody (Dako, Kyoto, Japan) at 2 μg/ml in PBS containing 1% normal goat serum and 1% bovine serum albumin (BSA) at 4°C. Envision+ (Dako) for rabbit immunoglobulin was added and incubated at room temperature for 30 min, and positive reactions were visualised with diaminobenzidine.

Morphometrical analysis

According to the methods of previous studies [21, 22], morphometrical analysis was performed for semi-quantitative evaluation of the immunohistochemical findings by two different investigators without prior knowledge. Briefly, 10 microscopic fields (magnification ×400) were randomly selected in each section, and the ratio of the number of intranuclear Ub-positive infiltrating cells (neutrophils, macrophages and fibroblasts) to the total number of infiltrating cells was calculated in each microscopic field. The average ratio of the 10 selected microscopic fields was evaluated in each wound specimen.

Statistical analysis

In each group, the mean values of the Ub-positive ratios and standard errors (SE) were calculated. Statistical analyses were performed using one factor analysis of variance (ANOVA) to determine whether differences existed among the group means, followed by Scheffé's F to identify the significantly different means.

Results

Animal experiments

In unwounded skin specimens, the nuclei of the epidermal cells and sweat glands showed intensive Ub-positive reactions, and the cytoplasm of those cells showed a weakly positive immunoreaction. After wounding, the infiltration of neutrophils with strong intranuclear Ub-positive reactions were observed in 12 -h-old wound sites. With increasing wound age, those neutrophils had disappeared from the wound site, and mononuclear cells (probably macrophages) showing intensive intranuclear positive reactions, were recruited at the wound sites. Furthermore, at 6 days after injury the new formation of granulation tissue was observed indicating the proliferative phase of skin wound healing, and spindle-shaped fibroblastic cells in addition to mononuclear cells showed intranuclear Ub-positive immunoreactions. Through the wound healing, a few leukocytes and fibroblasts showed faintly Ub-positive reactions in the cytoplasm. Morphometrical analysis demonstrated that the intranuclear Ub-positive ratio was most evident 6 days after injury (Fig. 1).

Human skin wounds

In the unwounded skin specimens, intranuclear Ub immunoreactivity was strongly detected in the keratinocytes

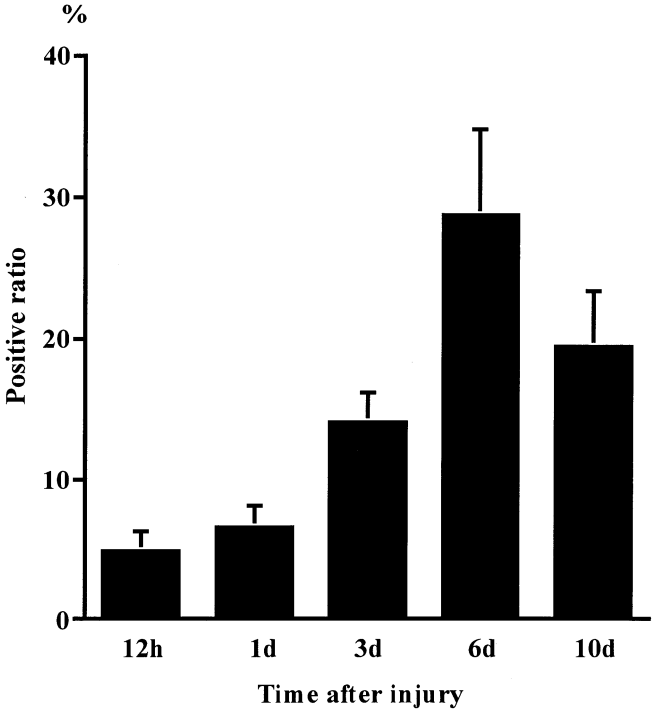

Fig. 1 Ratio of intranuclear Ub-positive infiltrating cells in wound healing of murine skin wounds

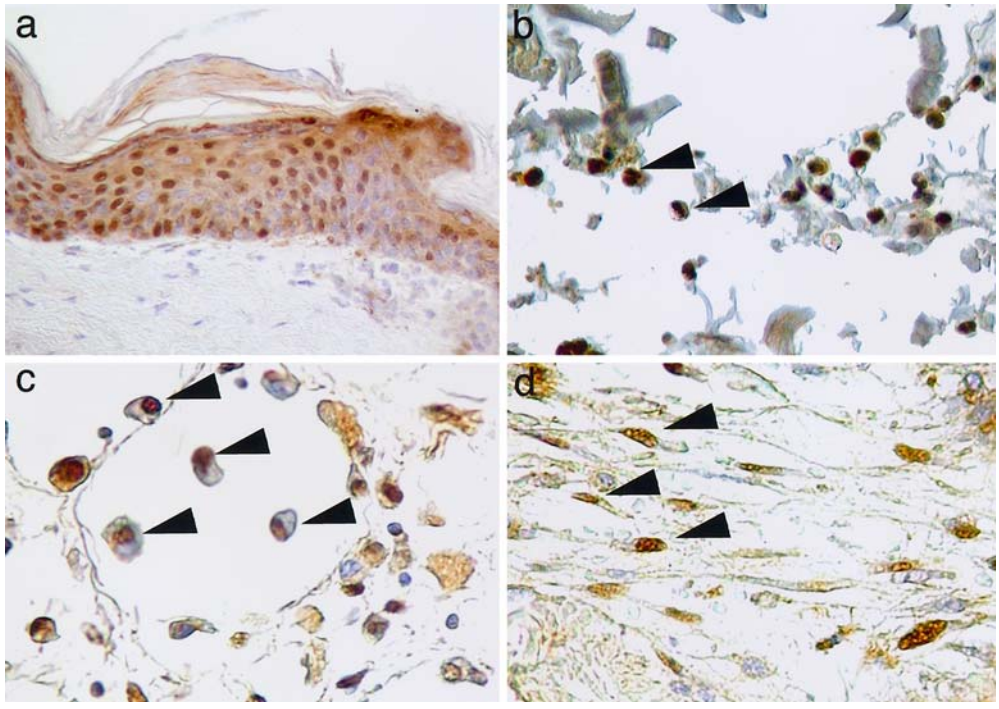

Fig. 2a Control skin samples showed an intensive intranuclear positive reaction of keratinocytes. **b** A 1-day-old skin wound with infiltration of neutrophils (*arrowheads*) showing positive reaction for Ub. **c** In this 4-day-old wound, phagocytic macrophages (*arrowheads*) are immunostained with anti-Ub antibody. **d** In this 14-day-old wound, spindle-shaped fibroblastic cells (*arrowheads*) were positively immunostained with anti-Ub antibody (**a** ×100, **b**–**d** ×400)

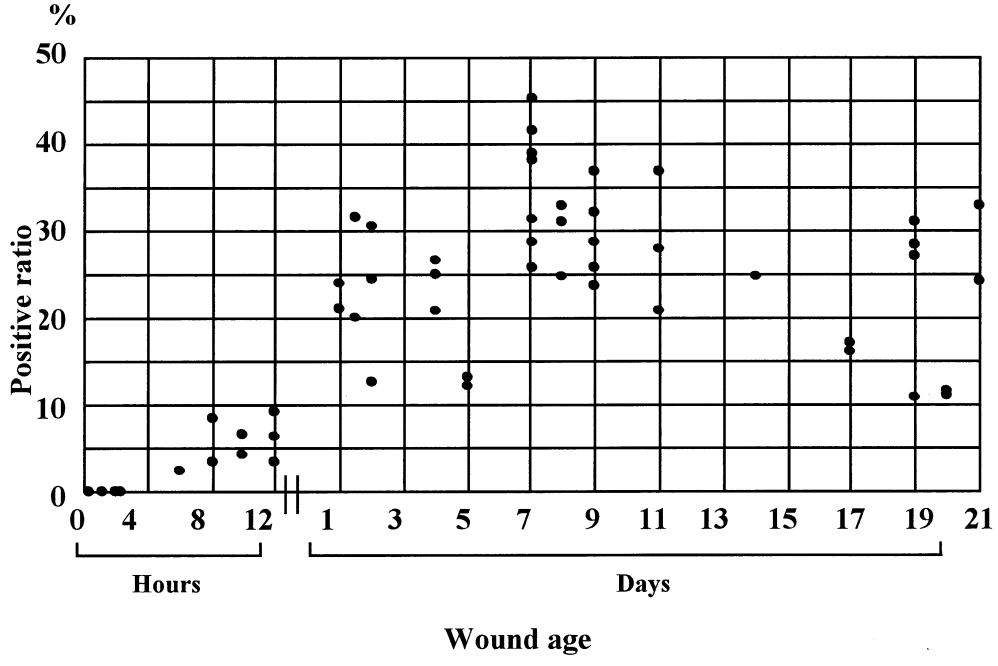

Fig. 3 Ratio of intranuclear Ub-positive infiltrating cells in relation to the wound age

and sweat gland cells, and more faint positive reactions were also observed in the cytoplasm (Fig. 2a). In the wound specimens aged 4 h–1 day, polymorphonuclear cells, probably neutrophils were mainly observed at the wound site, and some of them showed strong Ub-positive reactions in the nuclei (Fig. 2b). With the increase of the wound age, infiltration of round-shaped mononuclear cells (probably macrophages) was dominant against neutrophils and then a migration of spindle-shaped fibroblasts with granulation tissue formation and angiogenesis was also observed. Intensive Ub-positive reactions were observed in the nuclei of the macrophages (Fig. 2c) and fibroblasts (Fig. 2d). Moreover, a few of cells such as neutrophils, macrophages and fibroblasts showed faint positive reactions of the cytoplasm. There was consistency in the Ub-positive cell types between mouse and human skin wounds.

Figure 3 demonstrates the distribution of the ratios of intranuclear Ub-positive infiltrating cells in relation to wound age. The positive ratios of Ub were very low in

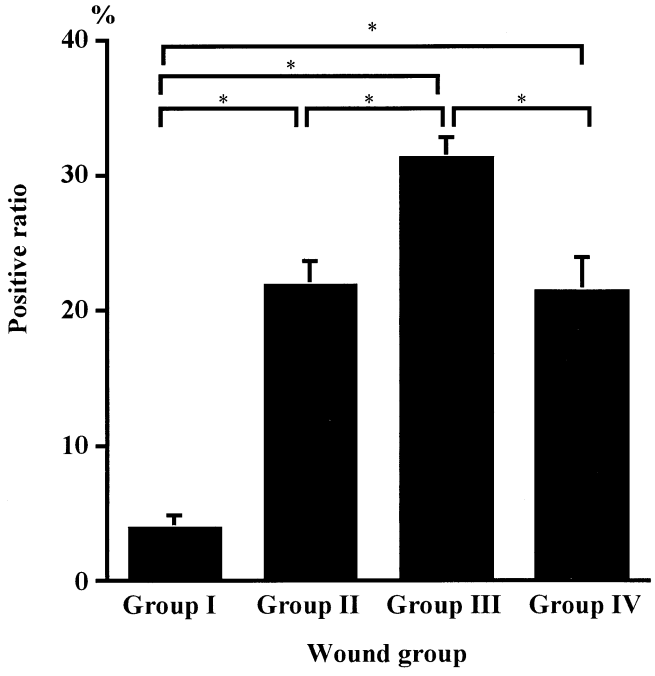

Fig. 4 Mean value and standard error of Ub-positive infiltrating cells in each wound group (*A significant difference was observed statistically, $p<0.05$).

group I, thus showing that all of the wound specimens in group I gave a value of less than 10% (mean±SE: 3.8±0.9%). However, in group II, the ratio of Ub-positive cells rapidly increased, and all wound specimens showed Ub-positive ratios of >10% (mean±SE: 22.4±9.2%). Moreover, the Ub-positive ratio gradually increased in group III, and all samples had a Ub-positive ratio of >20% (mean±SE: 31.3±1.6%). A 7-day-old wound in group III showed the maximum value (45.3%) among all of the 55 human skin wound specimens in the present study. Thereafter, the Ub-positive cell ratio in group IV in contrast, gradually decreased with increasing wound ages (mean±SE: 21.3±2.5%). Statistical analysis revealed that significant differences were found between group I and the three other groups, between groups II and III, and between groups III and IV (Fig. 4).

Discussion

In the present study, intensive positive reactions for Ub were found in the nuclei of the leukocytes and fibroblasts. Moreover, the cytoplasm of those cells also showed a faint Ub-positive reaction. Biochemical studies demonstrated that Ub can move from the cytoplasm into the nucleus, combine with denatured proteins to form macromolecular polymeric aggregates and finally degrade into the mononers [40, 41, 42]. It was also reported that Ub was expressed in the nuclei and the cytoplasm of the tubular epithelial cells and epidermal cells [36, 43]. Collectively, it was considered that the intranuclear Ub-positive reaction was more bioactive. Actually, the intranuclear positive reaction was evaluated in the previous studies [37, 38]. Thus, we evaluated leukocytes (neutrophils and macrophages) and fibroblasts with intranuclear Ub-positive reactions.

Recently, Ub expression has been examined in the field of forensic science. Shoji [39] demonstrated that the severity of tracheal burns was correlated with the intensity of Ub expression in the lungs. Ub overexpression in various kinds of organs was found in cases of sudden infant death, battered children, drug intoxication, multitrauma and neurogenic shock [44, 45, 46, 47]. Quan et al. [37, 38] demonstrated that Ub was induced in the pigmented substantia nigra neurons by severe stress such as asphyxia, drowning and burns. Shimizu et al. [36] showed that Ub was also induced by hypothermia. Briefly, Ub expression was significantly observed in the liver, kidney and pancreas, suggesting that Ub expression might be important in clarifying the cellular kinetics in death from hypothermia. These observations suggested that Ub was induced by various kinds of stimuli and its expression might be useful for the evaluation of causes of death in the forensic practice. However, to the best of our knowledge, there has been no study on Ub expression in human skin wounds from the viewpoint of forensic wound age determination.

According to the previous study [48] on mouse epidermis, mRNA of Ub was detected in the spinous and granular layers using in situ hybridisation. Moreover, the strongest reaction was observed in the hair bulb. In the present study, unwounded skin specimens of humans and mice showed Ub positive reactions in the nucleus of epidermal and hair follicle cells, being consistent with the previous study [48]. These observations may imply that the skin is always exposed to various external stimuli and needs a relatively rapid turnover. In the wounded skin specimens, Ub immunoreactivity was observed in the nuclei of neutrophils, macrophages and fibroblasts, which showed that Ub is induced in skin wounds. At present, the pathophysiological role of Ub in skin wound healing is not yet well understood. However, the results of the animal experiments showed that Ub expression was more intense in the proliferative rather than the inflammatory phase of wound healing, thus suggesting that Ub may play an important role in the proliferative phase of wound healing.

Moreover, the results from animal experiments also indicated that the Ub expression in skin wounds was likely to be useful for wound age determination, and this hypothesis could be confirmed in the study using human skin wounds of various wound ages. From the viewpoint of a forensic pathological application, the present study showed that Ub is suitable as a marker of wound age determination. The skin wound specimens in groups II and IV showed Ub-positive ratios of >10% (mean±SE: 22.4±9.2% in group II, 21.3±2.5% in group IV), and all samples in group III had Ub-positive ratios of >20% (mean±SE: 31.3±1.6%). These results suggest that Ub-positive ratios of >10% indicate a wound age of at least 1 day. Conversely, Ub-positive ratios of less than 10% in-

dicate a wound age of <1 day. Moreover, there was a significant difference in the Ub-positive ratio between group III and the other three groups. Thus, Ub-positive ratios, considerably exceeding 30%, possibly indicate a wound age of 7–14 days. With regards to the applicability in older wounds, it is not easy to determine wound age based only on the intranuclear Ub-positive ratio, since there was no significant difference between groups II and IV. However, leukocytes such as neutrophils and macrophages were the main cell type showing Ub-positive reactions in group II. On the other hand, fibroblasts and macrophages were dominant in group IV. Thus, although further investigation is required, the combined evaluation of Ub-positive ratio and positive cell type may be useful for the age determination of wounds with longer survival periods.

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