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ORIGINAL ARTICLE

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Forensic application of VEGF expression to skin wound age determination

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Abstract An immunohistochemical study combined with morphometry was carried out to examine the time-dependent expression of vascular endothelial growth factor (VEGF) using 53 human skin wounds with different wound ages (groups I: 0–12 h, II: 1–4 days, III: 7–14 days and IV: 17–21 days). In the human wound specimens aged 4–12 h, neutrophils recruited at the wound showed no positive signals for VEGF. With an increase in wound ages of ≥7 days, granulation tissue and angiogenesis were observed, with the migration of macrophages and fibroblasts of which the cytoplasm expressed VEGF-positive reactions. Morphometrically, the average VEGF-positive ratio was highest in group III, followed by that of group IV. In groups III and IV, 13 out of 26 wound samples had VEGF-positive ratios of more than 50%. However, all of the wound samples in groups I and II showed VEGF-positive ratios of less than 50%. With regard to the practical applicability and forensic validity, these observations suggest that a VEGF-positive ratio of more than 50% possibly indicates a wound age of 7 days or more.

Keywords Forensic pathology · Wound age determination · Immunohistochemistry · VEGF · Angiogenesis

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Introduction

Skin wound healing is a complicated but well-organized biological response composed of three phases including inflammation, proliferation, and maturation [1, 2]. Wound examination is one of the most important matters in the forensic practice. At present, various kinds of biological substances such as growth factors, cytokines and adhesion molecules are known to be closely involved in each phase of the wound healing process [1, 2]. Skin wound healing is a relevant aspect of forensic practice with respect to the determination of wound age and wound vitality. Thus, many studies on forensic wound age determination have been performed [3, 4, 5, 6, 7, 8, 9, 10]. Recently, biological substances such as growth factors, cytokines, and adhesion molecules have become useful markers for wound age determination [11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30].

Neovascularization is important for tissue repair and tumor growth, and the vascular endothelial growth factor (VEGF), which is induced under hypoxic conditions has been identified as a potent angiogenic factor [31, 32]. For example, overexpression of VEGF by virus vectors has been applied to the gene therapy of ischemic disease [33]. In skin wound healing, angiogenesis is a crucial event for formation of new granulation tissue in the proliferative phase [34, 35, 36]. In the present study, we immunohistochemically examined VEGF expression in human skin wounds with different wound ages, and discuss the practical availability of VEGF as a marker for wound age determination.

Materials and methods

Antibodies

The following monoclonal (mAbs) or polyclonal antibodies (pAbs) were used for immunohistochemical and immunofluorescence analyses in the present study: mouse anti-human CD31 mAb (clone JC/70A; Dako Cytomation,

Kyoto, Japan), mouse anti-human CD68 mAb (clone PG-M1; Dako Cytomation), mouse anti-α-smooth muscle actin (αSMA, clone 1A4; Dako Cytomation), rabbit anti-human VEGF pAb (SC-507; Santa Cruz, CA), cyanine dye 3 (cy3)-conjugated donkey anti-mouse IgG pAb, and FITC-conjugated donkey anti-rabbit IgG pAb (Jackson ImmunoResearch Laboratories, West Grove, PA).

Human skin wound specimens

A total of 53 human skin wounds with different post-infliction intervals ranging from a few minutes to 21 days (15 stab wounds, 8 incised wounds, 23 surgical wounds and 7 lacerations) were removed at forensic autopsies (Institute for Legal Medicine, University of Munich). The individual ages ranged from 8 to 75 years old (mean age 40.6 years old), and the postmortem interval was less than 3 days in each case. None of the cases had suffered from severe malnutrition, malignant diseases or metabolic disorders, and no substances such as cytostatic agents or glucocorticoids, which may possibly influence wound healing, were administered during medical treatment. The wound specimens were classified into 4 groups according to wound age as follows; I: 0–12 h (n=13), II: 1–4 days (n=14), III: 7–14 days (n=17) and IV: 17–21 days (n=9). Uninjured skin from the same individual was also taken as a control.

Immunohistochemistry

The wound specimens were fixed in 4% formaldehyde solution with phosphate-buffered saline (PBS pH 7.2) and embedded in paraffin, followed by sectioning at a thickness of 4 μm. After deparaffinization, the sections were immersed in 0.3% H_2O_2-methanol for 30 min and incubated with anti-VEGF pAb (1:100) or anti-CD31 mAb (1:100) at 4°C overnight. Thereafter, Envision+ (Dako Cytomation) for rabbit or mouse immunoglobulin was reacted at room temperature for 30 min, and positive reactions were visualized with diaminobenzidine.

Double-color immunofluorescence analysis

A double-color immunofluorescence analysis was also performed to determine the types of VEGF-expressing cells during skin wound healing, as described previously [37]. Briefly, deparaffinized sections were incubated with PBS containing 1% normal donkey serum and 1% BSA to reduce non-specific reactions. Thereafter, the sections were further incubated in pairs of anti-VEGF (1:100) and anti-CD68 (1:00) for human macrophages, or anti-VEGF (1:100) and anti-αSMA (1:100) antisera at 4°C overnight. After incubation with cy3-conjugated anti-mouse IgG pAb (1:50) and FITC-conjugated anti-rabbit IgG pAb (1:25) at room temperature for 1 h, the sections were observed under a fluorescence microscope.

Morphometrical analysis

According to the methods of previous studies [20, 21, 26], morphometrical analysis was performed for semi-quantitative evaluation of the immunohistochemical findings by two different investigators without prior knowledge. Briefly, in each section, 10 microscopic fields (magnification ×400) were randomly selected, and the ratio of the number of VEGF-positive infiltrating cells such as leukocytes 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 as the VEGF expression score in each wound specimen.

Statistical analysis

In each group, the mean values of the VEGF-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-test to identify significantly different means.

Results

Immunohistochemical and double-color immunofluorescence analyses

In unwounded specimens, VEGF-positive signals were detected in the epidermal cells (Fig. 1). In wound specimens with the ages of 4 h to 1 day, polymorphonuclear cells, probably neutrophils, were mainly observed at the wound site. However, these neutrophils showed no immunopositive reaction for VEGF. With an increase in wound age, the infiltration of round-shaped mononuclear cells was obviously dominant over neutrophils, after which a migration of spindle-shaped fibroblasts with angiogenesis was also observed. In particular, in serial sections, VEGF-positive mononuclear cells and fibroblastic cells were observed around the neovessels evidenced by CD31-positive areas (Fig. 2). Immunopositive signals for VEGF were localized in the cytoplasm of these mononuclear cells and fibroblastic cells (Fig. 3). Furthermore, we performed a double-color immunofluoresence analysis to determine the cell type producing VEGF using a combination of anti-VEGF and anti-CD68 (a marker for macrophages) or anti-VEGF and anti-αSMA (a marker for myofibroblasts). As shown in Fig. 4, macrophages and myofibroblasts were the main cellular sources of VEGF in human skin wounds.

Morphometry

Figure 5 demonstrates the distribution of the ratios of VEGF-positive infiltrating cells in relation to wound age.

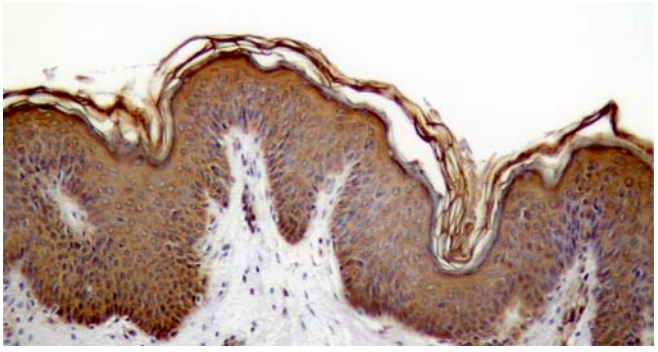

Fig. 1 Immunopositive reactions for VEGF detected in the keratinocytes of uninjured skin samples

VEGF-positive ratios were very low in wound samples aged 0–12 h (group I), with most of the wound specimens in group I giving values of less than 10% (mean±SE 2.9 ±0.8%). In wound specimens with postinfliction intervals of 1–4 days (group II), the ratio of VEGF-positive cells apparently increased (mean±SE 20.7±3.5%). Moreover, in wound samples aged 7–14 days (group III), the VEGF positive ratio considerably increased and in 15 out of 16 samples, the ratio was over 40% (mean±SE 55.9±2.8%). A 14-day-old wound in group III showed the maximum value (75.8%) among all of the 53 human skin wound specimens in the present study. Although, in the wound specimens with postinfliction intervals of 17–21 days (group IV), the VEGF-positive ratio was significantly lower compared with group III, it still remained high (mean±SE 40.3±3.5%). Statistical analysis demonstrated that significant differences were found between the four groups (Fig. 6). However, some of the wound samples in groups I and II also showed VEGF-positive ratios of 30–40% in individual cases.

Discussion

In the forensic practice, the determination of wound age including wound vitality is always required to clarify the relationship between wounds and the cause of death. Conventionally, the detection of hemosiderin deposits by Berlin blue staining was carried out for wound age determination. Wound age determination is closely involved in the pathophysiology of the wound healing process. With advances in biochemical and immunohistochemical techniques, several markers have been applied to wound age determination [3, 4, 5, 6, 7, 8] and immunohistochemical analysis is now exclusively utilized for wound age determination [9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21].

VEGF has been characterized as an angiogenic growth factor from media conditioned by bovine pituitary folliculostellate cells. VEGF is a heparin-binding growth factor specific for vascular endothelial cells and is able to induce angiogenesis in vivo. VEGFs are secreted proteins, in contrast to other endothelial cell mitogens such as acidic or basic fibroblast growth factors and platelet-derived endothelial cell growth factor [31, 32]. VEGF expression is up-regulated by cytokines and growth factors, and inflammatory cytokines such as interleukin (IL)-1 and IL-6 induce VEGF gene expression [38]. The expression of these inflammatory cytokines is enhanced in the early phase of skin wound healing process, whereas VEGF induction starts at a later phase. Consistently, our previous study demonstrated that IL-1α expression was considerably higher in wound specimens with postinfliction intervals of 4 h–1 day [20], and the present results show that the peak of VEGF expression was reached at a later phase, corresponding to the proliferative phase, histologically evidenced by angiogenesis and granulation tissue formation.

VEGF is also induced under hypoxic conditions [38], suggesting that VEGF expression is correlated with hypoxia-related substances such as hypoxia-induced factor (HIF) and oxygen-regulated protein 150 (ORP-150). There are HIF-binding sites in the VEGF promoter. The transcription levels of VEGF were found to be increased with binding HIF to the promoter region [38]. In other previous studies, the expression pattern of VEGF was almost parallel to that of ORP-150 in wound healing. Now, it has been clarified that ORP-150 is a molecular chaperone for the intracellular transport of VEGF from the endoplasmic reticulum to the Golgi apparatus [39]. Furthermore, p53 protein (product of the tumor suppressor gene, *p53*) also regulated the expression of VEGF. Wild-type p53 protein inhibited VEGF expression, whereas its mutant form promoted angiogenesis. Pathophysiologically, wild-type p53 protein increased during wound

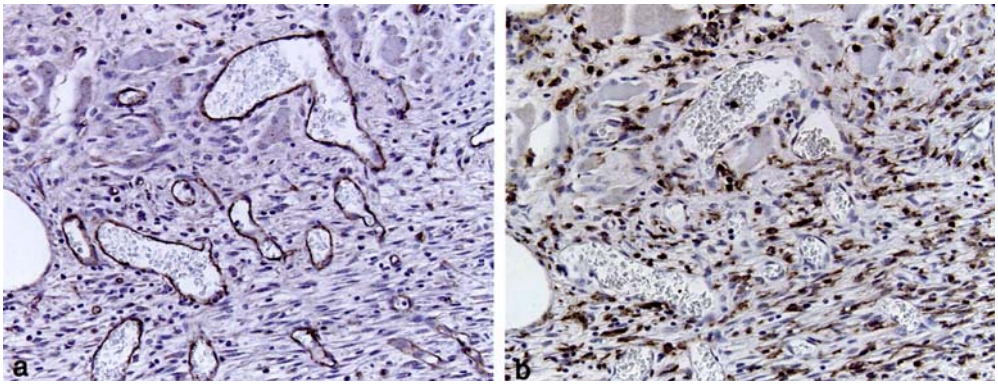

Fig. 2 Immunostaining for **a** CD31 and **b** VEGF using serial sections of a 14-day-old wound. Neovascularization was detected within the new granulation tissue **a**. In the serial sections, VEGF-positive cells were found around the neovessels **b** (original magnification ×100)

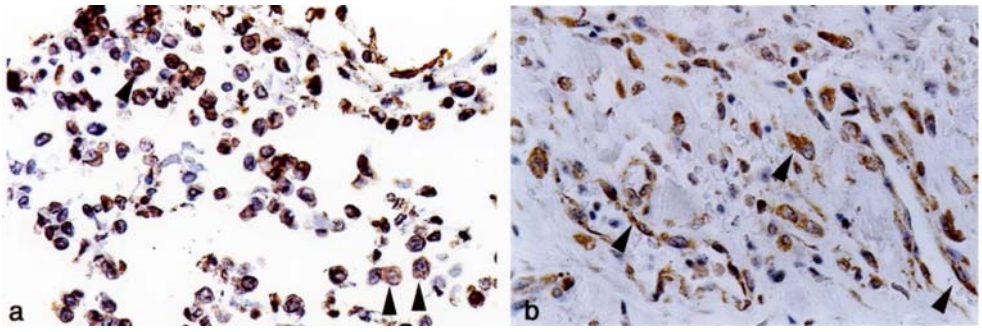

Fig. 3a In this 7-day-old wound, phagocytic macrophages (*arrowheads*) were immunostained with anti-VEGF antibody. **b** In this 14-day-old wound, spindle-shaped fibroblastic cells (*arrowheads*) were positively immunostained with anti-VEGF (original magnification ×400)

healing, which may indirectly control neovascularization. Hausmann et al. [40] demonstrated that the p53 protein was also a useful marker for wound age determination.

Consistent with the previous study, immunopositive signals for VEGF were detected in the cytoplasm of keratinocytes, macrophages, and myofibroblasts. In uninjured skin specimens, immunopositive reactions for VEGF were constitutively detected in the keratinocytes. It was quite difficult to evaluate the difference of immunohistochemical results of the keratinocytes between uninjured and injured skin samples. Thus, it is considered that VEGF-positive epidermal cells can provide no useful information for wound age determination, and infiltrating cells such as leukocytes and fibroblasts should be morphometrically analyzed in accordance with our previous studies [20, 21, 26].

According to the previous study [9], macrophages appeared at 3 days or later after wounding in human skin wounds. In skin wound healing, macrophage migration is indispensable, suggesting that macrophage-derived VEGF has essential roles in skin wound healing. Myofibroblasts are the other VEGF-producing cells. Betz et al. [41] examined the appearance of α-SMA-positive myofibroblasts in human skin wounds. Their results demonstrated that α-SMA-positive myofibroblasts were detected with the initial formation of typical granulation tissue in human skin wounds as early as approximately 5 days post-wounding. Based on the present results and Betz et al.'s study [41], myofibroblasts as well as macrophages are presumed to play a key role in angiogenesis due to the secretion of VEGF in skin wound healing

There is only one study focusing on VEGF in wound age determination. Takamiya et al. [42] demonstrated that VEGF was a possible marker for wound age determination by animal experiments using mice. Unfortunately, the practical availability of VEGF for wound age determination has still not been investigated. We used human skin wounds with known postinfliction intervals. From the

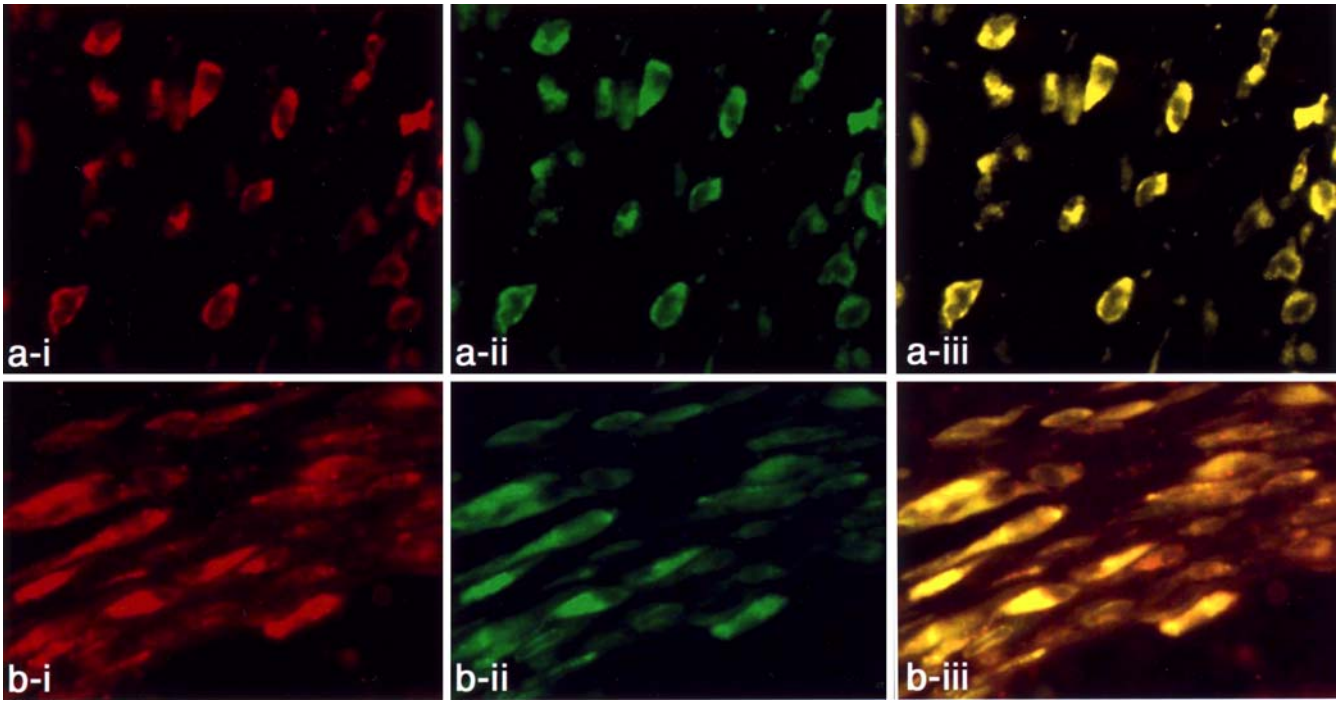

Fig. 4a-b A double-color immunofluorescence analysis was performed to determine the VEGF-expressing cell types. The samples were immunostained with anti-CD68 (**a-i** Cy3), anti-αSMA (**b-i** Cy3), or anti-VEGF (**a-ii** and **b-ii** FITC) as described in Materials and Methods and observed under a fluorescence microscopy. Signals in **i** and **ii** were digitally merged in panels **iii**. Representative results from three independent experiments are shown here (original magnification ×400)

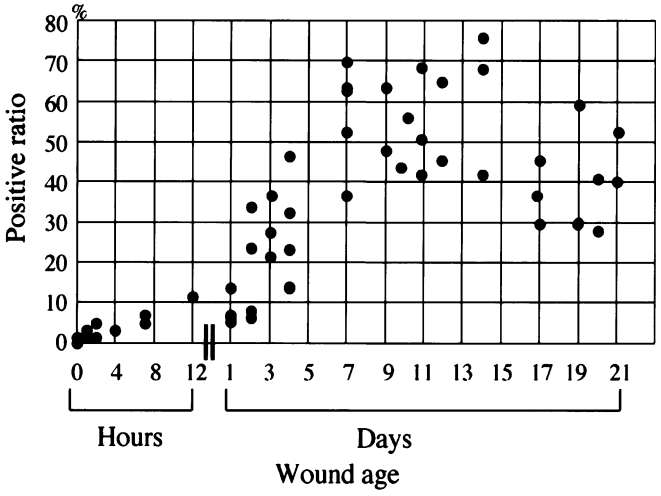

Fig. 5 Ratio of VEGF-positive infiltrating cells in relation to wound age

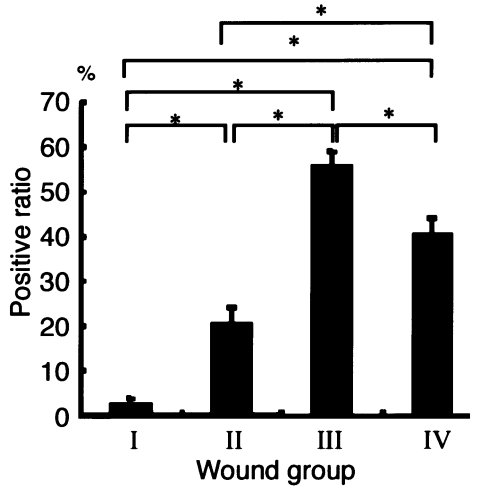

Fig. 6 Mean value and standard error of VEGF-positive infiltrating cells in each wound group. *A significant difference was observed statistically ($p<0.05$)

viewpoint of forensic pathological application, the present study shows that VEGF is available as a marker of wound age determination. However, it is very difficult to determine wound ages from 30–40% under the aspect of forensic validity, since some of wound samples aged a few days also showed similar VEGF-positive ratios in individual cases. Although all of the wound samples in groups I and II showed VEGF-positive ratios of <50%, 13 out of 26 wound samples in groups III and IV (wound age >7 days) had VEGF-positive ratios of >50%. Thus, with regard to practical applicability and forensic validity, these observations suggest that VEGF-positive ratios of >50% possibly indicate a wound age of >7 days.

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