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ORIGINAL ARTICLE

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Estimation of wound age and VCAM-1 in human skin

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Abstract To estimate the age of skin wounds, the endothelial adhesion molecule VCAM-1 (CD 106) was detected in paraffin sections after autoclaving and using the ABC technique. The percentage of VCAM-1 positive blood vessels was determined after the blood vessels had been marked with PECAM-1 (CD 31). Low positive staining reactions were observed for VCAM-1 on endothelial cells of uninjured skin in 18% of the samples. In injured skin, 51% of the cases investigated showed a VCAM-1 expression. Strong positive staining reactions were observed 3 h at the earliest and 3.5 days at the latest after the time of injury. The immunohistochemical results for VCAM-1 differed significantly between the injured and uninjured skin ($P < 0.01$). In a few cases VCAM-1 was detected ($n = 6$) at low intensity in postmortem skin wounds and a moderate to strong expression of VCAM-1 is indicative of the vitality of the wound. The detection of VCAM-1 can be used for estimating the age of wounds in forensic applications if the degree of expression of further adhesion molecules, especially that of selectins, is taken into account.

Key words VCAM-1 · Selectins · Wound age · Immunohistochemistry

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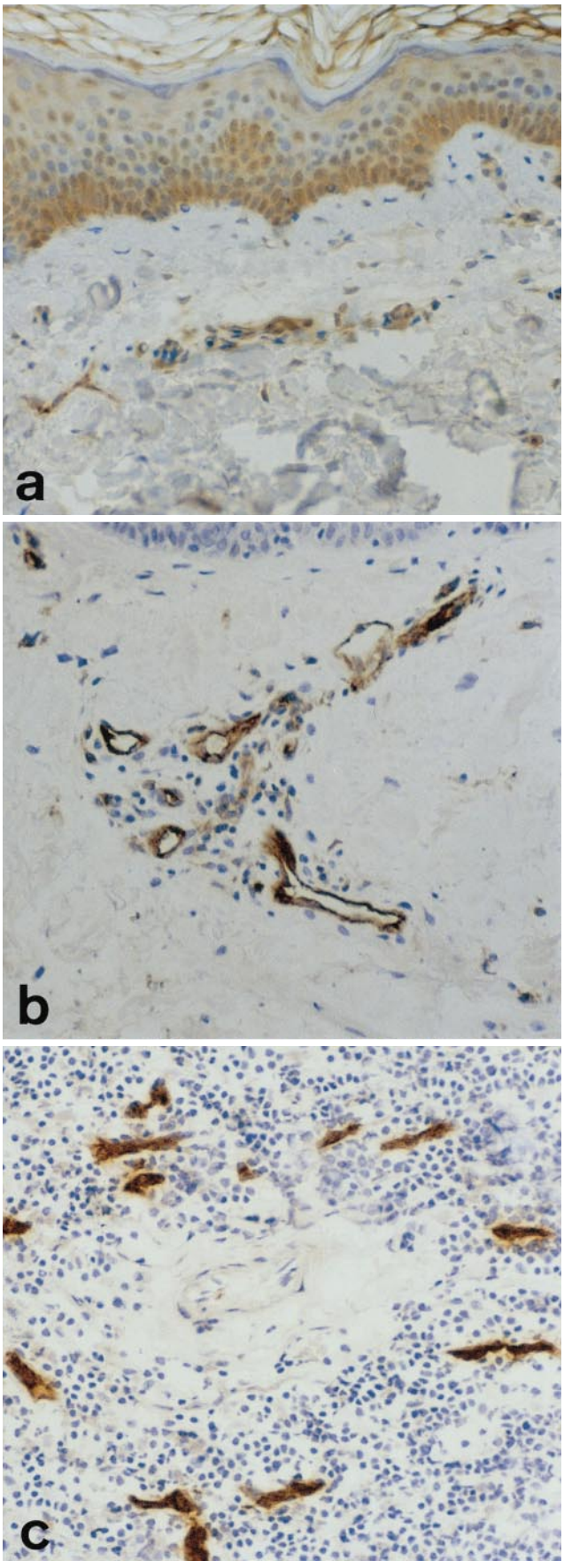
Introduction

The adhesion molecules identified in recent years can help to improve the estimation of the wound age, especially of injuries with a short survival time. This is also indicative of the vitality of the wounds [9, 21].

The vascular cell adhesion molecule-1 (VCAM-1, CD 106) is considered to belong to the immunoglobulin supergene family. It has a molecular weight of 100–110 kDa and has the ability of acting as a ligand for the β1-integrin very late activation antigen-4 (VLA-4) [11]. VCAM-1 is not expressed constitutively on endothelial cells [8, 12]. This would require the activation by lipopolysaccharides and cytokines, especially IL-1β and TNF-α, which are released in the wound healing process [17, 22]. These are the same inflammation mediators which are also responsible for the up-regulation of the intercellular adhesion molecule-1 (ICAM-1) [3]. VCAM-1 regulates the diapedesis of lymphocytes, monocytes and eosinophils from the blood vessels into the tissue [2, 6, 7, 18–20, 25]. VCAM-1 is not only observed on endothelial cells but also on macrophages and dentritic cells of the lymphatic tissue [24]. The up-regulation of VCAM-1 begins after 2–4 h and may last several days [1, 23]. In-vitro studies have shown that VCAM-1 could be found over a period of 6–48 h after cytokine stimulation [14]. Hence, VCAM-1 and ICAM-1 are activated at a slower rate as compared with the expression of the selectins [10, 22].

The aim of this study was to establish if there is a correlation between the occurrence of VCAM-1 and the wound age in injured skin and to ascertain whether this correlation can be used for estimating the time since injury. The occurrence of these adhesion molecules in skin wounds induced postmortem was also studied with a view to assessing the vitality of injuries.



Material and methods

The material investigated in this study originated from 197 skin wounds of which 97 were taken from autopsy material and 100 were excised during the surgical treatment of wounds of patients, after having duly informed them of the purpose and obtained their consent.

There were no indications of an immunosuppressive therapy such as the administration of cytostatics or glucocorticoids, or of metabolic disorders or forms of malnutrition that could be anamnestically evaluated.

In addition, 31 postmortem skin injuries were investigated. For this, an incised wound was introduced on the extensor side of the right thigh 1–2 h before the beginning of the autopsy. The autopsy samples were kept at 4° C in a cooling chamber for a maximum of 7 days.

The wound age varied between 3 min and 790 days. The time of injury was taken from the records of the criminal investigation department for the autopsy cases or from the anamnestic data of the patient concerned for the surgical cases.

The samples were prepared in the following steps: fixation in 4% PBS formaldehyde solution, extraction of 2–4 µm thick paraffin sections, staining with hematoxylin-eosin or according to the elastica van Gieson method, application of the indirect avidin-biotin complex method (ABC), dewaxing and hydration and autoclaving [4] at 120° C (Varioklav, H + P Labortechnik GmbH) in citrated buffer (10 mM, pH 6.0) for 10 min. The samples were rinsed with PBS buffer (10 mM, pH 7.4), endogenous peroxidase was blocked with 1% H_2O_2, initial incubation of the sections with normal serum (horse) at 37° C for 15 min, later at 37° C for 1 h using anti-VCAM-1 (monoclonal antibody, anti-mouse; Dako, Hamburg) at a 1 : 10 dilution. Incubation with biotin labelled secondary antibody (monoclonal antibody, anti-mouse; Vector, Heidelberg) at 37° C for 15 min, incubation with Vectastain "Elite" ABC peroxidase complex (Vector, Heidelberg) at 37° C for 15 min, staining with DAB at 20° C for 5–10 min followed by nuclear staining with haemalum.

The staining intensity was assessed semi-quantitatively using a four-category ordinal scale (0.0 = negative; 1.0 = low staining, 2.0 = moderate staining, 3.0 = strong staining). Furthermore, the number of blood vessels (capillaries, venules, arterioles) was visualized using CD 31 a marker of endothelial cells and determined microscopically under magnification × 180 for five visual fields. Of these, the percentage of blood vessels with positive brown staining reaction for the antibodies CD 106 was estimated.

A PBS buffer was substituted for the primary antibody in the negative control test. Tonsil samples from the person concerned, which were stained with the VCAM-1-specific antibody and ABC kit of the same batch, were used in the positive control experiment (Fig. 1 c).

Differences in the distribution of the variables of the findings between types of wounds and wound age classes were statistically tested by means of unadjusted Mann-Whitney-U rank sum tests. A multinomial logistic regression analysis [16] was used to generate a multivariate model for the prediction of the most probable wound age.

Fig. 1 a–c Staining intensity of VCAM-1 in injured skin and palatine tonsil tissue (paraffin, ABC; × 250) **a** VCAM-1: low positive reaction on keratinocytes of basal cells and some prickle cells of the epidermis, low positive reaction in capillaries of the dermis (19 h-old lacerated/contused knee wound) **b** VCAM-1: strong positive, membranous reaction of the endothelial cells of small subepidermal blood vessels, negative reaction of mononuclear perivascular cells (3 h-old surgical wound of the forearm **c** VCAM-1: strong positive cell detritus within the lymphatic tissue (palatine tonsil, positive control)

Results

VCAM-1 in intact skin

In uninjured skin, VCAM-1 was detected with a low intensity (1.0) on endothelial cells in 18% (n = 17) of the investigated blood vessels of the dermis and subcutis. Keratinocytes showed no immunohistological staining reaction in any sample.

VCAM-1 in injured skin

In injured skin, VCAM-1 showed an increase in the semiquantitatively determined intensity of the immunohistological staining reaction and a higher number of blood vessels with positive reaction.

VCAM-1 (Fig. 1 b) was found to be positive on endothelial cells of small and large blood vessels in 51% (n = 101) of the samples. It occurred on endothelial cells of the blood vessels investigated with low intensity (1.0) in 29% (n = 58), moderate intensity (2.0) in 17% (n = 34) and strong intensity (3.0) in 5% (n = 9) of the cases. The percentages of blood vessels with positive reaction in injured and uninjured skin differed significantly ($P < 0.01$). VCAM-1 was regularly found to be positive on macrophages and cell detritus. Perivascular inflammation cells showed a slight positive staining in 6% (n = 12) and keratinocytes in 3% (n = 6) of the samples (Fig. 1 a).

VCAM-1 in skin injured postmortem

VCAM-1 could not be detected in 81% of the samples taken from skin wounds induced postmortem and was found to occur with low (1.0) intensity in 6 cases on endothelial cells of the blood vessels. The comparison and distribution of immunohistological staining reactions of VCAM-1 of postmortem-induced skin wounds with vital injuries (autopsies and surgical case material) showed significant differences ($P < 0.01$).

Time dependence of VCAM-1 expression

Strong positive staining reactions were observed after 3 h at the earliest and in 3.5-day-old skin wounds at the latest.

The intensity of the expression of immunohistochemical reactions for the VCAM-1 was found to increase up to a wound age of 4–6 h (Fig. 2). For these wounds the mean staining intensity was 1.37. After that, a decrease in the staining intensity (arithmetic mean = 1.0) in the samples investigated was evident for VCAM-1.

Model for the prediction of the most probable wound age

The wound age and the percentage of positively reacting blood vessels were subdivided into four categories each and a multinomial logistic regression model for the prediction of the most probable wound age was formulated by varying both the points of distinction between the categories and the combination of variables. Optimum predictor variables were found in the VCAM-1 and the P- and E-selectins (CD62P/E). However, of the 64 possible combinations of the 3 predictor variables, only 25 were actually observed so that the predictor quality is still not satisfactory.

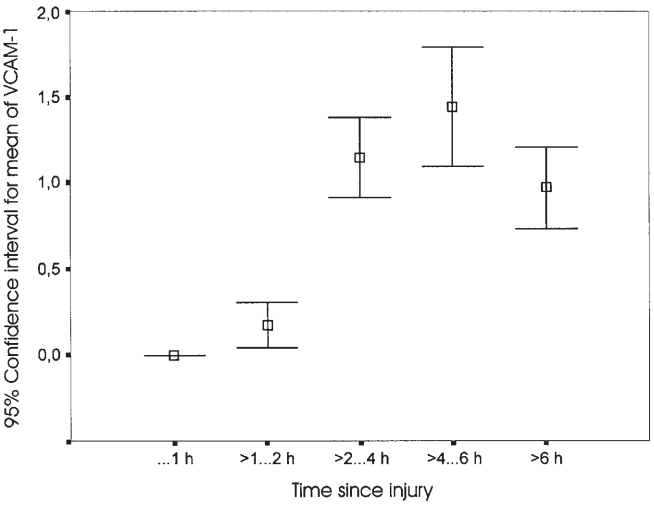


Fig. 2 Confidence interval (CI) for mean of the staining intensity of VCAM-1 (CD 106) vs time since injury

Discussion

Osborn et al. [22] detected VCAM-1 on endothelial cells after activation by lipopolysaccharides and cytokines, especially IL-1β and TNF-α. This regulates the diapedesis of monocytes and lymphocytes from the blood vessels into the tissue during the wound healing process. The present studies revealed that VCAM-1 could be detected on endothelial cells of the blood vessels in 51% of the skin injuries. In uninjured skin, low positive immunohistochemical reactions could be detected in 18% of the samples only. A constitutive expression of VCAM-1 could not be found in the material investigated. In conformity with the findings by Rice et al. [24], we observed VCAM-1 also on macrophages and cell detritus of the lymphatic control tissue (tonsil) and injured skin. Mononuclear cells of perivascular infiltrates showed low positive reactions in 6% of the injuries and Hausmann et al. [15] also detected VCAM-1-positive perivascular cell infiltrates in the stratum reticulare from 12 patients with dermatomyositis.

According to Pober and Cotran [23] and Abe et al. [1], the up-regulation of VCAM-1 begins after 2–4 h and can last several days. In-vitro experiments conducted by Gemmell et al. [14] showed that VCAM-1 could be detected over a period of 6–48 h. Strong positive staining reactions were also observed after 3 h at the earliest and in 3.5-day-old skin wounds at the latest in our study.

Compared with the control samples, VCAM-1 in injured skin showed a statistically significant difference ($P < 0.01$) of the semi-quantitatively recorded intensity of the immunohistological staining reaction and an increased number of blood vessels with positive reaction. What is of forensic interest for estimating the age of a wound is the earliest, regular and latest detectability of reactions in skin wounds [5]. A detection threshold had to be introduced for VCAM-1 because it was found at low intensity on endothelial cells of the blood vessels of uninjured skin. The earliest and latest detectability of VCAM-1 relates to the identification of a strong immunohistochemical staining reaction. In the injured skin samples studied, a regular occurence of VCAM-1 could not be defined for any wound age interval.

The intensity and distribution of immunohistological staining reactions for VCAM-1 of skin wounds induced postmortem was significantly different from vital injuries ($P < 0.01$). Hence, VCAM-1 is a suitable marker to allow the vitality of the injury to be assessed.

The samples taken from the wounds of autopsy cases were 7 days old at the most. The immunohistochemical tests conducted during the cold storage period revealed no significant difference in the VCAM-1 staining intensity. Fieguth et al. [13] found that lysocymes of the skin are also relatively resistant to autolysis.

The detection of an increased VCAM-1 expression alone is not sufficient for estimating the age of a wound with the accuracy needed for forensic purposes. Nonetheless, a multinomial logistic regression model [16] can be used to reduce the observed percentage of positively reacting blood vessels for VCAM-1 and the P- and E-selectins (CD62P/E) to the predicted probability of a wound age. This allows VCAM-1 to be used for estimating the age of wounds if the degree of the expression of other adhesion molecules, especially that of the selectins, is taken into account.

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