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ORIGINAL ARTICLE

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Enhanced expression of selectins in human skin wounds[1]

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Abstract The aim of the study was to characterize the vitality and age of skin wounds by the detection of selectins. A prospective study was conducted for this purpose in which 197 vital human skin wounds (time since injury ranging from 3 min to 790 days) were investigated immunohistologically. Of the samples tested, 97 were taken from autopsy material and 100 from patient material from the department of surgery at the university hospital. The selectins were detected in paraffin sections after autoclaving and using the ABC technique. The intensity was rated by a semi-quantitative evaluation using a four-stage ordinal scale. Strong positive immunohistochemical reactions were observed for the P-selectin 3 min at the earliest and 7 h at the latest after the time of injury. For the E-selectin a positive staining was evident 1 h at the earliest and 17 days at the latest from the time the skin was injured. The staining intensity decreased significantly after an interval of 12 h from the time of injury ($P < 0.05$). The L-selectin was regularly detected on leukocytes in the samples of injured skin. The immunohistochemical results for the P- and E-selectins were significantly different between injured and uninjured skin ($P < 0.01$). The expression of the selectins is indicative of the vitality of the wound. P-selectin was detected in a few cases ($n = 4$) at low intensity while E-selectin could not be found in the control samples ($n = 31$) of postmortem skin wounds. The use of P- and E-selectins for forensic purposes can help to achieve better estimates of the age of wounds with short survival times.

Key words Selectins · Wound age · Immunohistochemistry

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Introduction

The age of a wound and thus the vitality of an injury cannot be easily diagnosed, especially in the initial stage of the would healing process, because apart from the detection of fibronectin and the migration of leukocytes there are hardly any findings of forensic relevance [2, 7].

Selectins are molecules which initiate the rolling and later adhesion of the leukocytes to the activated microvascular layer of endothelial cells. Leukocyte rolling takes place at a velocity of about 50 μm/s [15]. The leukocytic L-selectin (LECAM) has a C-terminal lectin domain, a transmembrane region and a short intracellular domain [5, 11, 14, 23, 27] and a molecular weight of 75–80 kDa [16]. The L-selectin is able to recognize carbohydrate-containing neuroamidase-sensitive ligands on the endothelium which causes the transient adhesion of the leukocytes [15]. The endothelium expresses the P-selectin (PADGEM) and E-selectin (ELAM-1) which may be induced by cytokines, especially tumor necrosis factor-α, interleukin-1β and lipopolysaccharides. The endothelial P- and E-selectins are characterized by a high sequence homology with LECAM [10]. Through their interaction with sialysized derivatives of LewisX-oligosaccharides on the leukocyte surface, they contribute to initiating the leukocyte rolling [9, 17, 18, 20]. The molecular weights are 140 and 115 kDa respectively [16].

The P-selectin is found in the α-granules of thrombocytes and in the Weibel-Palade's bodies of the endothelial cells [19]. On the stimulation of the endothelial cells, a degranulation process occurs in which the P-selectin is

expressed instantly at the luminal cell surface, i.e. within 5–30 min [13, 26].

The E-selectin is responsible for the adhesion of polymorphonuclear neutrophils [3], eosinophils and basophils [4], monocytes [6] and memory T-lymphocytes [25]. The expression of E-selectin begins 2–6 h after the inflammatory irritation and ends within 2 days [26]. L-selectin is always expressed on leukocytes [29].

The aim of the study was to establish if there is a correlation between the occurrence of selectins and the wound age in injured skin and to ascertain whether this correlation can be used for estimating the time since injury. With a view to assessing the vitality of injuries, it should also be verified whether these adhesion molecules can be found in postmortem skin wounds.

Material and methods

The material investigated in this study originated from lacerated/contused wounds, incised wounds, surgical wounds and excoriations (n = 197, *males* = 128, *females* = 69). Of the samples, 97 were taken from autopsy material and 100 were extracted during the surgical treatment of wounds (excision) of patients.

Each autopsy sample was compared with a sample of intact skin taken from a corresponding region of the body of the same patient. 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 befor the beginning of the autopsy. The autopsy samples were kept at 4 °C in a cooling chamber for a maximum of 9 days.

The wound age varied between 3 min and 790 days. The age distribution of the patients covered by the study is shown in Table 1.

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, rinsing with PBS buffer (10 mM, pH 7.4), autoclaving [7, 24] at 120 °C (Varioklav, H+P Labortechnik GmbH) in citrate buffer (10 mM, pH 6.0) for 20 min and blocking of the endogenous peroxidase with 1% H_2O_2. Initial incubation of the sections with normal serum at 37 °C for 15 min, later at 37 °C for 2 h and at 4 °C overnight using the antibodies at the following concentrations: anti-P-selectin 1:10 (monoclonal antibody, anti-mouse; Dianova, Hamburg), anti-E-selectin 1:50 and anti-L-selectin 1:30 (monoclonal antibodies, anti-mouse; Dako, Hamburg). 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 Mayers haemalum.

The staining intensity was assessed semi-quantitatively using a four-category ordinal scale (∅ = no staining; + = low staining, ++ = moderate staining, +++ = strong staining). Furthermore, the number of blood vessels (capillaries, venules, arterioles) was visualized using a marker of endothelial cells CD 31 and determined microscopically under magnification × 180 for five visual fields. Blood vessels which were positively stained for the antibodies anti-P-selectin, anti-E-selectin and anti-L-selectin were estimated as a percentage.

A PBS buffer was substituted for the primary antibody in the negative control test. Tonsil samples from the person concerned were stained with the selectin-specific antibody and ABC kit of the same batch and used for the positive control.

All samples were independently evaluated by two investigators and the results statistically analysed by means of unjusted Mann-Whitney rank sum test[2].

Table 1 Age distribution of the patients covered in the study

	Surgery cases	Autopsy cases	Postmortem cases
n	100	97	31
Arithmetic mean	39.2	49.5	52.0
Standard deviation	1.8	2.2	2.6

Results

Selectins in intact skin

In uninjured skin, P-selectin (Fig. 1 a) was detected with a low intensity (+) on endothelial cells in 15% of the investigated blood vessels of the dermis and subcutis. Thrombocytes were positive in all samples and were used as the positive internal control test.

E-selectin (Fig. 1 b) was not detected on endothelial cells in 99% of the cases with only one sample showing a low reaction for 11% of the blood vessels investigated. Keratinocytes were negative in all cases.

L-selectin (Fig. 1 c) was observed on 27% of the intravascular leukocytes but occurred on endothelial cells of the blood vessels in only 1% of the samples. Basal keratinocytes showed low positive immunohistological reactions in 15% of the samples.

Selectins in injured skin

In injured skin, the P- and E-selectins showed both an increase in the semi-quantitatively determined intensity of the immunohistological staining reaction and a higher number of blood vessels with a positive reaction.

[2] The Mann-Whitney rank sum test is used to test the null hypothesis that two samples were not drawn from populations with different medians. The rank sum test is a nonparametric procedure which does not require assuming normality or equal variances

Fig. 1 a–c Staining intensity of selectins in uninjured skin of the head (Paraffin, ABC; × 250) **a** P-selectin: low positive diffuse reaction of the endothelial cells (arrowhead) of small subepidermal blood vessels, **b** E-selectin: endothelial cells (arrowhead) of dilated blood vessels of the dermis are negative, **c** L-selectin: single epidermal and subepidermal granulocytes (arrowhead) with positive reaction. Endothelial cells of the subepidermal blood vessels are negative ▶

Fig. 2 a–c Four-hour-old lacerated/contused wound of the face (Paraffin, ABC; × 250) **a** P-selectin: strong positive membranous reaction of the endothelial cells (arrow) of small subepidermal blood vessels, **b** E-selectin: strong positive membranous reaction of the endothelial cells (thick arrow) of dilated blood vessels of the dermis. Adhesion and emigration of numerous granulocytes. Single perivascular granulocytes (thin arrow) with low positive reaction, **c** L-selectin: numerous positive intravascular and extravascular granulocytes (thin arrow). Endothelial cells of the subepidermal blood vessels are negative

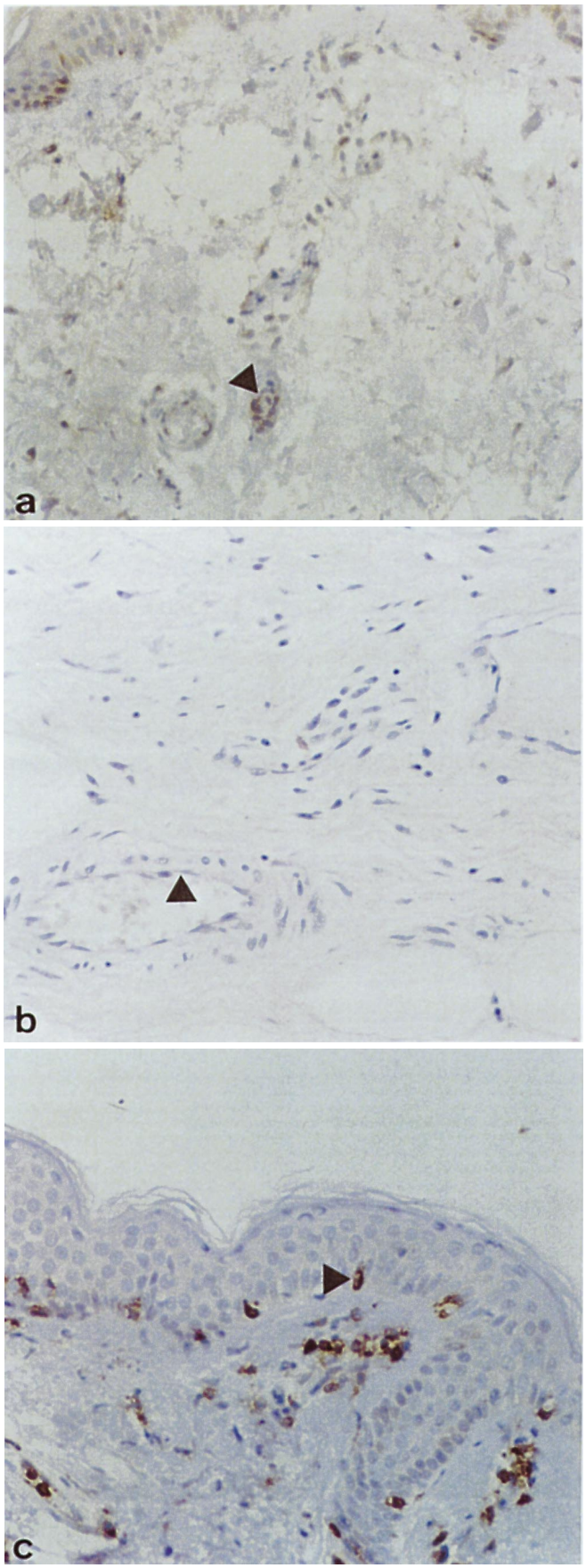

Fig. 1a–c

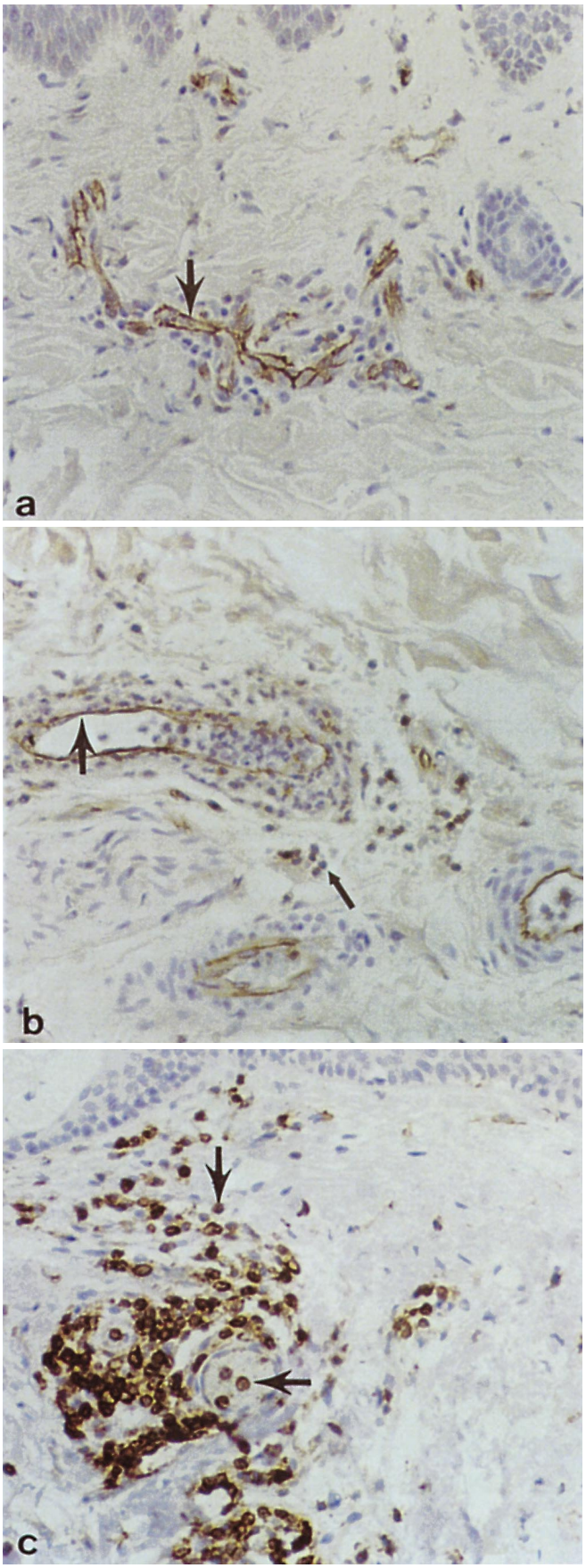

Fig. 2a–c

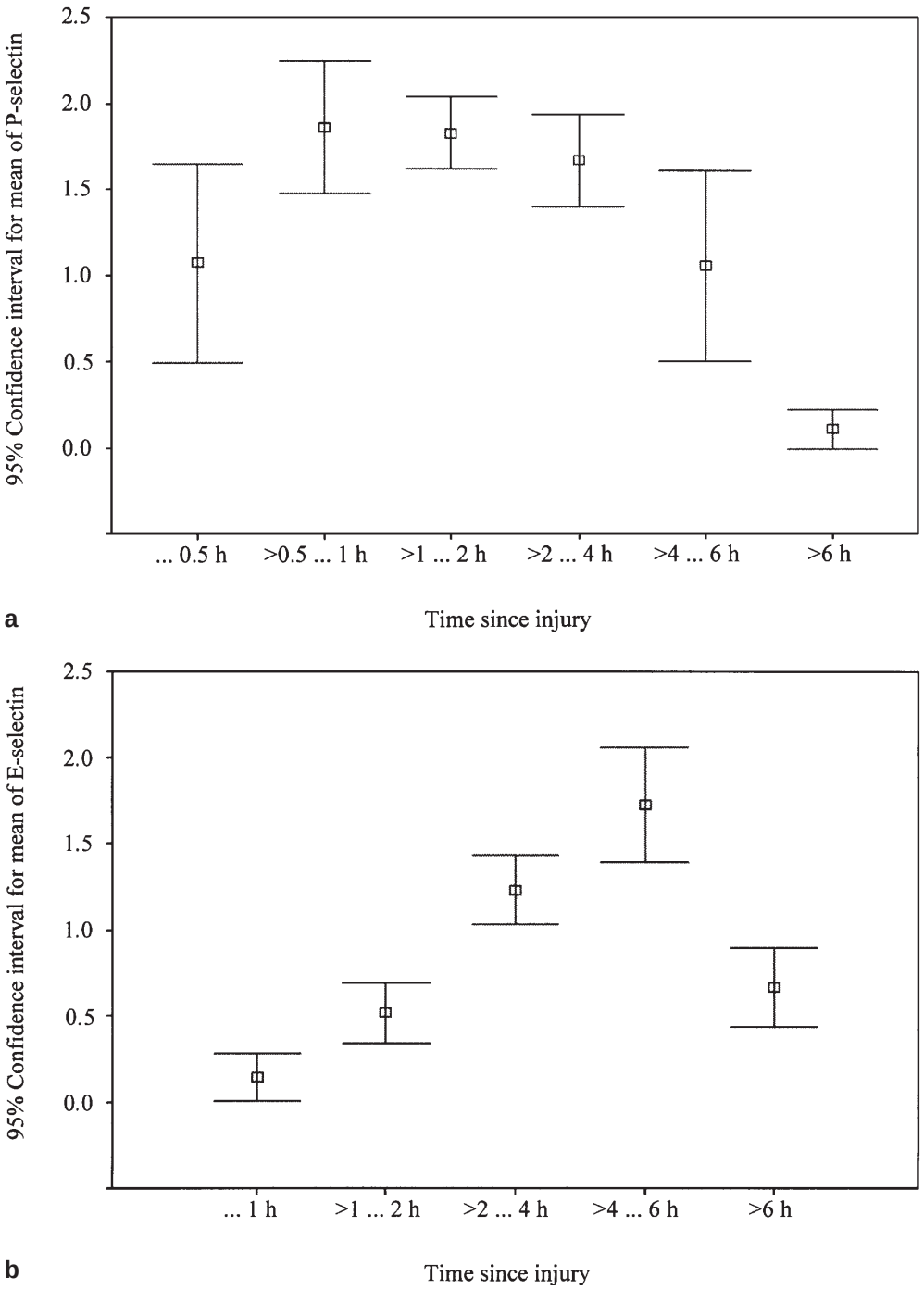

Fig. 3 a, b Confidence interval for mean of the staining intensity of P- and E-Selectin vs. time since injury, **a** P-selectin, **b** E-selectin

P-selectin (Fig. 2 a) was found to occur with moderate (++) or strong (+++) intensity on endothelial cells of 36% of the blood vessels investigated. The percentage of blood vessels with a positive reaction in injured and uninjured skin differed significantly ($P < 0.01$). The staining intensity of P-selectin on endothelial cells of the blood vessels was found to be elevated, especially in the vicinity of inflammatory infiltrates. In 37% of the wounds investigated, leukocytes with low to moderate positive reaction were observed.

E-selectin (Fig. 2 b) could be detected with low (+) to moderate (++) staining on endothelial cells in 51% of the cases, which was significantly higher ($P < 0.01$) compared with uninjured skin. Low immunohistological reactions of the perivascular leukocytes were observed in 8% of the wounds.

Microscopic epidermal and subepidermal inflammatory infiltrates were detected in 79% of the wounds. L-selectin (Fig. 2 c) was present on leukocytes in all of these cases, whereas only 4% of the samples showed low positive reactions on the endothelial cells of the blood vessels. Statistically, this result does not differ ($P = 0.174$) from the few blood vessels with positive reaction in uninjured skin.

The immunohistological reactions of the selectins showed a stronger expression in the margins and bottom areas of the wound than in the adjacent uninjured tissue. A comparison of the distributions of the staining intensity in the skin wounds of the autopsy cases with those in the surgical cases revealed no significant difference for the P- and L-selectins ($P = 0.134$ and 0.824) but a significant difference for the E-selectin ($P = 0.017$).

Selectins in skin injured postmortem

Selectins could not be detected in 90% of the samples taken from skin wounds induced postmortem. Only P- and L-selectins were found to occur with low (+) intensity on endothelial cells of 13% of the blood vessels and 16% of intravascular leukocytes respectively. The comparison and distribution of immunohistological staining reactions of the selectins of skin wounds induced postmortem with vital injuries (autopsies and surgical case material) showed significant differences ($P < 0.01$).

Time dependence of selectin expression

Strong positive immunohistochemical reactions for P-selectin were observed earliest in wounds after 3 min and at the latest after 7 h. A positive staining reaction for the E-selectin was found in skin wounds aged 1 h at the earliest and 17 days after injury at the latest; the intensity of the staining reaction was seen to decrease significantly 12 h after injury ($P < 0.05$). L-selectin could regularly be detected on leukocytes in injured skin. The migration of the leukocytes could be observed at the earliest in 15-minute-old wounds.

The intensity of the expression of immunohistochemical reactions for P-selectin was found to increase up to a wound age of 1 h. The same trend was shown by the "confidence belt" made up of the confidence intervals (Fig. 3a). After that, a decrease in the staining intensity in the samples investigated was evident for P-selectin. In 68 skin wounds with a time since injury in excess of 6 h, the mean of the staining intensity was 0.15. For the E-selectin, the intensity of the immunohistological reactions was found to increase up to a wound age of between 4 and 6 h (Fig. 3b). Older skin wounds clearly showed a decrease in the staining intensity (arithmetic mean = 0.60).

Discussion

McEver et al. [19] detected P-selectin in the α-granules of thrombocytes and in the Weibel-Palade's bodies of endothelial cells. A degranulation in which P-selectin is expressed on the luminal cell surface may occur after 5–30 min from the stimulation of the endothelial cells [13, 26]. Through the discharge of proinflammatory cytokines [12], injuries will lead to an up-regulation of the adhesion molecules on the endothelial cells. In the injured skin studied, a moderate or strong expression of P-selectin was found on 36% of the blood vessels investigated. In uninjured skin, however, 15% of the blood vessels showed a low expression of P-selectin on endothelial cells. Strong positive immunohistochemical reactions were observed for the P-selectin earliest 3 min after injury and latest after 7 h. These findings are in concordance with the results reported by Silber et al. [28] and Ohnishi et al. [22], who observed the expression of P-selectin within few minutes after intradermal injection of endotoxin or anti-ovalbumin (Arthus reaction) in animals. Ortmann and Brinkmann [24] reported a strong immunohistochemical staining reaction for P-selectin on blood vessels in the lungs from autopsy cases where death had occurred rapidly (hanging, carbon monoxide and cyanide intoxication), but a decrease of stainability of the endothelial cells of the blood vessels in cases with protracted death as a result of pneumonia and septic shock. The causes discussed are the metabolization of P-selectin after reinternalization and enzymatic cleavage on the endothelial cell membrane. These biochemical processes are also considered as possible causes for the down-regulation of the selectins on the blood vessels of injured skin.

Pober and Cortan [26] found out that E-selectin is expressed on blood vessels 2-6 h after an inflammatory irritation and could be detected for a period of up to 2 days. This process is a new synthesis because E-selectin does not occur preformed in the endothelial cells. In the studies we undertook, E-selectin could not be detected in uninjured skin in 99% of the cases; but E-selectin could be found in skin wounds in low or moderate expression on endothelial cells in 51% of the cases. The expression of E-selectin was evident in one-hour-old skin wounds at the earliest and 17 days after injury at the latest. The intensity of the staining reaction was observed to decrease significantly 12 h after injury. The time dependence of E-selectin expression we found is more or less in line with the conclusions of Bevilacqua et al. [3], Nwariaku et al. [21] and Abe et al. [1].

L-selectin is unanimously mentioned as an adhesion molecule [29] and is inherently expressed by leukocytes. L-selectin was regularly detected on leukocytes when inflammatory infiltrates occurred in the investigated samples of injured and uninjured skin. The immunohistochemical staining correlates with the density of the leukocyte infiltrates.

Compared with the samples of uninjured skin, the P- and E-selectins in injured skin showed a statistically significant difference ($P < 0.01$) of the semi-quantitatively recorded intensity of the immunohistological staining reaction and an increasing 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 [2]. E-selectin alone does not occur in the blood vessels of uninjured skin and the earliest and latest times for detection could be established in our study. A detection threshold had to be introduced for P-selectin because it was found in low intensity on endothelial cells of the blood vessels of uninjured skin. In the injured

skin samples studied, a statistically significant "regular" level P- and E-selectin expression could not be defined for any wound age interval. However, L-selectin could be detected regularly on leukocytes, which were found to migrate in 15-min-old wounds at the earliest.

The comparison of immunohistological staining reactions of the selectins of skin wounds induced postmortem with vital injuries showed significant differences ($P < 0.01$) and allow the vitality of the injury to be assessed.

The samples taken from the wounds of autopsy cases were 9 days old at the most. Although it was originally suspected that the selectins might decompose by autolysis during cold storage, this could not be substantiated. Fieguth et al. [8] found that lysozymes of the skin are also relatively resistant to autolysis.

In conclusion, it can be said that the detection of an increased expression of P- and E-selectins can improve the wound age assessment in injuries with short survival times. L-selectin is not suitable for estimating the age of a wound with the accuracy needed for forensic purposes.

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