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Time dependence of the expression of ICAM-1 (CD 54) in human skin wounds

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Abstract To characterize the vitality and age of skin wounds by means of the ICAM-1 pattern, 157 intravital human skin wounds (time since injury ranging from 5 min to 730 days) were immunohistochemically investigated. ICAM-1 was detected in paraffin sections after autoclaving and using the ABC technique in 86% of the wounds investigated. The correlation between ICAM-1 expression and the degree of wound inflammation is weak. Strong positive staining was observed 1.5 h at the earliest and 3.5 days at the latest after the time of injury. ICAM-1 also appeared at low concentrations in samples of uninjured skin (n = 65), on keratinocytes and the endothelial cells of blood vessels. Moderate to strong ICAM-1 expression is a valuable indication of the vitality of the wound. However, at present the detection of ICAM-1 *alone* is not sufficient to fix the wound age with the accuracy which is required for forensics applications.

Key words ICAM-1 · CD 54 · Wound age · Immunohistochemistry

Introduction

The survival time of the deceased following injury is often unknown at the time of autopsy. Using conventional histological methods, the age of a wound and thus the vitality of the injury can only be assessed to a certain degree, primarily because of the lack of markers in the initial state of the wound healing process [6, 17].

Adhesion molecules identified in recent years initiate a cascade of bonding reactions leading to the emigration of lymphocytes, monocytes and granulocytes from the blood vessels into the tissue [23]. The intercellular adhesion molecule 1 (ICAM-1, CD 54) is an integral membrane glycoprotein with a molecular weight of 85–110 kDa [10, 33] with an extracellular part comprising 453 amino acids and consists of 5 immunoglobulin domains [33]. It is considered to belong to the immunoglobulin supergene family and is capable of being a ligand for the leucocyte-function-associated antigen 1 (LFA-1) [22].

ICAM-1 has been found on the surface of human keratinocytes and endothelial cells of inflamed skin [9–11, 14, 19, 34] and several pro-inflammatory cytokines may up-regulate ICAM-1 expression [8, 11, 20, 21, 31, 32]. In animal experiments, Nwariaku et al. [27] observed that in burn injuries the immunohistochemical staining intensity of ICAM-1 increased after 2 h and decreased after 24 h. In their in-vitro and in-vivo studies, Griffiths et al. [15] and Norris et al. [26] found that the up-regulation of ICAM-1 on endothelial cells begins some 4 h after injury and may last several days. Krutmann et al. [18] saw an increase in ICAM-1 surface expression in cytokine-induced keratinocytes after 4 h and the maximum was reached after 16–24 h. According to Behrends et al. [2] ICAM-1 expression increases at the earliest 3 h after irradiation.

The aim of the present paper is to establish a correlation between ICAM-1 expression and wound age in the healing process of injured skin and to ascertain whether the results can be used for more precise estimation of the time since injury.

Materials and methods

The investigated material originated from lacerated wounds, incised wounds and excoriations taken from 157 individuals (male = 108, female = 49). A total of 65 samples originated from our au-

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Table 1 Age distribution of the cases covered by the study (age in years)

	Autopsy cases		Surgery cases	
	Male	Female	Male	Female
n	42	23	65	27
Median	42.8	57.6	32.0	54.3
Arithmetic mean	46.9	50.9	34.2	53.6
95% Confidence interval of mean	[41.6, 52.3]	[40.2, 61.5]	[31.0, 37.4]	[44.6, 62.5]

topsy cases, whereas 92 samples were taken during the surgical treatment of wounds after the patients had been informed of the purpose of the sampling and given their consent.

The autopsy samples were taken in the course of routine injury diagnosis. Each sample was compared with a sample of intact skin taken from the same part of the body. The autopsy cases had been kept at 4 °C in a cooling chamber for up to 7 days.

The wound age varied between 5 min and 730 days. The time of injury was taken (for the autopsy cases) from the records of the criminal investigation department or (for the surgical cases) from the anamnestic information of the patients themselves.

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.

The age distribution of the cases covered by the study is shown in Table 1.

The age distribution of the female autopsy cases did not significantly differ from that of the surgical cases. This, however, was not true for the male cases as the confidence intervals for average age (Table 1) do not overlap.

The specimens were prepared in the following way: after being fixed in 4% PBS-formaldehyde solution, 2–4 µm thick paraffin sections were cut and stained with hematoxylin-eosin or by the van Gieson method. For application of the indirect avidin-biotin complex method (ABC) the sections were dewaxed, hydrated and rinsed with PBS buffer (10 mM, pH 7.4). Endogenous peroxidase was blocked with 1% H_2O_2 followed by autoclaving for 10 min at 120° C (Varioklav, H+P Labortechnik GmbH). The sections were initially incubated with normal serum at 37° C for 15 min and then covered with a ready-for-use primary antibody solution (monoclonal mouse anti-human ICAM-1, Biogenex, Hamburg) at 37° C for 2 h and at 4° C overnight. This was followed by incubation with pre-diluted 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, and staining with DAB at 20° C for 5–10 min, followed by nuclear staining with haemalum.

The specimens immunohistochemically prepared under standardized conditions at 180 × magnification. The staining intensity was assessed semi-quantitatively using a four-category ordinal scale (∅ = negative; + = low, ++ = moderate, +++ = strong expression). Expression was negative (∅) if the tissue investigated showed no positive brown staining reaction. In the (+) category, up to 30% of endothelial cells, keratinocytes and leukocytes showed a positive brown staining. Samples in which 30–60% of these cells showed positive reactions were (++) rated, and for a (+++) rating more than 60% of the cells had to show a strong positive immunohistochemical staining reaction.

A PBS buffer was substituted for the primary antibody in the negative control test, and either lung tissue (with known radiation fibrosis [16]) or tonsil samples from the person concerned (the samples were stained with the ICAM-1-specific antibody and ABC kit of the same batch) were used for the positive control experiment.

All specimens were independently evaluated by two investigators.

Table 2 Distribution of the staining intensity of ICAM-1 in intact skin (%)

ICAM-1	(∅)	(+)	(++)	(+++)
PIC	87.7	12.3	0.0	0.0
bKC	66.2	33.8	0.0	0.0
KC	73.8	26.2	0.0	0.0
EC	66.2	33.8	0.0	0.0

PIC = perivascular infiltration cells, bKC = basal keratinocytes, KC = keratinocytes, EC = endothelial cells

Results

ICAM-1 in intact skin

In uninjured skin (Table 2), ICAM-1 was detected to a minor extent (+) on endothelial cells (EC) of the vessels in the dermis and subcutis and on keratinocytes (bKC, KC) in the epidermis. Leukocytes of perivascular infiltrates (PIC) showed a low positive (+) staining reaction in 12% of the samples. No moderate (++) or strong (+++) immunohistological staining was observed on the cells investigated.

ICAM-1 in injured skin

In injured skin (Fig. 1), moderate or strongly positive staining reactions were observed on the keratinocytes (cell surface and cytoplasm) of all three layers of the epidermis (basal cells, prickle cells and granular cells). No ICAM-1 was found on the Langerhans cells.

ICAM-1 staining was more intensive on endothelial cells, particularly near inflammatory infiltrates. The granulocytes and lymphocytes in the perivascular infiltrates showed positive staining reactions (Fig. 2a, b).

In the skin wounds investigated, a moderate or strongly positive staining reaction for ICAM-1 was observed in 15%–35% (n = 157) of the cells (Table 3). The reaction was stronger at the wound edge and bottom than in the adjacent uninjured tissue.

The ICAM-1 expression patterns for PIC, bKC and KC in injured and uninjured skin from the autopsy cases differed significantly ($P < 0.001$). No significant differences were found for EC in this sample group ($P = 0.054$). The results were tested by the Mann-Whitney rank sum test[1] at a significance level of $\varepsilon = 0.95$.

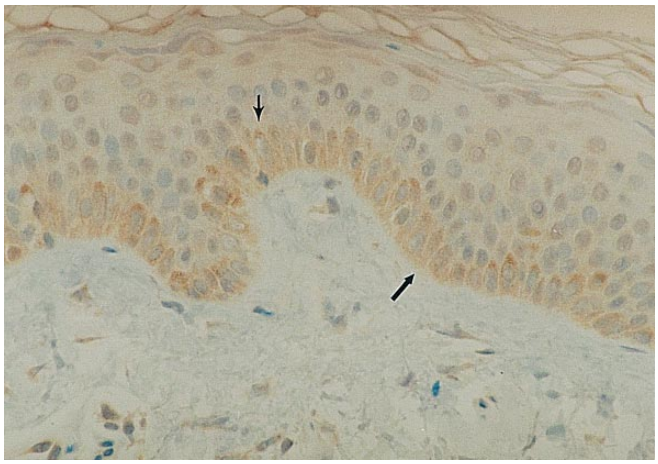

Fig. 1 Two-hour-old lacerated/contused scalp wound (case # 33; paraffin, ABC; 360 ×): Epidermis with strong ICAM-1 expression on keratinocytes of basal cells (*thick arrow*) and prickle cells (*thin arrow*)

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. The *P* value is the probability of falsely concluding that the two groups differ (i.e. the probability of falsely rejecting the null hypothesis, or committing a Type I error). The smaller the *P* value, the greater the probability that the samples are drawn from different populations.

Comparison of the distributions of the staining intensities in the skin wounds of autopsy cases with those in the surgical cases (Table 3) revealed no significant differences for PIC, bKC and KC ($P = 0.085$, 0.096 and 0.095), but a significant difference ($P = 0.002$) for EC. This also applies to the overall ICAM-1 distribution:

- comparison of autopsy and surgical cases: $P = 0.019$ (significant difference)
- autopsy cases, comparison of injuries and intact skin: $P < 0.001$ (significant difference).

Microscopic intraepidermal and subepidermal inflammatory infiltrates were observed in 71% of the wounds investigated. They consisted almost exclusively of neutrophilic granulocytes, isolated monocytes and lymphocytes. Hemorrhages under the skin were present in 92% of the skin wounds.

Time dependence of ICAM-1 expression

The time dependence of the ICAM-1 staining reaction in injured skin is shown in Table 4. The results obtained for the intact skin samples and those obtained for injured skin samples are shown in the same line (columns 7 to 11).

The earliest emigration of inflammatory cells was observed in 15-min-old wounds. The granulocytes and leukocytes in the perivascular infiltrates showed the first strong positive (+++) staining reaction 2 h after injury.

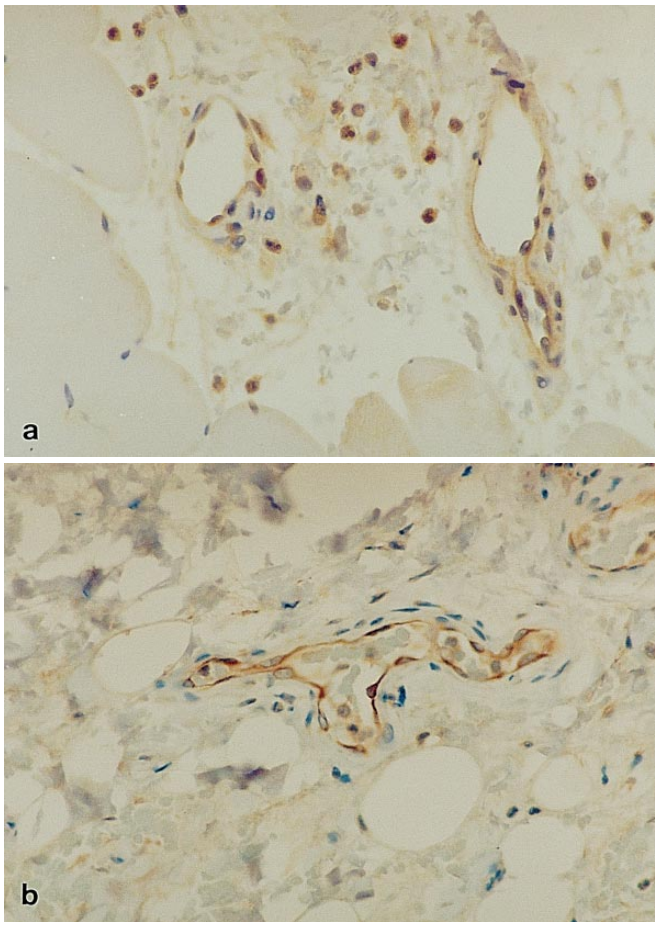

Fig. 2 Four-hour-old surgical wound of the hand (case # 118; paraffin, ABC; 360 ×). **a** ICAM-1 strongly positive endothelial cells of dilated subepidermal blood vessels with strongly positive leucocyte adhesion and emigration. **b** ICAM-1 strongly positive endothelial cells of a venule

Table 3 Distribution of the staining intensity of ICAM-1 in injured skin (%)

Sample	ICAM-1	(∅)	(+)	(++)	(+++)
Autopsy cases ($n = 65$)	PIC	52.3	32.3	13.8	1.5
	bKC	26.2	53.8	10.8	9.2
	KC	60.0	20.0	10.8	9.2
	EC	52.3	26.2	12.3	9.2
Surgery cases ($n = 92$)	PIC	37.0	41.3	19.6	2.2
	bKC	9.8	64.1	20.7	5.4
	KC	20.7	44.6	25.0	9.8
	EC	21.7	44.6	28.3	5.4

PIC = perivascular infiltration cells, bKC = basal keratinocytes, KC = keratinocytes, EC = endothelial cells

[1] This test and the tests referred to hereinafter are standard tests as used in mathematical statistics and described by Müller [24] and Rasch [29]. The numerical treatment of the tests was performed by the SigmaStat software from Jandel Scientific GmbH, Erkrath, Germany ("SigmaStat Statistical Analysis System" V1.02 [1992])

Table 4 Frequency of positive ICAM-1 reactions vs. wound age

Wound age [h]	Autopsy cases										Surgery cases				
	n	Injured skin				*n*	Intact skin				*n*	Injured skin			
		(∅)	(+)	(++)	(+++)		(∅)	(+)	(++)	(+++)		(∅)	(+)	(++)	(+++)
0… 1 h	6	2	4	0	0	6	5	1	0	0	2	0	2	0	0
1… 2 h	8	2	6	0	0	8	5	3	0	0	19	1	11	6	1
2… 3 h	7	2	3	1	1	7	5	2	0	0	16	0	5	8	3
3… 4 h	4	0	4	0	0	4	2	2	0	0	17	2	8	6	1
4… 5 h	3	1	0	1	1	3	1	2	0	0	15	0	8	4	3
5…24 h	6	0	1	2	3	6	4	2	0	0	10	0	3	5	2
1… 2 d	3	1	0	1	1	4	2	2	0	0	0	0	0	0	0
2… 3 d	7	0	3	4	0	6	5	1	0	0	0	0	0	0	0
3… 4 d	3	0	2	0	1	3	1	2	0	0	1	0	1	0	0
4… 5 d	4	2	1	1	0	4	3	1	0	0	1	1	0	0	0
5…10 d	8	2	5	1	0	8	5	3	0	0	1	1	0	0	0
> = 10 d	6	2	4	0	0	6	5	1	0	0	10	3	4	3	0
Sum	65	14	33	11	7	65	43	22	0	0	92	8	42	32	10

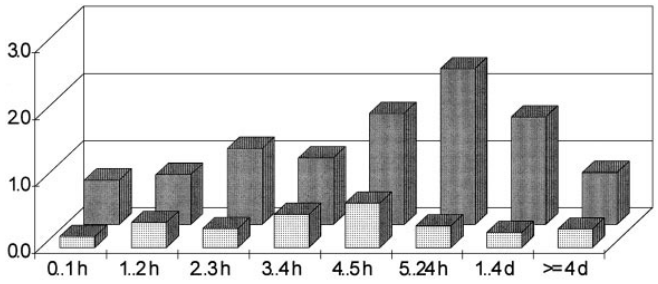

Fig. 3 Staining intensity of ICAM-1 (autopsy cases) during the time after injury (*in front:* intact skin)

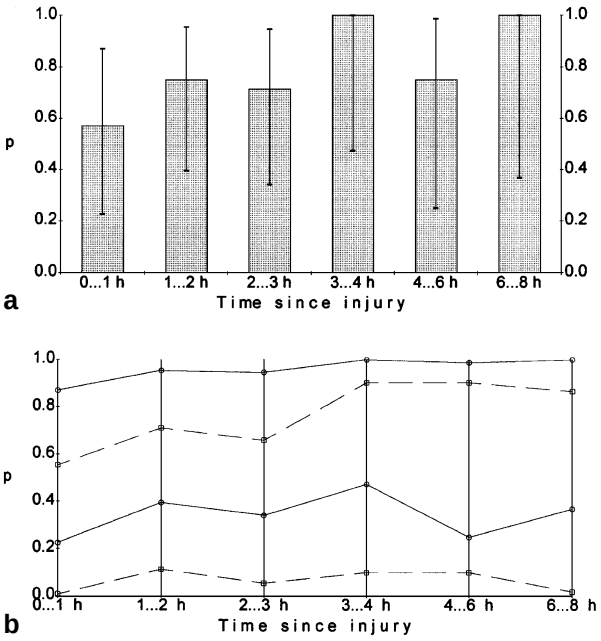

Fig. 4 **a** Confidence interval estimation [25] of the frequency (*p*) of positive ICAM-1 reactions in the injured skin of autopsy cases vs. time since injury. **b** Confidence belts for the frequency (*p*) of positive ICAM-1 reactions in injured skin (*solid lines*) and intact skin (*dashed lines*) vs. time since injury

A strong positive ICAM-1 staining reaction (+++) on endothelial cells and keratinocytes was observed 1.5 h after injury at the earliest. The oldest wound with a strong positive (+++) staining reaction was 3.5 days old.

Assuming the number of cases with j "+" ratings for the ICAM-1 expression to be n_j (j = 0, 1, 2, 3), the frequency p of positive reactions is obtained as

$$p = (n_1 + n_2 + n_3) / n,\ n = n_0 + n_1 + n_2 + n_3.$$

The mean intensity i of the ICAM-1 expression can be defined by:

$$i = (n_1 + n_2 + n_3) / n.$$

Figure 3 shows the intensity (i) as a function of time since injury for the autopsy cases. As (++) or (+++) ratings did not occur for intact skin in the autopsy cases (Table 4), the numerical values of frequency (p) and intensity (i) are equal.

Since the distributions of positive ICAM-1 expression in injured skin differs widely between surgical and autopsy cases, these groups were not pooled. It is remarkable in this connection that the wound age distribution of the ICAM-1 positive surgical cases differed significantly from that of the autopsy cases (median of wound age for the surgical cases: 3.0 h, median of wound age for the autopsy cases: 12.8 h; Mann-Whitney rank sum test, P = 0.005).

The frequency of positive reactions and the intensity of expression increased perceptibly for up to 4 h after injury. This trend can be statistically verified insofar as the "confidence belt" made up of the confidence intervals also shows the same trend (Fig. 4a).

If the sampling frequency p is considered to be the estimation of the "true" but unknown frequency π of an event, then the confidence interval is the estimated range

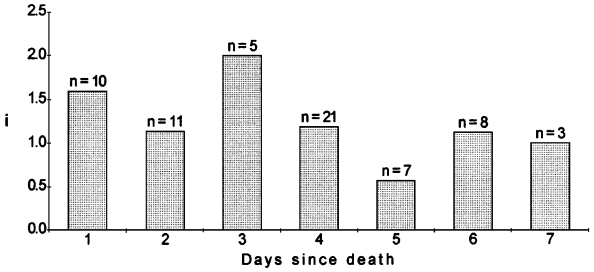

Fig. 5 Staining intensity (*i*) for ICAM-1 versus time after death

in which the true frequency will fall for a percentage $(1–\alpha)$ of all possible samples drawn from the population; α is the error probability given.

A first impression as to whether and to what extent ICAM-1 expression can be used as an indicator of the time since injury is obtained by the graphic representation of the confidence belts for the ICAM-1 frequencies in injured and intact skin (Fig. 4b).

Figure 5 shows the mean staining intensity as a function of the time of sampling after death; the maximum time was 7 days.

Discussion

Nwariaku et al. [27] detected an increase in ICAM-1 expression after skin injury whereby the up-regulation of ICAM-1 in the skin is a wound healing reaction [18, 19]. In the study described here, moderate to strong ICAM-1 expression was detected on 15%–35% of the investigated cells in injured skin. However, 27% of the samples taken from uninjured skin also showed some ICAM-1 expression on the endothelial cells of blood vessels, keratinocytes and leukocytes. This is in concordance with results reported by Dustin et al. [11] who state that these cells can express ICAM-1 even without inflammatory irritation.

The expression patterns on keratinocytes and perivascular inflammatory cells (granulocytes, lymphocytes and monocytes) in injured and uninjured skin differ significantly. The forensic interest is in the earliest, "normal", and latest detectability of the reaction in skin wounds for estimating wound age [4, 5]. The emigration of inflammatory cells was observed in 15-min-old wounds at the earliest. Their presence was not rated as positive until at least three neutrophilic granulocytes were found outside the blood vessels, i.e. not in hemorrhages [3, 5, 13].

The immunohistological staining reaction for ICAM-1 correlates only weakly with the density of the leukocyte infiltrates.

In our investigations, strong immunohistochemical reactions for ICAM-1 were not found until 1.5 h after injury, presumably owing to time-dependent up-regulation of ICAM-1 in injured skin [18, 27]. A relationship between the infiltration of the wound with leukocytes and the ICAM-1 expression is suspected [27].

The detection threshold we found is more or less in line with the conclusions of Nwariaku et al. [27], but contradicts those of Behrends et al. [2], Griffiths et al. [15], Norris et al. [26] and Krutmann et al. [18], all of whom claim that ICAM-1 surface expression increases later.

In the injured skin samples we studied, a statistically significant "normal" level of ICAM-1 expression could not been defined for any wound age interval. The oldest wound with strong ICAM-1 expression was 3.5 days old. One possible source of the discrepancy between the reported variations in time of ICAM-1 expression might lie in the uncertainty of the anamnestic information concerning the time of injury. In our study, this information was obtained from police records for the autopsy cases, but for the surgical cases we had to rely on unverifiable information imparted by the patients themselves. The fact that relatively few wounds were investigated shortly after injury, so that individual errors could not be "averaged out", adds to the uncertainty.

The differences between the results published in the literature and and those of our study may conceivably also be caused by differences in materials (in our case paraffin sections, compared to cryostatic sections or cell culture) and the methods (immunochemistry in our case compared to flow cytometry). Moreover, most of the results given in the literature were obtained in animal experiments (mouse, rat), although the skin morphology and circulatory systems of these species are known to differ from those of man [7].

Surgical wounds were included in our study to build up the broadest possible database for wound age estimation as the amount of autopsy material is restricted. Unfortunately, analysis of the data revealed that the distribution of positive ICAM-1 reactions in surgical cases differs significantly from that for autopsy cases. The most probable cause of this seems to be the difference between the wound age distributions for the autopsy cases (median of wound age = 12.8 h) and surgical cases (median of wound age = 3.0 h). Further possible causes include differences between the structures of the material donor populations and differences in sampling collection.

Male autopsy cases were significantly younger than the male surgical patients, and healing times are known to depend not only on the type of trauma and topography of the wound, but also on the age and general condition of the subject [28]. Moreover, the skin samples from the patients were taken in the course of regular surgical procedures, whereas they were taken from autopsy cases without surface treatment.

The samples taken from the wounds of autopsy cases were 7 days old at the most. Although it was originally suspected that the ICAM-1 might decompose by autolysis during cold storage [12, 30], this could not be substantiated. Our immunohistochemical methods revealed no significant difference in the ICAM-1 staining intensity which with respect to wound age determination, is a desirable effect.

The semi-quantitive approach used to assess the immunohistochemical staining intensity for ICAM-1 allows the vitality of the wound to be assessed. What stands in the way of determining wound age from the intensity of ICAM-1 staining is that the confidence belts for the de-

tection frequency in injured and intact skin partly overlap (Fig. 4b).

In a first approximation, the width of a confidence interval is inversely proportional to the square root of the sample size. Only further experiments involving more samples can show whether and to what extent the confidence belts can be separated. However, the different results obtained for autopsy and surgical cases suggest that the "natural" (residual) scatter in the parent population is considerable.

The immunohistochemical detection of ICAM-1 does not make excessive demands on laboratories. The pretreatment (autoclaving) of the paraffin sections should pose no problems [1], and all antibodies and reagents are commercially available. It is important, however, that the experiments are made under standardized conditions, i.e. with monoclonal primary antibodies and a standardized staining kit.

In conclusion it can be said that detection of increased ICAM-1 expression alone is currently not sufficient for estimating the age of a wound with the accuracy needed for forensic purposes. Nonetheless, its use as an aid in immunohistochemical wound age assessment is thought to be useful and practicable.

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