Analysis of inflammatory cells and mediators in skin wound biopsies to determine wound age in living subjects in forensic medicine

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ABSTRACT

Objective: In forensic medicine it is important to determine the age of skin wounds in living subjects. The aim of this study was to assess whether analysis of inflammatory cells and inflammatory mediators in skin biopsies of wounds from living subjects could improve wound age determination.

Methods: Biopsies (n=101), representing the superficial border area of a skin wound, were taken from skin injuries of known wound age (range: 4.5 hours-25 days) of living subjects. All biopsies were analysed for 3 inflammatory cell markers (MPO, CD45 and CD68) and 4 inflammatory mediators (MIP-1, IL-8, CML and vitronectin). For quantification, biopsies were subdivided in 4 different timeframes: 0.2-2 days, 2-4 days, 4-10 days and 10-25 days old wounds. Subsequently, a probability scoring system was developed.

Results: MPO, CD45, MIP-1, IL-8 (inflammatory cell markers) and N(epsilon)-(carboxymethyl)lysine (CML) positivity was maximal in wounds of 0.2-2 days old and then decreased in time. Remarkably, CD45, CD68 and CML showed a minor but non-significant increase again in 10-25 days old wounds. MPO and CD68 positivity was significantly lower in 4-25 days old wounds compared to 0.2-4 days old wounds. MPO positivity was also significantly lower in 10-25 days old wounds compared to 0.2-10 days old wounds. For CD45, MIP-1, IL-8 and CML no significant differences between the age groups were found. In case of vitronectin positivity in the extravasate or when the number of MIP-1 or IL-8-positive cells was more than 10 cells/mm² the probability that the wound was more than 10 days old was 0%. A probability scoring system of all analysed markers can be used to calculate individual wound age probabilities in biopsies of skin wounds of living subjects.

Conclusions: We have developed a probability scoring system of inflammatory cells and mediators that can be used to determine wound age in skin biopsies of living subjects.
**INTRODUCTION**

In forensic medicine it is essential to determine wound age. In the Netherlands, when victims of physical abuse report a crime, they are referred to a forensic physician for an assessment of the injury, including wound age estimation. It is however known that the macroscopical description of a wound to determine wound age is inadequate. Hence there is need for a more adequate method to determine wound age in living subjects. In forensic autopsies, immunohistochemical analysis is used to determine wound age more precisely in skin excisions containing epidermis, dermis and (part of) the subcutis, and the wound itself. Since it is not ethical to excise skin wounds in living subjects, we wondered whether superficial skin biopsies, representing the border area of skin injuries, could also be used for (immuno) histochemical analysis to determine wound age. In this study different inflammatory markers were analysed, that were mostly studied in previous autopsy studies. Although, this is the first study on living subjects not going anesthesia, in other studies skin samples were taken from living patients undergoing surgical interventions.

The first inflammatory cells that invade the site of injury are neutrophilic granulocytes. They can be detected extravascular in human skin wounds as soon as 20-30 minutes after infliction. Lymphocytes, which are attracted to the wound by chemokines, can be found as soon as 5-6 hours post wound infliction. Also macrophages can be detected as early as 5-6 hours after wounding, although they have been described to peak at 1-2 days in wounds, as studied in both human skin and animal models. Kondo et al. showed that neutrophils also express the chemokines MIP-1 and IL-8, important mediators of the immune response, as early as 4-12 hours after wounding in autopsy skin wounds. With increasing wound age macrophages and fibroblasts were positive for MIP-1 and IL-8 as well. The inflammatory mediator vitronectin has an inhibiting role in the complement pathway and is upregulated in human fibroblasts on day 3 and day 6 after wound infliction. Finally, an important proinflammatory mediator of activated endothelium is N(epsilon)-(carboxymethyl)lysine (CML), an advanced glycation endproduct. Its role in wound age determination however is unknown.

We have now quantified these inflammatory cells and mediators in skin biopsies of living subjects, to analyse whether they can be helpful to determine wound age in living subjects.

**MATERIALS AND METHODS**

We have collected 101 human skin biopsies from patients being referred to a forensic physician of the Public Health Service in Amsterdam from May 2008 until November 2009. Patients with skin injuries of known age were asked permission to obtain a wound biopsy. The
mean age of the population was 37 years (range: 17-80 years), consisting of 52 males (51%) and 49 females (49%). Mean wound age, as reported by the victim, was 5.4 days (range: 4.5 hours-25 days). 3 cases were excluded, as there was not enough material. The distribution of wound age in the other 98 cases was as follows: 0.2-2 days (n=27), 2-4 days (n=28), 4-10 days (n=28) and 10-25 days (n=15). The wounds were identified macroscopically. The different types of injuries were bruises (69%), abrasions (19%), bites (3%), stabs (2%), scratches (2%), unknown (3%) and firework (1%). Permission for this study was given by the Medical Ethical Commission (METC) of the VUmc.

**Histochemistry and immunohistochemistry**

Skin biopsies were fixed in 4% formaldehyde solution and embedded in paraffin. For histochemical analysis, 4 µm-thick tissue sections were stained with Hematoxylin/Eosin (H&E) according to standard methods. For immunohistochemical analysis tissues were deparaffinized and rehydrated and incubated in methanol/H2O2 (0.3%) for 30 minutes to block endogenous peroxidases. Antigen retrieval was performed by either boiling slides for 10 minutes in a citrate pH 6.0 buffer (MPO, CD68 and vitronectin), or Tris-EDTA pH 9.0 (IL-8) solution or by incubation with pepsine/HCl (0.1%) (MIP-1, CML) for 30 minutes at 37°C. Sections were incubated with either rabbit anti-human myeloperoxidase (1:700, Dako, Glostrup, Denmark), mouse anti-human CD45 (1:50, Dako, Glostrup, Denmark), mouse anti-human CD68 (1:400, Dako, Glostrup, Denmark), rabbit anti-human MIP-1 (1:50, Genetex, Atlanta, USA), rabbit anti-human IL-8 (1:100, Novus Biologicals, Littleton, USA), rabbit anti-human CML (1:500, CLB, Amsterdam, The Netherlands) or mouse anti-human Vitronectin (1:2000, Abcam, Cambridge, UK) antibody for 1 hour at room temperature (RT). Next sections were incubated with anti-mouse/rabbit Envision (Dako, Glostrup, Denmark) for 30 minutes at RT. Staining was visualized using 3,3′-diaminobenzidine (0.1 mg/ml, 0.02% H2O2). Sections were then counterstained with haematoxylin, dehydrated and covered. As a control, the same staining procedure was used, but then the primary monoclonal or polyclonal antibody was replaced by phosphate buffered saline. These tissue slides were negative (not shown).

**(Immuno)histochemical analysis**

Slides were evaluated by light microscopy. The biopsies reflected the superficial border zone area of a wound, containing the epidermis and dermis. In total 8 variables were scored as follows:

*Inflammatory cells:*

- MPO (neutrophilic granulocytes), CD45 (lymphocytes), CD68 (macrophages): number of extravascular cells/mm².
Inflammatory cells and mediators to determine wound age

*Inflammatory mediators:*
- MIP-1: number of MIP-1-positive extravascular neutrophils and macrophages/mm²
- IL-8: number of IL-8-positive extravascular neutrophils and macrophages/mm²
- IL-8: intensity of the expression of the epidermis:
  - Score 0: no staining
  - Score 1: minor staining
  - Score 2: moderate staining
  - Score 3: strong staining.
- CML: each positive blood vessel was given an intensity score of:
  - Score 1: minor staining
  - Score 2: moderate staining
  - Score 3: strong staining
  Each score was multiplied with the number of positive blood vessels with that particular intensity score, divided by the total surface area, resulting in a final immunohistochemical intensity score of CML/mm²².
- Vitronectin expression in extravasate of erythrocytes and/or inflammatory cells: yes/no.

The total surface of each sample was measured using QPRODIT²².

Statistical analysis
Statistical analysis was performed with SPSS (Windows version 20, IBM corp., Armonk, NY). Differences in the variables between different wound age groups were tested with the chi-square test (dichotomous or categorical variables) or the Kruskal-Wallis and the Mann-Whitney test (non-normal continuous variables). P-values <0.05 were considered significant. Post-hoc analysis to compare two different wound age groups were corrected for multiple testing via the Bonferroni correction, in which case P-values <0.017 were considered significant.

Probability scores
For quantification wound age was divided into four groups: 0.2 to 2 days old, 2 to 4 days old, 4 to 10 days old and 10 to 25 days old. To compute the probability scores, the MPO, CD45, CD68, MIP-1, IL-8 and CML counts first had to be binned into 4 groups of approximately equal size. Then, ordinal regression analyses were applied to compute the probability that a variable has a certain value (e.g. an epidermal IL-8 expression score of 2 or no vitronectin expression) with wound age as factor. Finally, by Bayes’ rule, these probabilities could be inverted to obtain the probability that a wound has a certain age given the value of the parameter, i.e. the probability score.
RESULTS

Inflammatory cells
the number of neutrophilic granulocytes (analysed via MPO staining) (figure 1A/2A) was maximal in 0.2-2 days old wounds and then declined gradually in time, with a significant difference between the wound age groups (p=0.021). After dividing wound age in two groups (0.2-4 and 4-25 days old), the number of neutrophilic granulocytes was significantly higher in wounds of 0.2-4 days old compared to wounds of 4-25 days old (p=0.004). The same was true for wounds of 0.2-10 days old compared to wounds more than 10 days old (p=0.016). The highest number of CD45-positive lymphocytes (figure 1B/2B) was also found in wounds of 0.2-2 days old, which declined in wounds up to 10 days old. Wounds older than 10 days however showed a minor increase in the number of lymphocytes. No significant differences between the wound age groups were found (p=0.633). The highest number of CD68-positive macrophages (figure 1C/2C) was found in wounds of 2-4 days old and then declined in time, with a significant difference between the wound age groups (p=0.014). The number of macrophages in 0.2-4 days old wounds was significantly higher compared to 4-25 days old wounds (p=0.002). Also here, wounds older than 10 days showed a small increase in the number of macrophages.

Probability scores (table 1)- The probability that a wound was 0.2-2 days old was 23% in case ≤10 MPO-positive cells/mm² were found, the probability that a wound was 0.2-4 days old or 0.2-10 days old was 45% and 75%, respectively. These probabilities increased when the number of MPO-positive cells/mm² increased: 32, 63 and 93%, respectively, in case of 10-50 MPO-positive cells/ mm², and 34, 68 and 95% or 36, 72 and 96% in case of 50-100 or >200 MPO-positive cells/mm². The cumulative wound age probabilities increased for an increasing number of CD45-positive cells. The probabilities that a wound was 0.2-2 days old were 22, 26, 31 and 37% for ≤ 25, 25-50, 50-100 and >100 CD45-positive cells/mm², respectively. The probability that a wound was 0.2-4 days old ranged from 49 to 65% and that a wound was 0.2-10 days old from 83 to 88% for these four categories of the number of CD45-positive cells/mm². Except for 0.2-2 days old wounds, the cumulative probabilities increased for an increasing number of CD68-positive cells: from 48 to 75% for the probability that a wound was 0.2-4 days old and from 82 to 93% for the probability that a wound was 0.2-10 days old. The probability that a wound is 0.2-2 days old varied between 28 and 31%.

Inflammatory mediators
The highest number of inflammatory cells positive for MIP-1 (figure 1D/3A) and IL-8 (figure 1E/3B), were found in 0.2-2 days old wounds and showed a strong decline for older wounds. No significant differences between the wound age groups were found (p=0.117 for MIP-1 and p=0.191 for IL-8). In all age groups most biopsies showed a minor intensity (score 1)
of IL-8 expression in the epidermis (*figure 1F/3C*), albeit no relation with wound age was found (p=0.492). The advanced glycation end product N(epsilon)-(carboxymethyl)lysine (CML) (*figure 1G/3D*) had the highest blood vessel intensity score in wounds of 0.2-2 days old, with a decline for wounds 2-4 and 4-10 days old, and a small increase in wounds older than 10 days. No significant differences between the wound age groups were found (p=0.099). Finally, vitronectin expression (*figure 1H/3E*) was only found in skin injuries up to 8 days old. No significant differences between the wound age groups were found (p=0.058).

**Probability scores (table 1)** - In case ≤10 MIP-1-positive cells/mm² were found the probabilities that a wound was 0.2-2, 0.2-4 or 0.2-10 days old were 22%, 51% and 81%, respectively. The wound age probabilities were higher and more or less equal independent of the number of cells in case 10-50, 50-150 or >150 MIP-1-positive cells/mm² were found. Furthermore, the probability that a wound was older than 10 days is 0% in case of >10 MIP-1-positive cells/mm². The probability scores of IL-8-positive inflammatory cells (divided in groups as follows: ≤10, 10-100, 100-200 and >200) were comparable to the MIP-1 scores. The lowest probabilities were found when ≤10 IL-8-positive cells/mm² were found (25, 52 and 80% for respectively the probability that a wound was 0.2-2, 0.2-4 and 0.2-10 days old) with higher and more or less equal probabilities with a higher number of IL-8-positive cells. Also here, the probability that a wound was older than 10 days is 0% in case of >10 IL-8-positive cells/mm². The probability that a wound was 0.2-2 days old is comparable for all scores of epidermal IL-8 expression, ranging from 27 to 29%. When the epidermis showed no IL-8 expression it was more likely that the wound was more than 4 days old (56%) whereas it was more likely that a wound was less than 4 days old in case of minor, moderate or strong IL-8 expression (57, 68 and 70%, respectively).

The cumulative probabilities for CML increased with an increasing immunohistochemistry score. The probability that a wound was 0.2-2 days old was 23, 29, 32 and 34% when the CML immunohistochemistry score was ≤25, 25-50, 50-75 and >75, respectively. For these scores, the probability that a wound was 0.2-4 days old was 43, 56, 64 and 70%, respectively, and 74, 86, 91 and 94% that a wound was 0.2-10 days old.

When vitronectin was expressed in the extravasate of erythrocytes or inflammatory cells (n=24) the probability that a wound was 0.2-2 days old was 38%, that a wound was 0.2-4 days old was 76% and that a wound was 0.2-10 days old was 100%. When the extravasate did not have vitronectin expression the probabilities were lower, namely 23% (0.2-2 days old), 49% (0.2-4 days old) and 78% (0.2-10 days old).
Figure 1: Immunohistochemical pictures of the 7 different markers:

a) A 2 days old skin wound with MPO-positive cells (arrow).

b) A 1 day old skin wound with CD45-positive cells (arrow).

c) A 3 days old skin wound with CD68-positive cells (arrow).

d) A 1 day old skin wound with MIP-1-positive cells (arrow).

e) A 4.5 days old skin wound with IL-8-positive cells (arrow).

f) A 2 days old skin wound with a moderate IL-8 expression of the epidermis (arrow).

g) A 2.5 days old skin wound with CML-positive blood vessels. The arrow shows a vessel with intensity score 3.

h) Vitronectin expression of the extravasate in a 1.6 days old skin wound (arrow).

Original magnification: (a-e, g) 400x; (f, h) 200x.
Figure 2: Analyses of inflammatory cells/mm$^2$:
The number of a) MPO, b) CD45, and c) CD68-positive cells in the different groups. Wounds of 0.2-2 and 2-4 days old combined are significantly different ($p=0.004$ for MPO and $p=0.002$ for CD68) compared to wounds of 4-10 and 10-25 days old combined. For MPO also wounds of 0.2-2, 2-4 and 4-10 days old combined are significantly different ($p=0.016$) compared to 10-25 days old wounds.
Figure 3: Analyses of inflammatory mediators:
The number of a) MIP-1 and b) IL-8-positive cells in the different groups. c) The intensity of IL-8 expression of the epidermis. d) The CML blood vessel intensity score in the different groups. e) Vitronectin expression in the extravasate of erythrocytes and/or inflammatory cells. Each dot represents 1 skin wound.
Table 1. Probability scores of the 4 wound age groups for MPO, CD45, CD68, MIP-1, IL-8, CML and Vitronectin. The probabilities in bold denote cumulative probabilities, i.e. the probability that the wound is 0.2-2, 0.2-4 and 0.2-10 days old.

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<td>23%</td>
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DISCUSSION

An additional method to determine wound age in living subjects is needed, since macroscopical assessment of an injury is inadequate. In this study inflammatory cells and inflammatory mediators were evaluated in the superficial border zone of skin wounds. They discriminated
in wound age determination in human skin biopsies: vitronectin was only found in wounds of 0.2-2, 2-4 and 4-10 days old while positivity of MPO, CD45, MIP-1 IL-8 and CML was maximal in wounds of 0.2-2 days old and then decreased in time. However, for CD45, IL-8 and CML a small, non-significant increase in the oldest age group (10-25 days) was found. For MPO a significant difference was found between 0.2-4 and 4-25 days old wounds and between wounds 0.2-10 and 10-25 days old wounds. Also CD68 showed a significant difference between 0.2-4 and 4-25 days old wounds. Epidermal IL-8 expression was not discriminating.

Our finding that the highest number of inflammatory cells was found in wounds up to 2 (MPO, CD45) or 4 (CD68) days old with a decline thereafter, is consistent with the literature, where inflammatory cells are described to invade the site of injury within hours after wounding, achieving their maximum, depending on the type of inflammatory cell, from 1 to 7 days post wound infliction, studied in both animal and human post mortem material. The highest scores of the inflammatory mediators MIP-1 and IL-8 in neutrophils and macrophages were found the first 2 days post-infection. This is comparable to the findings of Kondo et al., who analysed MIP-1 and IL-8 expression in neutrophils, macrophages and fibroblasts and found a positive ratio of >40% for MIP-1 and >50% for IL-8 indicating a wound age of at least 1 day, albeit this was studied in the wound itself in autopsy material. The maximum positive ratio of MIP-1 and IL-8-positive neutrophils, macrophages and fibroblasts was found on day 2, gradually decreasing with wound age. Also CML showed the highest score in 0.2-2 days old wounds. To the best of our knowledge, no time dependant study on the upregulation of CML in healing processes of the skin was executed until now.

It has to be stressed that our study is not directly comparable to other studies in this field. In the aforementioned studies the markers were assessed within the wound and in post mortem material whereas we evaluated the parameters in the border zone area of wounds from living subjects, since it is not ethical to excise the wound in living subjects.

For three markers, MIP-1, IL-8 and vitronectin expression, the probability that a wound was more than 10 days old was 0% for certain groups (i.e. in case of vitronectin positivity in the extravasate or when the number of MIP-1 or IL-8-positive cells was more than 10 cells/mm$^2$). It has to be emphasized that, although only 51% of the biopsies showed erythrocyte extravasation (i.e. haemorrhage), the biopsies were also scored positive for vitronectin in case the extravasate of inflammatory cells showed expression. Relatively low probabilities were found for ≤ 10 MPO-positive cells/mm$^2$ compared to the other groups (i.e. 10-50, 50-200, >200). The highest probability was found for >200 positive cells/mm$^2$, giving a probability of 96% that an injury was inflicted 10 days ago or less. For both CD45 and CD68 increasing probability scores were found with an increasing number of positive cells, with highest probabilities of 88% and 93% that an injury was inflicted 10 days ago or less when >100 CD45-positive cells/mm$^2$ and >50 CD68-positive cells/mm$^2$ were found, respectively. Also the probability scores of epidermal IL-8 expression and CML more or less increased
with increasing intensity of the epidermal expression and an increasing blood vessel intensity score, respectively. Highest probabilities were found in case of strong IL-8 expression or a CML intensity score of >75, namely 91% and 94% that a wound was 0.2-10 days old respectively. The probability scoring system can be used to determine the individual likelihood of wound age for all markers.

We did not look specifically to differences on inflammatory reaction between the different types of injuries (limitation of the study). However, the vast majority of the wounds were bruises (69%) and abrasions (19%), a possible difference on inflammatory reaction in the other wound types should not affect the overall picture.

To the best of our knowledge, this is the first study assessing the use of inflammatory cell markers and inflammatory mediators in wound age determination of human skin biopsies from living subjects. It was shown that they can be used to determine wound age in forensic medicine.
REFERENCES