A new method to determine wound age in early vital skin injuries: a probability scoring system using expression levels of Fibronectin, CD62p and Factor VIII in wound haemorrhage


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ABSTRACT

Background: In forensic autopsies it is important to determine the age of early vital skin wounds as accurate as possible. In addition to inflammation, coagulation is also induced in vital wounds. Analysis of blood coagulation markers in wound hemorrhage could therefore be an important additional discriminating factor in wound age determination. The aim of this study was to develop a wound age probability scoring system, based on the immunohistochemical expression levels of Fibronectin, CD62p and Factor VIII in wound hemorrhage.

Methods: Tissue samples of (A) non injured control skin (n = 383), and samples of mechanically induced skin injuries of known wound age, (B) injuries inflicted shortly before death (up to a few minutes old) (n = 382), and (C) injuries inflicted 15-30 minutes before death (n = 42) were obtained at autopsy in order to validate wound age estimation. Tissue slides were stained for Fibronectin, CD62p and Factor VIII and were subsequently scored for staining intensity (IHC score) in wound hemorrhage (1 = minor, 2 = moderate, 3 = strong positive). Finally, probability scores of these markers were calculated.

Results: In at most 14% of the non-injured control samples, hemorrhage was found, with mean ± standard deviation IHC scores of 0.1 ± 0.4, 0.2 ± 0.4 and 0.2 ± 0.5 for Fibronectin, CD62p, and Factor VIII, respectively. Expression of these markers significantly increased to mean IHC scores 1.4 ± 0.8 (Fibronectin), 1.2 ± 0.6 (CD62p), and 1.6 ± 0.8 (Factor VIII) in wounds inflicted shortly before death (few minutes old) and to 2.6 ± 0.5 (Fibronectin), 2.5 ± 0.6 (CD62p), and 2.8 ± 0.4 (Factor VIII) in 15-30 minutes old wounds. The probabilities that a wound was non vital in case of an IH score 0 were 87%, 88% and 90% for Fibronectin, CD62p, and Factor VIII, respectively. In case of an IHC score 1 or 2, the probabilities that a wound was a few minutes old were 82/90%, 82/83% and 72/93%. Finally, in case of an IHC score 3, the probabilities that a wound was 15-30 minutes old were 65%, 76% and 55%.

Conclusion(s): Based on the expression of Fibronectin, CD62p and Factor VIII in wound hemorrhage, we developed a probability scoring system that can be used in forensic autopsies to improve wound age estimation in early skin injuries.
**INTRODUCTION**

In forensic pathology it is not only important to determine whether skin wounds are vital or not but also to give an estimation of wound age [1-10]. In the past, it was suggested that histological characteristics could classify a vital wound. For example hemorrhage, i.e. the extravasation of erythrocytes after damage of blood vessels, was postulated to represent a vital wound characteristic. Hemorrhage, however, can also occur in non vital wounds, e.g. due to mechanical manipulation of the body [11]. The same is true for swelling, which may occur in loosely arranged tissue, independent of wound infliction [3,12-14]. Therefore a pure morphological description to determine a vital wound is inadequate.

Enzyme histochemical methods were also used to define wound age in tissue sections (e.g. esterase, acid phosphatase, ATPase). However, these methods proved to be too unreliable and showed a high rate of negative cases [2].

Immunohistochemistry of wounds has been studied extensively, especially related to inflammatory cells or extracellular matrix-associated markers (e.g. TNF-α, TGF-β, Fibronectin, IL-6). However, in addition to inflammation coagulation is also induced in vital wounds [17]. We hypothesize that induction of the coagulation cascade proteins Fibronectin, P-selectin (CD62p) and Factor VIII [18-27] in wound hemorrhage, are important additional discriminating factors in wound age determination, especially in early wounds.

First, Glucose transporter 1 (GLUT-1) was used to determine the area of extravasated erythrocytes (i.e. hemorrhage). GLUT-1 is an integral membrane protein that facilitates the transport of glucose across the plasma membranes, including in erythrocytes [28]. Next, Platelet endothelial cell adhesion molecule (PECAM-1), also known as cluster of differentiation 31 (CD31), was used to demonstrate the presence of thrombocytes, i.e., to verify whether coagulation indeed was induced in the area of hemorrhage. CD31 is found on the surface of thrombocytes and it is part of a large portion of endothelial cell intercellular junctions [29].

Fibronectin is deposited at the site of injury, forming a blood clot. In addition, it promotes the spreading of platelets at the site of injury. It also plays a role in the migration of neutrophils, monocytes, fibroblasts and endothelial cells into the wound region, and later on in the migration of epidermal cells to restore skin injury [23,30-33]. In uninjured skin, strong immunohistochemical Fibronectin expression was not only described in the epidermal basement membrane and around skin appendages but also in endothelial cells of blood vessels. Furthermore, in 20-40 minutes old vital skin injuries, strong immunohistochemical Fibronectin expression was found in wound hemorrhage also [23,30,34-36].

CD62p on the other hand is naturally located within granules of endothelial cells and platelets and plays an essential role in the early binding of leukocytes to endothelium during inflammation and in the recruitment and aggregation of platelets at areas of vascular injury. CD62p has also been found on
activated thrombocytes, already a few minutes subsequent to wound induction [18-22,26]. However, until now, CD62p was not analyzed in wound hemorrhage. Finally, Factor VIII is a blood coagulation factor that is normally bound to von Willebrand Factor, a multimeric glycoprotein. It mediates the adhesion of thrombocytes to subendothelial connective tissue. Like CD62p, Factor VIII is naturally present in endothelial cells and has been described to localize at the surface of endothelial cells also a few minutes after wound infliction. Like CD62p, Factor VIII has not been described in wound hemorrhage yet [18,24,25,27].

The aim of this study was to develop a new method to determine the age of human skin wounds by analyzing Fibronectin, CD62p and Factor VIII expression in wound hemorrhage in early vital wounds (up to 30 minutes old). Additionally, we wanted to develop a so-called probability scoring system based on the expression levels of these markers in wound hemorrhage, to determine the probability that a wound has a certain age. This because in daily practice of forensic autopsies, it is necessary to estimate wound age as accurate as possible. For this purpose tissue samples of mechanically induced skin injuries of known wound age, up to a few minutes old and injuries inflicted 15-30 minutes before death, were obtained at autopsy. Tissue slides were stained for Fibronectin, CD62p and Factor VIII and intensity of staining was quantified in wound hemorrhage. Finally, probability scores of these markers were calculated.

MATERIALS AND METHODS

In total 322 victims are included in this study, of whom one or more human skin wound samples and non-injured control skin samples were obtained at forensic autopsies (807 human skin samples in total). Only skin wound samples that are the result of “blunt force trauma” were included in this study. Non-injured control skin samples were taken from the same victims, from areas of suspected post-mortem changes. There may be differences in histology within a wound. For that reason all the samples were taken from the center of the wounds. Standardization regarding the location of sampling is important to compare between the wounds. All samples were collected up to maximal 2 days after death. Wound samples were examined to verify witness statements related to wound age, and as such were part of the diagnostic process.

These samples were divided in three different groups:

Group A) Controls: non-injured skin, consisting of 383 skin samples from 163 autopsies (age of victims ranged from 0 years old up to 95 years old).

Group B) “Very early” vital injuries: injuries inflicted shortly before death (a few minutes old), consisting of 382 skin samples from 122 autopsies (age of victims ranged from 0 years old up to 94 years old).
Group C) “Early” vital injuries: injuries inflicted 15 to 30 minutes before death, consisting of 42 skin samples from 37 autopsies (age of victims ranged from 2 years old up to 84 years old).

**Immuno**histochemistry

Preservation method: After collection the tissues were fixated in buffered 4% formalin for 2 to 3 days and subsequently embedded in paraffin. Then, the paraffin embedded tissues were sliced (4 µm) for microscopic investigation, whereafter standard Hematoxylin-Eosin (HE) staining was performed. For immunohistochemistry, tissues were dewaxed and rehydrated in xylene and alcohol (100%) followed by incubation in a methanol/H$_2$O$_2$ 0,3% solution to block endogenous peroxidases. Antigen retrieval was performed by either boiling slides in a citrate pH 6.0 (CD31) or a Tris-EDTA pH 9.0 (Factor VIII and GLUT-1) solution in a microwave for 10 minutes or by incubation with pepsine/HCl 0,1% (Fibronectin and CD62p) for 30 minutes at 37°C. Next, sections were incubated with 1:100 polyclonal rabbit anti-human GLUT-1 (Thermo Scientific, UK) to visualize erythrocytes in hemorrhage, 1:40 monoclonal mouse anti-human CD31 (Dako cytation, Denmark) to visualize thrombocytes in hemorrhage, 1:200 monoclonal mouse anti-human CD62p (Serotec, UK), 1:18000 polyclonal rabbit anti-human Fibronectin (Dako cytation, Denmark) or 1:2000 rabbit polyclonal anti-human Factor VIII (Dako cytation, Denmark) for 1 hour at room temperature. Sections were then incubated with undiluted goat anti-mouse/rabbit envision (Dako cytation, Denmark) for 30 minutes at room temperature. Staining was visualized using 3,3-diaminobenzidine (DAB, 0,1 mg/ml, 0,02% H$_2$O$_2$). Sections were counterstained with hematoxylin, dehydrated and covered. As a control, the same staining procedure was used, but instead of the primary monoclonal or polyclonal antibody, 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS) solution was used. These skin tissue slides were found to be negative (data not shown).

**Immunohistochemical analysis**

HE and GLUT-1 (identifying erythrocytes) stained slides were used to determine the microscopical wound area, based on extravasated erythrocytes. Immunohistochemical staining of the different markers was scored in this particular area of the wound. To this end, the dominant appearance of an immunohistochemical score (IHC score) for each marker (see below) within the particular wound did define the final score per wound. From each wound, one representative slide was analyzed. All stainings were verified by a second assessor and consensus was achieved for each scoring. In case differences were found between the observations of the investigators (i.e. variation in the IHC score), the investigators analyzed the slides together in order to reach an agreement.

**The scoring system**

**Fibronectin:**
• Score 0: No staining in hemorrhage and/or no hemorrhage (see figure 1A).
• Score 1: Minor staining in hemorrhage (see figure 1B).
• Score 2: Moderate staining in hemorrhage (see figure 1C).
• Score 3: Strong staining in hemorrhage (see figure 1D).

Figure 1. Examples of negative (IHC score 0), minor (IHC score 1), moderate (IHC score 2) and strong (IHC score 3) staining (arrows) of Fibronectin. No staining of Fibronectin in hemorrhage (arrow), i.e. IHC score 0 (A). Minor staining of Fibronectin in hemorrhage (arrow), i.e. IHC score 1 (B). Moderate staining of Fibronectin in hemorrhage (arrow), i.e. IHC score 2 (C). Strong staining of Fibronectin in hemorrhage (arrow), i.e. IHC score 3 (D). (Magnification 63x).

CD62p:
• Score 0: Diffuse staining of endothelial cytoplasm. No staining in hemorrhage
and/or no hemorrhage (see figure 2A).

• Score 1: Minor linear membrane staining of endothelial cells and/or vascular intraluminal staining. No staining in hemorrhage (see figure 2B).

• Score 2: Minor staining in hemorrhage (see figure 2C).

• Score 3: Strong staining in hemorrhage (see figure 2D).

Figure 2. Examples of negative (IHC score 0), minor (IHC score 1), moderate (IHC score 2) and strong (IHC score 3) staining (arrows) of CD62p. Diffuse staining of endothelial cytoplasm and no staining of CD62p in hemorrhage (arrow), i.e. IHC score 0 (A). Minor linear membrane staining of endothelial cells and vascular intraluminal staining of CD62p (arrow), i.e. IHC score 1 (B). Moderate staining of CD62p in hemorrhage (arrow), i.e. IHC score 2 (C). Strong staining of CD62p in hemorrhage (arrow), i.e. IHC score 3 (D). (Magnification 63x).

Factor VIII:

• Score 0: No staining in hemorrhage and/or no hemorrhage (see figure 3A).
• Score 1: Minor staining in hemorrhage (see figure 3B).
• Score 2: Moderate staining in hemorrhage (see figure 3C).
• Score 3: Strong staining in hemorrhage (see figure 3D).

Figure 3. Examples of negative (IHC score 0), minor (IHC score 1), moderate (IHC score 2) and strong (IHC score 3) staining (arrows) of Factor VIII. No staining of Factor VIII in hemorrhage (arrow), i.e. IHC score 0 (A). Minor staining of Factor VIII in hemorrhage (arrow), i.e. IHC score 1 (B). Moderate staining of Factor VIII in hemorrhage (arrow), i.e. IHC score 2 (C). Strong staining of Factor VIII in hemorrhage (arrow), i.e. IHC score 3 (D). (Magnification 63x).

Statistical analysis
Statistical analysis was performed with SPSS (Windows version 20.0, IBM corp., Armonk, NY). IHC scores between different groups were compared by a linear-by-linear association. P-values < 0.05 were considered significant. IHC scores are described as mean ± standard deviation (SD).

Probability scores
To obtain the probability scoring system, first the probability that the IHC score was 0, 1, 2 or 3 was estimated via ordinal regression with group as factor. By Bayes’ rule, these probabilities could be inverted to obtain the probability that the tissue is sampled from a certain group given the IHC score, i.e. the probability score.

RESULTS

Expression of the individual immunohistochemical markers in autopsy wounds

Immunohistochemical staining of Fibronectin, CD62p and Factor VIII was scored in areas of extravasated erythrocytes (i.e. hemorrhage), visualized using HE and GLUT-1 staining (figure 4A and B).

To verify whether in hemorrhage coagulation indeed was induced, we first stained slides with CD31 to visualize thrombocytes (figure 4C). Subsequently, we indeed found that CD31 positive areas coincide with the earlier mentioned coagulation markers Fibronectin, CD62p and Factor VIII. It has to be noticed that these markers did not always stain the total area of the wound hemorrhage.

A

B

C

CD31

HE

Glut-1
Figure 4. Extravasation of erythrocytes (i.e. hemorrhage) and CD31 positive areas (thrombocytes) in human skin wound. HE and Glut-1 staining (arrows) was used to visualize extravasation of erythrocytes (i.e. hemorrhage) (A and B). CD31 positivity of thrombocytes (C) in hemorrhage (arrow) of the injured human skin (>30 minutes old wound). (Magnification 40x).

In at most 14% of the non-injured control samples (group A), hemorrhage coincided with minor immunohistochemical positivity for Fibronectin, CD62p and/or Factor VIII (figure 5). The mean IHC scores in these control samples were 0.1 ± 0.4 (Fibronectin), 0.2 ± 0.4 (CD62p), and 0.2 ± 0.5 (Factor VIII). For all three markers, in wounds of a few minutes old (group B) a significant increase (p < 0.001) in the IHC score was found compared with non-injured control skin samples (group A), mean IHC scores were 1.4 ± 0.8 (Fibronectin), 1.2 ± 0.6 (CD62p), and 1.6 ± 0.8 (Factor VIII). Furthermore, the IHC scores for all three markers significantly increased even more (p < 0.001) in 15-30 minutes old wounds with a mean IHC score 2.6 ± 0.5 (Fibronectin), 2.5 ± 0.6 (CD62p), and 2.8 ± 0.4 (Factor VIII) (group C).
Figure 5. Mean IHC score of Fibronectin, CD62p and Factor VIII expression in autopsy wound samples of different wound ages. The expression of (A) Fibronectin, (B) CD62p and (C) Factor VIII in group A: Control (n = 383), group B: a few minutes old wounds (n = 382) and group C: 15-30 minutes old wounds (n = 42). The error bars in this figure are the Standard Errors of the Mean (SEM). + = significant difference (p < 0.001) between group A and C. # = significant difference (p < 0.001) between group A and B. * = significant difference (p < 0.001) between group B and C.

Probability scores

In daily practice, a forensic pathologist has to estimate the time frame of a certain wound sample. For this we subsequently calculated a so-called probability score for each particular marker in time (table 1).

In case the IHC score was 0, the probability that the sample was derived from uninjured skin was the highest: 87% (Fibronectin), 88% (CD62p), and 90% (Factor VIII). In case the IHC score was 1 or 2, the probabilities that a wound was a few minutes old were the highest: 82/90% (Fibronectin), 82/83% (CD62p), and 72/93% (Factor VIII). Finally, in case the IHC score was 3, it was most likely that the wound was 15-30 minutes old, with probabilities equal to 65% (Fibronectin), 76% (CD62p), and 55% (Factor VIII).

We did not find a 100% probability score for any of the markers. However, we did find that the probability that the wound is 15-30 minutes old for an IHC score of 0 or 1 was 0% for all three markers. Moreover, in case of an IHC score 3 the probability that tissue is non injured skin tissue was 1%, 0%, and 1% for Fibronectin, CD62p and Factor VIII, respectively.
Table 1. Probability score of the immunohistochemical scores 0-3 for Fibronectin, CD62p and Factor VIII. The probability (in percentages) is depicted that tissue is sampled from one of the groups (control, a few minutes old and 15-30 minutes old wounds) given that the score is 0, 1, 2, or 3 for Fibronectin, CD62p or Factor VIII. Bold = Highest probability score.

<table>
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<tr>
<th>Marker</th>
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<th>Score 2</th>
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<td>18</td>
<td>3</td>
<td>0</td>
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<td>Few min.</td>
<td>12</td>
<td>82</td>
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<td>76</td>
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<td>Control</td>
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<td>1</td>
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<td></td>
<td>15-30 min.</td>
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<td>0</td>
<td>3</td>
<td>55</td>
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DISCUSSION

In forensic pathology it is important to determine wound age [1-10]. In this study we developed a new method in order to estimate wound age in early post-traumatic vital skin wounds up to 30 minutes old by analyzing immunohistochemical expression of Fibronectin, CD62p and Factor VIII in wound hemorrhage. The markers described in this study have different roles both in coagulation and inflammation. Fibronectin promotes the spreading of platelets at the site of injury and forms a blood clot. It also plays a role in the migration of neutrophils, monocytes, fibroblasts and endothelial cells into the wound region. CD62p on the other hand plays an essential role in the early binding of leukocytes to endothelium during inflammation and in the recruitment and aggregation of platelets at areas of vascular injury. Finally Factor VIII is a blood coagulation marker that mediates the adhesion of platelets to subendothelial connective tissue.

The expression pattern of these three markers was very similar. Therefore, the combined use of these markers with regard to their expression in wound hemorrhage will strengthen the estimation of wound age of early skin injuries.

We found a significant increase (p < 0.001) in expression in wound hemorrhage in time for all three markers. In maximal 14% of the non-injured control samples, minor expression was found of Fibronectin, CD62p and Factor VIII, that significantly increased in wounds inflicted shortly before death and even more in wounds of 15-30 minutes old. For all three markers, in case of an IHC score 0, the probabilities that a wound was non-vital were highest. In case of an IHC score 1 or 2, the probabilities that a wound was a few minutes old were highest for all three markers. Finally in case of an IHC score 3, the probabilities that a wound was 15-30 minutes old were highest.
Different studies have already described the use of Fibronectin, CD62p and/or Factor VIII expression for skin wound age estimation [36], although CD62p and Factor VIII were not described in wound hemorrhage until now. Fieguth et al., Betz et al., and Ortiz-Rey et al., found in wounds of 20-40 minutes old “strong” immunohistochemical Fibronectin expression in wound haemorrhage [23,30,33]. We also found this in our study in 15-30 minutes old wounds. Additionally, we also found minor-moderate (IHC score 1-2) Fibronectin expression in wounds of a few minutes old, which was significant increased compared with non-injured control skin. With respect to the markers CD62p and Factor VIII, different results were published related to their discriminating role in wound age estimation. Several studies described translocation of CD62p and Factor VIII from granules to the surface of endothelial cells already after a few minutes of infliction [20-22,25,27,36-41]. However, this was debated by others who found that these markers were continuously present on the surface of endothelial cells, even in post-mortem injuries, resulting in (minor) positive staining [26]. We now found minor linear membrane CD62p staining of endothelial cells in wounds of a few minutes old, strong enough to discriminate from non-injured control samples with cytoplasmic CD62p staining. Furthermore, we found differential CD62p and Factor VIII expression within wound hemorrhage in time, indicating that this discriminates additionally in wound age determination.

Firstly, the area of wound hemorrhage should be defined. For this a GLUT-1 staining can be helpful. The staining intensity of the described markers then should be quantified in this area of wound hemorrhage. On the other hand, expression of these markers in wound hemorrhage, coinciding with thrombocytes, suggests a coagulation related expression induction [18,42]. We, however, can not exclude a contribution of fragmented endothelial cells, as CD31 is also expressed herein. Albeit, their contribution will be minor in comparison to the large hemorrhage area. In addition, passive leakage of these markers from damaged blood vessels can also not be excluded [18,21,23,26,27,30,31,39-41]. In at most 13% of non-injured control samples, we found minor positivity of Fibronectin, CD62p and Factor VIII in hemorrhage and moderate/strong positivity in 1% of the control tissue. At this moment we do not have a clear explanation for this. However, we can not exclude that inaccurate or incorrect statements of witnesses could explain these outliers within the control group.

In conclusion, the present study describes a new method to determine wound age in early vital skin injuries. In daily practice of forensic autopsies, it is necessary to estimate wound age as accurate as possible. Hence, we developed a probability scoring system to determine the probability that a wound has a certain wound age dependent on the expression levels of Fibronectin, CD62p and Factor VIII in wound hemorrhage. This system can be used in forensic autopsies to improve wound age estimation in early skin injuries.
However, a limitation of our study was that we were dependent of witness statements with regard to the wound ages. Therefore, inaccurate or incorrect witness statements could have resulted in aberrant estimations in this study.

REFERENCES


