Usefulness of Fibronectin and P-selectin as markers for vital reaction in uncontrolled conditions

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Abstract: The purpose of this article is to analyze the usefulness of fibronectin and P-selectin in detecting the vitality of the wounds in everyday legal medicine practice, in uncontrolled conditions, and to see whether they can be used for estimating the age of the lesions. We used 17 cases with a short interval from the production of the wounds to death. On the integuments studied from the cases selected in the interest group, fibronectin (FbN) was positive at the dermis - epidermis junction relatively frequently, in the superficial dermis focally and in the deep dermis peri-axially (sudoriparous glands, sebaceous glands) with a sometimes marked reaction; it was also interstitially positive around hemorrhagic areas in the hypodermic conjunctive and adipose tissue. P-selectin (CD-62P) was positive variably and focally in the extracellular matrix and the vessels, with a low intensity and a fibrillar pattern. The P-selectin expression in the extracellular matrix was equal to the P-selectin expression in the capillaries. Between the expression Fb and CD-62-P and the postmortem interval there was no correlation. The fibroblasts in the extracellular matrix showed variable positivity. For forensic practice the usefulness of fibronectin and P-selectin is debatable. If they are very useful in detecting the vitality of the wound, for estimating the age there is a need for a much larger scale study, that must take into consideration factors like CPR, individual pathologies, and so on. The two used antibodies have a moderate sensitivity and low specificity, and as such they have to always be correlated with the Perls staining, and in a histopathological context only.

Key Words: P-selectin, postmortem interval, Fibronectin.

In legal medicine a correct identification of the vitality of the lesions and a correct dating of their age is of a paramount importance. Wound healing of the skin includes a lot of biological processes like inflammation, proliferation, cellular migration, protein synthesis, maturation, and so on [1]. Currently there are a lot of methods of quantifying the age of a lesion using methods from classical histology, histochemistry, immunohistochemistry of molecular biology [1-27]. Three of the most promising immunohistochemistry markers for vital reaction were found to be fibronectin and P-selectin [8, 28-30]. The purpose of this article is to analyze the usefulness of fibronectin and P-selectin in detecting the vitality of the wounds in everyday legal medicine practice, in uncontrolled conditions, and to see whether they can be used for estimating the age of the lesions.

MATERIALS AND METHODS

Selection of cases and set up of the study group

The cases were selected from the archives of the "Mina Minovici" National Forensic Institute. The study (interest) group was represented by 17 patients, out of
which 10 males and 7 females (sex ratio M/F = 1.42), aged between 0-94 years-old (53.70±26.07). A number of 17 skins harvested from different areas have been studied: 3 lacerations produced by blunt trauma at the scalp, 2 thigh wounds, one calf wound, 2 forearm wounds, 4 stabbed wounds of the anterior thorax, 1 self-inflicted stabbed wound in the forearm, 1 stabbed wound in the posterior thorax, 2 surgical incisions in the abdominal skin, 1 thigh wound caused by dog bites in an abandoned newborn. The control group consisted of 10 cases of integuments without skin lesions (See Table 1 for details).

**Methods**

**Histology.** The optical microscopy methods used were standard histopathological staining (HE - hematoxylin eosin and van Gieson), Perls staining, and immunohistochemical tests. The histopathological analysis was performed on integument fragments fixed in 10% formalin (kept ~ 24-48 hours) and sections (3-5 μm) embedded in paraffin for the histopathological and immunohistochemical staining mentioned above.

**Immunohistochemistry.** We used the three-stage avidin-biotin-peroxidase complex (ABC) indirect technique [31, 32]. The technique was applied on fragments of tissue embedded in paraffin and sectioned at 3 μm thickness. The sections were displayed on slides treated with poly-L-lysine (See Table 2 for details).

The evaluation of the intensity of the IHC reaction on the investigated tissues from the study group cases was qualitative, with absolute values from 0-3 conventionally. We used a modified Quick score (2002), which takes into account the intensity and distribution of positivity, as it follows: negative (lack of reaction) = 0; weak (reaction visible in a high magnification field: x40) = 1, moderate (reaction visible in a low magnification field x10) = 2; strong (positivity marked in a low magnification field or with a magnifying glass) = 3. Furthermore, in order to ensure the validity of the IHC reactions, an internal quality control was performed according to a quality management system certification (ISO 9001/2008).

**Statistical methods.** For the statistical analysis of the uniformly distributed (symmetric) data, the Student’s t-test was used, the "paired samples for means" ("one group two-tails") option. The data were statistically analyzed using the Analysis Tool Pak program in Microsoft Excel 2003 of the Windows XP Professional. For correlation between variables was used the Kendall’s tau test (done using the SPSS v20 for Mac software). A value of p <0.05 was considered statistically significant.

**RESULTS**

Histopathologically, on the examined tissues from the selected cases in the interest group, we found various hemorrhagic aspects including: interstitial perivascular hematic extravasation, recent or relatively recent hemorrhages with partial blood lysis, or old hemorrhagic foci, with hemosiderin present (cases 9 and 10), and sometimes with interstitial siderophage macrophages.

The hemorrhagic foci were of various sizes, from isolated microhemorrhages to medium hemorrhagic foci or even hematomas. Sometimes the hemorrhagic foci tended towards confluence or a diffuse pattern.

The location of the hemorrhage was interstitial and perivascular for the superficial papillary dermis, interstitial and peri-axially for the deep reticular dermis.

**Table 1.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>Circumstances</th>
<th>Survival time (min)</th>
<th>CPR</th>
<th>FbN-Mx</th>
<th>FbN-Fb</th>
<th>CD62-Mx</th>
<th>CD62-Vs</th>
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<td>24</td>
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<td>1</td>
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</table>

**Table 2.** The antibodies utilised in the study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Dilution</th>
<th>Producer</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin (CD62-P)</td>
<td>C34</td>
<td>1:100</td>
<td>Novocastra</td>
<td>Activated endothelia</td>
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<td>Fibronectin</td>
<td>568</td>
<td>1:200</td>
<td>Novocastra</td>
<td>Extracellular matrix</td>
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</tbody>
</table>

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or focal interstitial for the hypodermis. Sometimes we noted intraepidermal hematic extravasations.

The Perls staining was positive in 7 cases out of 17 (41.17%). Toluidine blue staining was negative in the investigated cases, so mast cells in hemorrhagic foci could not be highlighted.

On the integuments studied from the cases selected in the interest group, fibronectin (FbN) was positive at the dermis - epidermis junction relatively frequently, in the superficial dermis focally and in the deep dermis peri-axially (sudoriparous glands, sebaceous glands) with a sometimes marked reaction; it was also interstitially positive around hemorrhagic areas in the hypodermic conjunctive and adipose tissue. The fibroblasts in the extracellular matrix showed variable positivity. The pattern of the IHC reaction was linear, reticular (Figure 1). The expression of the fibronectin in the extracellular matrix was higher than the fibronectin expression in fibroblasts (Figure 2).

P-selectin (CD-62P) was positive variably and focally in the extracellular matrix and the vessels, with a low intensity and a fibrillar pattern. The P-selectin expression in the extracellular matrix was equal to the P-selectin expression in the capillaries (Figures 3-5).

The IHC markers were variably positive in the extracellular matrix, fibroblasts and blood vessels, as follows: FbN was positive in ~ 94% of cases in fibroblasts...
and the matrix, and CD-62 was positive in ~ 82% of cases in the matrix.

A high correlation (of direct proportionality), statistically significant, was noted between the IHC expression of the FbN in the matrix and fibroblasts (r = 0.83, p < 0.0001). Also, the calculated t-Stat was ~ 3.5 times higher than the t-Critical table values, which shows a higher sensitivity and specificity of the FbN in the matrix than in fibroblasts.

In the study group there was no statistically significant correlation between the FbN expression and the CD62-P expression in the matrix; as a result, the two markers are independent. However, the calculated t-Stat was ~ 2 times higher than the t-Critical table values (p = 0.0006), which suggests that fibronectin is more specific for the extracellular matrix than the p-selectin on the tissues that were studied in the interest group.

In the study group there was a statistically significant positive correlation (of direct proportionality) between the FbN expression in the matrix and the CD62-P expression in the capillaries (r = 0.4, p <0.0001); also, the calculated t-Stat was ~ 3.5 times higher than the t-Critical table values, with a statistical significance.

There was no statistically significant correlation or difference between the IHC expression of the fibronectin in fibroblasts and of the p-selectin in the matrix (r = 0.2, p = 0.3). But there was still a statistically significant reasonable correlation between the IHC expression of the FbN in the fibroblasts and of the CD62-P in the capillaries (r = 0.46, p = 0.004), which suggests a direct proportionality between the fibronectin expression in fibroblasts and of the p-selectin in the capillaries. A directly proportional and statistically significant relation was also noted (r = 0.7, p = 0.04) - which shows a good positive correlation, between the CD62 expression in the matrix and CD62-P in the capillaries.

In order to test whether Fb and CD-62-P can be used to quantify the postmortem interval we used the Kendall’s tau test that showed no correlation (see Table 3).

**DISCUSSIONS**

There have been many studies analyzing the usefulness of immunohistochemistry in detecting the vitality of a wound and/or its age [28, 29, 33]. Most of them were however done in controlled conditions and/or on animal models [3, 8].

P-selectin (CD62-P) is an adhesion cell molecule present on the surface of activated endothelial cells, and activated platelets, playing essential roles in the initial recruitment of the leukocytes at the site of injury during inflammation. The IHC reaction appears as positive in vascular endothelia activated in inflammations or in trauma in capillaries with surrounding hemorrhages. In wounds P-selectin appears in minutes and disappears in about seven hours [33, 34]; this short interval makes it useful for detecting a very recent lesion. In our study P-selectin was constantly present, irrespective of the survival time. There were only three cases in which P-selectin was negative, both in the matrix and in the endothelium – two cases with a total survival time of about 10 minutes, and one case with a survival time of two and a half hours. The intensity of the staining was independent upon the estimated wound age (Kendall’s tau=0.075, not-statistically significant). However, the most intense staining was identified in wounds of about 30 minutes.

Fibronectin is an adhesion glycoprotein from the extracellular matrix which is secreted by fibroblasts and deposited interstitially; the IHC reaction can have a reticular pattern in the extracellular matrix, but sometimes nonspecific positive reactions may occur in the striated muscles. It has functions in cell adhesion, migration, growth or differentiation, being crucial in wound healing. It is mainly located in the basement membrane [8] but during inflammation is widely distributed the affected areas. In wounds it appears in about 10-20 minutes and is still present after 17 days [28]. In our study fibronectin was constantly present, with a negative reaction in only one case with a survival of about 10 minutes (case in which P-selectin was also negative) The intensity of the staining was independent upon the estimated wound age (Kendall’s tau=0.284 in the matrix and 0.216 in fibroblast, both not-statistically significant).

The two used antibodies have a moderate sensitivity and low specificity, and as such they have to always be correlated with the Perls staining, and in a histopathological context only.

The inflammatory cells, acute (PMN) or chronic (lymphocytes, plasma cells, macrophages) can be considered to be additional elements, that are helpful, but not mandatory, in supporting a vital reaction.
Limits of the study

A limited number of cases. The study was aimed to be a pilot, to see whether these markers are useful in everyday forensic practice.

Difficult quantification of the exact age of the wounds in practice. The wound age was estimated at the level of minutes, but an exact quantification is not possible in forensic practice, as this would mean that the physician is there when the person dies.

However, if a person is not dead the physician has the duty to perform CPR measures, that would alter the pattern of expression.

CONCLUSION

For forensic practice the usefulness of fibronectin and P-selectin is debatable. If they are useful in detecting the vitality of the wound, for estimating the age there is a need for a much larger scale study, that must take into consideration factors like CPR, individual pathologies, and so on.

The two used antibodies have a moderate sensitivity and low specificity, and as such they have to always be correlated with the Perl's staining, and in a histopathological context only.

References


