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VASCULAR ADHESIVE PEPTIDE-1 (VAP-1) EXPRESSION IN WOUNDS – A NEW VITAL REACTION MARKER?

Sorin Deacu[1,*], Lavinia Neculai-Cândea[2], Ştefan Pricop[2], Mariana Aschie[3], Liliana Mocanu[3], Marius Florentin Popa[2]

[1]"Ovidius" University, Faculty of Medicine, Dept. of Pathology, Forensic County Service, [2]"Ovidius" University, Faculty of Medicine, Dept. Of Forensic Pathology, Forensic County Service, [3]Constanta County Emergency Hospital, Pathology Service, Constanta, Romania

Abstract: *Introduction.* Few topics in forensic medicine have spilled as much ink as that of vital response in wounds. We join these efforts and aim to study a new marker included in the group of adhesion molecules, Vascular Adhesive Peptide-1 (VAP-1) *versus* an already extensively studied marker, P-selectin.

Material and method. For this prospective study, skin samples were collected from autopsied cadavers at the County Clinical Service of Forensic Medicine Constanta (SCJML Constanta). The main inclusion criteria were: accessible chronological documentation of the lesion and the absence of inflammatory, neoplastic, or liver conditions. Between 2018 and 2021, 122 cases fell within these criteria and were divided into 5 groups based on survival time. Each fragment from the wound was accompanied by a control fragment harvested from the site of an autopsy incision. Routine Hematoxylin-Eosin (HE) and Perls staining, as well as immunohistochemistry with VAP-1 and P-selectin markers, were performed on each fragment and a score based on staining intensity was established.

Results. Routine staining was not useful in assessing vitality in the segment with a survival time of less than 5 minutes, a segment that was also defined by the absence of inflammatory infiltrate. Immunohistochemically, both markers showed significantly increased values compared to control values ($p<0.001$). VAP-1 was found to have a statistically significant ($p<0.001$) peak of positivity in the under 5 min segment and between 3-24 h ($p<0.05$) compared to P-selectin.

Conclusions. It can be concluded that VAP-1 can be a reliable alternative for establishing the timing of the injury, at least for the segment with a short survival time of a few minutes.

Keywords: forensic, vital reaction, immunohistochemistry, VAP-1.

INTRODUCTION

In forensic medicine there is a topic that has been long debated and still has no validated answer, being open to some novel elements, namely the vital reaction and the existence of a possible microscopic marker that can help differentiate between wounds sustained shortly before death and those sustained shortly after death [1].

The element of certainty that proves a wound was sustained during life is the existence of inflammatory infiltrate. This phenomenon can occur between 10 to 20 minutes and usually peaks at 1 to 2 hours [2].

It goes without saying that there are some intravital wounds in which there is no inflammatory infiltrate. For these cases, numerous studies have attempted to demonstrate the efficacy of coagulation cascade factors [3-5], adhesion molecules [6-10], inflammatory markers [11-15] or growth factors [16-18].

To be attracted to the site of injury, leukocytes must initiate a process called diapedesis. This process is facilitated, in particular, by a group of molecules called adhesion molecules [19, 20].

The idea of the present study started from the need to identify a molecule that triggers and maintains post-traumatic inflammatory infiltration. One such candidate appears to be VAP-1 [19].

VAP-1 is a dual role molecule: it performs an intercellular adhesion function and oxidizes amines with the help of the copper ion in its structure [21].

*Correspondence to: Sorin Deacu, "Ovidius" University Constanta, Faculty of Medicine, Dept. of Pathology, Forensic County Service, 2 Zmeurei Alley, Constanta, Romania, E-mail: deacu.sorin@gmail.com

VAP-1 has a low expression on some endothelial cells in unaffected skin and tests positive in smooth muscle fibers and adipocytes [22].

During the inflammation process, VAP-1 expression is induced on endothelial cells and the protein is translocated from cytoplasmic vesicles to the plasma membrane, by a process similar to that of P-selectin [22].

P-selectin is an older acquaintance of pathologists with vast experience in wound age estimation. It has been studied in numerous articles and so far has been considered the flagship representative of adhesion molecules when it comes to vital reaction [19]. It is stored in endothelial cells, in Weidel-Palade granules [8, 23]. When endothelial cells are stimulated, translocation of P-selectin to the cell surface occurs (in about 10 to15 minutes). It is essential for leukocyte binding and is not influenced by cytokine synthesis [19]. Experimental studies have shown the increased presence of endothelial cells at a minimum time of 1 minute, with a marked increase at 10 to 15 minutes [8]. However, cases of its presence in post-mortem wounds have also been documented [9]. However, currently, it is still considered a useful marker in estimating the lesional age [19].

This study aims to evaluate the expression of VAP-1 in wounds of different ages and the comparison with P-selectin, in order to integrate these data in the field of forensic pathology.

MATERIAL AND METHOD

For this study, skin fragments were collected from forensic cases of violent deaths, autopsied in the Clinical County Service of Forensic Medicine Constanta, with a survival time (ST) of maximum 24 hours following the injury. In each case, a fragment of the skin-breached lesion was collected, as well as a control skin fragment. The samples included one of the wound margins, avoiding any aesthetic deficits.

Inclusion criteria were:

- Known/documented time of injury (investigation data, video evidence, records of emergency services call).

- Autopsy performed within 48 hours of death.

Exclusion criteria were:

- Inflammatory diseases discovered by autopsy/ microscopic examination.

- Liver disease (hepatitis, cirrhosis).

- Treatments with chemotherapy, corticosteroids, or blood thinners.

- History of psoriasis, lupus, or another inflammatory skin disease.

Thus, between 2018 and 2021, out of the 567 violent deaths with wounds present, a number of 122 cases met the criteria required by the study.

A total of 122 skin fragments were collected from both wounds and incisions made during the forensic autopsy. The fragments were divided into 4 groups according to survival interval:

- Group I: ST under five minutes;

- Group II: ST between 5 minutes and 60 minutes;

- Group III: ST between 60 minutes and 180 minutes;

- Group IV: ST between 180 minutes and up to 24 hours.

In addition to the 122 skin fragments, 9 fragments from postmortem wounds (Group V) with a documented time interval ranging from 17 minutes to 6 hours were also examined.

The characteristics of the study group are presented in Table 1.

Each skin fragment was placed in a container which was labeled with name, date and time of collection, and the anatomical area from which it originated. The samples were kept in a preservative agent (4% buffered formaldehyde) for 24 to 48 hours, after which the fragments were processed with Bioptika automated tissue processor. The paraffin-embedded blocks thus obtained were then sliced and mounted on slides on which routine He and Perls staining was performed.

Immunohistochemical technique

We used Anti VAP-1 antibodies (clone E10, dilution 1:150) from SantaCruz Biotechnology and P-selectin (clone AK-6, dilution 1:250) from Invitrogen,

Table 1. Characteristics of the study group

Survival time (ST)	Number of samples	Gender (M/F)	Age	Medical care (no. of cases)	Flaying injuries	Lacerations	Slash wounds
< 5 minutes	64	53/11	53±18.21	0	19	31	14
5-60 minutes	19	16/3	56.45±20.77	7	0	13	6
60-180 minutes	16	12/4	32.1±22.57	14	3	8	5
180 minutes-24 hours	23	17/6	43.31±15.95	23	2	15	6
Postmortem	9	6/3	60.4±16	3	3	2	4

ThermoFisher Scientific. Antigenic heat demasking was performed and for each marker, we followed the protocol recommended by the supplier. As a chromogen we used 3,3'diaminobenzidine (DAB), resulting in a brown color, considered to be a positive reaction. Mayer hematoxylin was used for counterstaining.

One representative slide was examined from each specific wound and a score was established, based primarily on the intensity of endothelial cell staining. The score has been used in other studies [24-26] and has 4 categories:

0-No positive endothelial cells are identified;

1-Weak endothelial membrane positivity (visible under x40 magnification)

2 - Moderate endothelial membrane positivity (visible under x20 magnification)

3 - Intense endothelial membrane positivity (visible under glass magnification and x10).

Statistical methods

All data were registered into a Microsoft Office Excel 2007 file and were statistically analyzed using the Analysis Tool Pak program in Microsoft Excel 2007 of the Windows 11 Professional Edition. The means and standard deviations (SDs) were calculated for all data. Statistical significance was evaluated by the T-test with $P < 0.05$ considered significant.

RESULTS

Routine staining: Hematoxylin-Eosin staining revealed areas of hemorrhage. No acute inflammatory infiltrate was identified on any of the fragments from wounds classified in the first group. Inflammatory infiltrate was apparent in 14 of the 19 cases from group 2 and on all other fragments in the other groups. Perls staining was ineffective in most cases, except in one case with a survival time of about 23 hours, where it was weakly positive.

Immunohistochemistry: For the control samples, similar values were obtained for the two markers: 0.25±0.43 (VAP-1) and 0.3±0.46 (P-selectin). For both markers, a distinct increase ($P<0.001$) was found in all segments compared to the control, except for the postmortem group. In the latter, values were close to the control group values ($P>0.05$) (Figs 1-4).

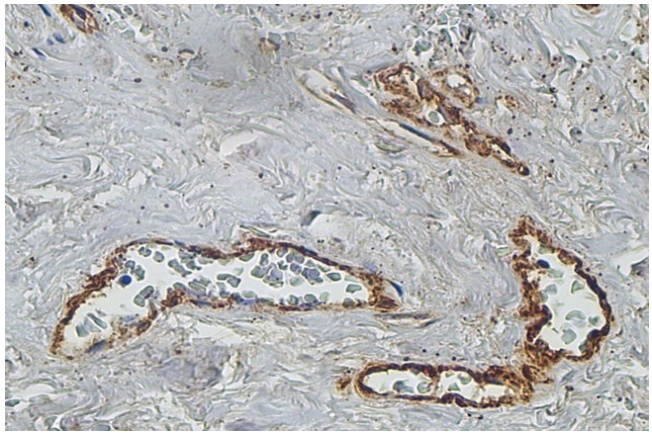

Figure 1. VAP-1, IHC score 3, ST< 5 minutes.

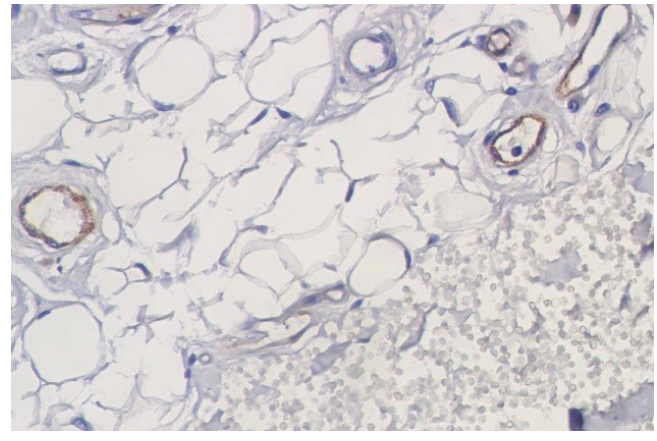

Figure 2. P-selectin - IHC score 2, ST < 5 minutes.

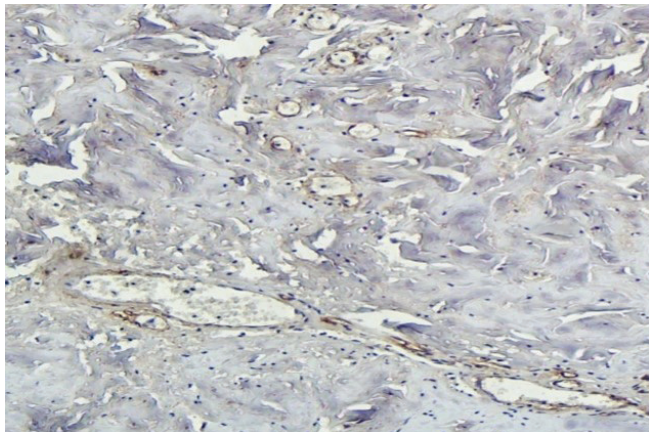

Figure 3. VAP-1, IHC score 1, ST-45 minutes.

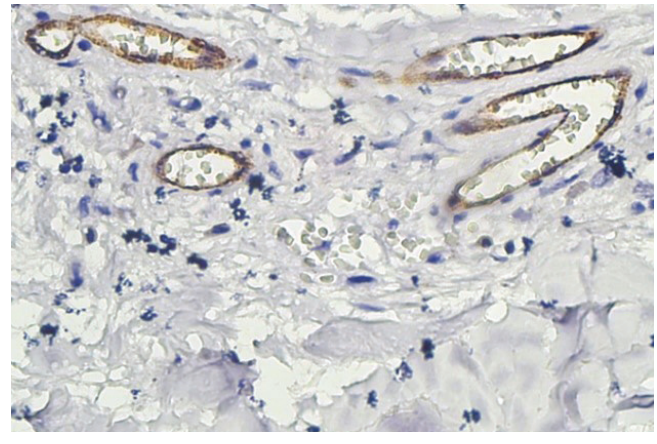

Figure 4. P-selectin- IHC score 3 , ST - 37 minutes.

Comparing the 2 markers, we noticed that VAP-1 had a statistically significant peak of positivity in both Group 1($p<0.001$) and Group 2($p<0.05$). On the other two segments, Groups 3 and 4 (5-60 minutes and 60-180 minutes), the values were not statistically significant (Fig. 5).

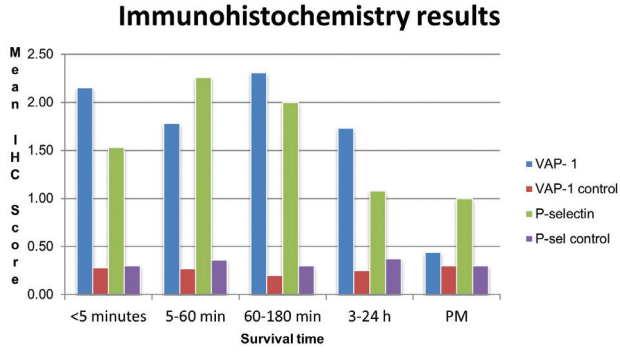

Figure 5. The mean IHC scores obtained by the two markers correlated with the survival time.

DISCUSSION

In all violent deaths, one of the objectives set by the authorities is for the coroner to determine whether the wound was inflicted antemortem or postmortem, and also to establish its timeline.

The presence of hemorrhage alone is not a sufficient criterion to classify a wound as an antemortem because numerous studies have shown that a degree of hemorrhage can occur even in autopsy incisions [27-29].

Whatever injury occurs, the body is programmed to heal it, to repair it. This process is accomplished with the aid of inflammatory cells.

The inflammatory response involves the extravasation of immune system cells outside the vessels. It is a highly coordinated process, called leukocyte transmigration, consisting of several sequences (margination, rolling, adhesion, diapedesis, and chemotaxis) each of which is controlled by the expression of molecules on the endothelial cell surface as well as on the leukocyte surface [30]. Leukocyte-dependent VAP-1 transmigration has been previously documented [31, 32]. This molecule mediates the diapedesis rate and also the rolling and firm adhesion of leukocytes to the vascular endothelium [31].

Studies using mice with inhibited VAP-1 gene expression have shown that the rate of leukocyte rolling on the endothelium is therefore increased and the process of diapedesis is reduced in the cremasteric vessels, compared to mice in which gene expression has not been inhibited [33]. Thus, we observe the meaningful role of this molecule in the initiation of inflammation. In cases of chemically induced cutaneous inflammation followed by intravenous administration of the anti-VAP-1 antibody, this antibody bound to those endothelial cells in which VAP-1 expression was increased as a result of the inflammation. In those endothelial cells from skin vessels that were not exposed to chemical stimulation, no binding of the antibody was found [22].

VAP-1 contributes to leukocyte binding to the vascular endothelium through its role as an adhesion molecule, a role independent of enzyme activity. This process will produce $2HO_2$ which triggers and regulates the synthesis and expression of endothelial adhesion molecules including P-selectin [35].

Our results obtained in group I (samples from wounds with ST <5 minutes) revealed that VAP-1 managed an average IHC score of 2.15 (Figure 4) vs 1.53 of P-selectin ($p<0.001$), suggesting that indeed VAP-1 seems to induce P-selectin expression. The results in group II (ST 5 to 60 minutes) resulted in a reversal of the ratio, with a mean P-selectin score of 2.26 (Figure 3). This confirms the data found in literature describing a peak in positivity after 10 minutes, falling off after 7 hours (mean score of 1.08 in group IV).

As for fragments harvested from postmortem lesions, both markers discriminated them from intravital lesions, although there have been studies showing P-selectin positivity in the former.

A shortcoming of our research is that, by being positive in smooth muscle fiber, endothelial VAP-1 expression is rather difficult to evaluate in arterioles and venules. Also, the limited number of postmortem lesions may be a drawback.

In conclusion, we have tested for the first time, after extensive research, the expression and potential applicability of VAP-1 in the field of vital response in forensic pathology. Although future and further studies are needed, we can consider VAP-1 as a marker of injuries produced shortly before death.

Conflict of interest

The authors declare that they have no conflict of interest.

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