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The postmortem activation status of platelets

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Abstract Platelets are activated by substances from the subendothelial matrix in endothelial lesions or by factors in the plasma coagulation cascade. Conversely, activated platelets are potent activators of this cascade. Only activated platelets express the adhesion molecules Gp53, GMP140 and thrombospondin on the plasma membrane. The postmortem activation status of platelets, therefore, can be determined immunoelectron microscopically by immunogold labeling of antibodies against these glycoproteins. Our studies revealed that the vast majority of these antigens were located within the granules postmortem, hence the platelets had not been activated. Thrombin-induced activation of platelets in vitro was only possible in the early postmortem interval, as demonstrated by labeling of the adhesion molecules on the plasma membrane. Later, such activaton was no longer possible even though thrombin-induced fibrin formation gave the appearance of "coagulated blood". In forensic medicine, these findings can possibly be applied to distinguish intravital clotting from the postmortem coagulation phenomena and intravital hematomas from postmortem hematomas.

Key words Postmortem platelet activation status
Platelets · Immunoelectron microscopy · Glycoproteins
Gp53, GMP140, thrombospondin

Zusammenfassung Thrombozyten werden durch Substanzen der subendothelialen Matrix in Endothelläsionen oder aktivierte Faktoren der Plasmagerinnungskaskade aktiviert. Umgekehrt aktivieren aktivierte Thrombozyten die plasmatische Gerinnung. Nur aktivierte Thrombozyten exprimieren die Adhäsionsmoleküle Gp53, GMP140 und Thrombospondin auf der Plasmamembran, bei ruhenden Thrombozyten liegen sie intragranulär. Nach Markierung der Glykoproteine mit monoklonalen Antikörpern und Immunogoldlabeling waren Gp53, GMP140 und Thrombospondin an Thrombozyten im Leichenblut elektronenmikroskopisch ganz überwiegend intragranulär darzustellen. Der intragranuläre Nachweis der Glykoproteine zeigt, daß die Thrombozyten im Leichenblut nicht aktiviert waren. Die Thrombozyten im Leichenblut waren nur im frühen postmortalen Intervall aktivierbar, sie exprimierten dann die Markierungen der Adhäsionsmoleküle auf der Plasmamembran. Später waren sie in vitro auch dann nicht mehr aktivierbar, wenn durch Zugabe von Thrombin der Ausfall von Fibrin provoziert wurde und der Aspekt "geronnenen Blutes" entstand. In der forensischen Medizin könnten diese Befunde hilfreich sein zur Differenzierung intravitaler und postmortaler Gerinnungsvorgänge.

Schlüsselwörter Thrombozyten · Aktivierungsstatus
Postmortem · Immunelektronenmikroskopie
Glykoproteine Gp53, GMP140, Thrombospondin

Introduction

At autopsy, intravascular blood is found in the fluid state either because coagulation has not occurred or because fibrinolysis has taken place following postmortem coagulation. Previous studies on the aggregation status of postmortem blood, on the clotting factors that trigger the coagulation cascade, and on postmortem fibrinolysis have produced conflicting results or interpretations [for review see: 1–3]. We approached this problem by studying the activation status of platelets in cadaver blood.

Activation of the coagulation cascade leads to the generation of thrombin, the most potent agonist of platelet activation [4]. Once stimulated, platelets release substances which in their turn activate the coagulation cascade [5–10]. The activation status of platelets, therefore, is an indicator of whether or not blood has coagulated after death.

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In resting platelets the glycoproteins Gp53 and GMP140 and thrombospondin (TSP) are harbored in storage granules within the cell. Only after activation are they expressed on the surface membrane. They can thus serve as markers of platelet activation.

Gp53 is a 53-KDa glycoprotein occurring in lysosomes of resting platelets [11–14]. After platelet stimulation the protein is expressed on the surface membrane. The biological function of Gp53 is unknown.

GMP140 is a 140-KDa glycoprotein harbored in α-granules of resting platelets [15–17]. Like Gp53, GMP140 is expressed on the plasma membrane only after activation [18–20]. GMP140 plays a decisive role in the cell to cell interaction of platelets with monocytes, granulocytes and T-lymphocyte subsets (CD4, CD18) [20, 21].

TSP is a 450-KDa glycoprotein that is a major constituent of platelet α-granules which, on activation, is translocated to the platelet surface. Some evidence suggests that TSP may interact simultaneously with both its platelet membrane receptor CD36 and with macromolecules such as fibrinogen, heparin, collagen, fibronectin, plasminogen, laminin, fibrin, and the urokinase plasminogen activator. Thus, TSP may play a role in tissue remodeling, development, and in hemostasis [for review see 22, 23].

The localization of these glycoproteins – whether within the cell or on the plasma membrane – is an indicator of the platelet activation status. In cadaver blood, the activation status of platelets shows whether the blood has remained fluid without coagulation or has coagulated postmortem and undergone subsequent fibrinolysis.

Materials and methods

Aliquots of whole blood from healthy donors (n = 2; 180 µl) were added fresh to each of 2 Eppendorf vials (Eppendorf Reaktionsgefäße 72.690.600). The same amount of blood was added to 2 vials with 1:3 of Capture-P (Capture-P wash and conservation solution for platelets; Immucor 003001) to prevent spontaneous coagulation. One vial each of fresh whole blood and blood with Capture-P was then activated at 37° C for 60 sec by thrombin (Sigma T-7009) at a final concentration of 1.0 U/ml. The remaining samples were not exposed to thrombin. The fresh blood sample was left at room temperature until spontaneous coagulation had occurred.

From each sample 3 × 60 µl of fluid blood and 3 × 8 mm^3 of coagulated blood in 60 µl Capture-P were incubated for 12 h at 4° C in equal volumes of each of the following 3 monoclonal antibodies (mAb) diluted 1:5 in Capture-P:

mAb IgG1 (mouse) CD63 (Dianova 0768) [24]
mAb IgG1 (mouse) CD62 (Dianova 0767) [25]
mAb IgG1 kappa (mouse) anti TSP (Dianova 0257) [26].

For controls the primary antibodies were replaced by heat-denaturated antibody solutions or mouse serum. After 2X washing with Capture-P, the samples were incubated for 1 h at 18° C in equal volumes of immunogold (goat anti-mouse IgG H + L chain labeled with 12 nm gold particles; Dianova 115–205–146) diluted 1:3 in Capture-P. After renewed washing with Capture-P, the samples were fixed for at least 24 h in highly purified 25% glutardialdehyde (Serva 23114) diluted 1:5 in 0.1 M phosphate buffer (pH 7.4). The specimens were then rinsed in phosphate buffer, incubated for 1 h and 20° C in 2% aqueous osmium tetroxide (Baker 1425), and embedded in araldite (Ciba-Geigy 23857.9). Ultrathin sections were contrasted as described by Reynolds [27] and evaluated on a Philips 300 electron microscope equipped with 35 mm and 70 mm rollfilm. Cadaver blood (n = 10) was collected at intervals ranging from 1 to 48 hours postmortem (hpm) (Table 1). Fluid blood was taken from subjects with known time of death, no signs of shock, no anticoagulant treatment, and no multiple injuries. Blood was obtained by gravity flow from the median veins of extremities. In the event of repeated punctures, the cephalic and basilic veins were also used. Autopsy was performed 2 days after the taking of blood samples. Subjects who died of epidural hematoma or asthma had fluid blood in the entire vascular system. In cases of myocardial infarction, fluid blood was found in peripheral and central vessels, but a buffy coat had formed in the left ventricle of the heart. The case of breast cancer had fluid blood in small vessels, but slightly clotted blood in large vessels. Cadaver blood was processed in the same manner as whole blood from healthy donors. In addition, a sample of spontaneously coagulated whole blood was left at room temperature until spontaneous lysis occurred.

Table 1 Hours postmortem (h.p.m.) up to collection of blood samples, sex, age and cause of death. * = multiple samples taken

h.p.m.	Sex	Age	Cause of death	Agony
1	f	37	Epidural haematoma	1 h
2	m	58	Myocardial infarction	2 h
2.5	m	64	Asthma death**	30 min
3	m	70	Myocardial infarction*	5 min
4.5	m	64	Asthma death**	30 min
8,5	m	64	Asthma death**	30 min
10.5	m	64	Asthma death**	30 min
11	f	51	Breast cancer	24 h
24	m	70	Myocardial infarction*	5 min
48	m	70	Myocardial infarction*	5 min

After incubation with the antibodies, araldite blocks were prepared from 48 intravital and 150 postmortem samples and 5–10 ultrathin sections were cut from each block for immunoelectron microscopy.

Results

Platelets from healthy donors

Activation status. Platelets in aliquots of whole blood from healthy donors showed intragranular immunogold labeling when spontaneous coagulation and thus platelet activation was blocked by suspension in Capture-P. Sparse labeling on the surface membrane did occur, becoming increasingly likely the more often the blood had been centrifuged during washing. Intragranular labeling, however, clearly predominated in every sample.

Stimulation studies. Following in vitro stimulation of the platelets by thrombin, dense immunogold labeling was observed on the plasma membranes, especially in areas of cell-to-cell contact, preferentially the pseudopods. Labeling on the surface membrane was greatest for Gp53, somewhat less for GMP140, and much less for TSP. Labeling was equally intense regardless of whether the platelets had been activated in vitro by thrombin or during spontaneous clotting. Surface labeling did not diminish after spontaneous fibrinolysis of the clot. Occasional soli-

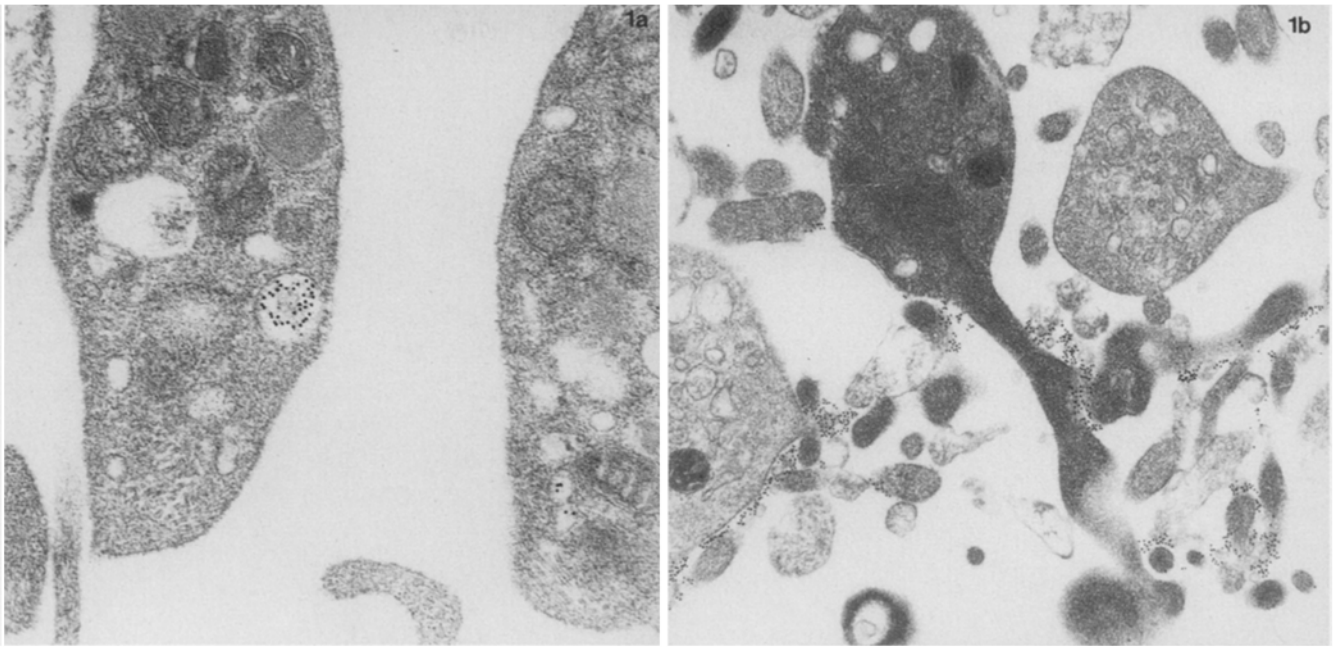

Fig. 1 Cadaver blood, early postmortem interval (a and b). Platelets exhibit dense intragranular labeling in the storage granules (a), indicating that they are not activated (CD63, immunogold labeling ×57640). After stimulation with 1.0 U/ml thrombin, the glycoproteins are expressed on the platelet surface membrane (b), hence the platelets are activated. (CD63, immunogold labeling ×36630)

tary intragranular immunogold labeling was noted, but remained rare.

Cadaver blood (early postmortem interval)

Activation status. In the early postmortem interval (1–4 hpm), platelets exhibited dense intracellular labeling in the storage granules or in the extended "surface connected canalicular system" [28]. Labeling on the surface membrane was rare (Fig. 1a). Shortly after death, therefore, the activation status of platelets in cadaver blood was comparable to that of platelets in blood from healthy donors. In both instances the glycoproteins were located within the cell and not on the surface membrane. In the early postmortem interval the platelets were not activated, indicating that blood in the intact vascular system does not coagulate at this time without the addition of a further stimulant. This applies to all of the cases studied regardless of duration of agony or type of illness.

Stimulation studies. Following in vitro thrombin-induced or spontaneous coagulation in the early postmortem interval, Gp53, GMP140 and TSP were expressed on the platelet surface membrane (Fig. 1b). Hence, platelets in cadaver blood were as capable of being activated shortly after death as platelets in blood from healthy donors. In both instances, the glycoproteins were expressed on the cell surface. In the early postmortem interval, therefore, cadaver blood is capable of coagulation with corresponding activation of platelets. The staining pattern in cadaver blood was the same regardless of whether coagulation had occurred spontaneously or was induced in vitro by thrombin. Even after spontaneous fibrinolysis, the glycoproteins could be demonstrated of the platelet surface.

Cadaver blood (late postmortem interval)

Activation status. In the late postmortem interval (4.5–11 hpm), the glycoproteins could still be detected in markedly dilated storage granules or in the extended "surface connected canalicular system" and not, or only rarely, on the surface membrane (Figs. 2a, 3a and 3b). Some platelets exhibited blunt, worm-like pseudopods (Figs. 3a, 3b), as described in previous electron microscope studies [29]. The intragranular localization of the glycoproteins indicated that the platelets had not been activated.

Stimulation studies. Unlike platelets in the early postmortem period, platelets in the late postmortem interval possessed intragranular glycoproteins even after thrombin or spontaneous blood coagulation had induced the formation of a macroscopically evident fibrin mesh (Fig. 2b). The platelets located within this "clot" did not express the glycoproteins on their plasma membranes, and hence were not activated. Although the macroscopically evident fibrin mesh produced the appearance of "coagulated blood", the glycoprotein expression typical of activated platelets was lacking. In native cadaver blood, Gp53 and GMP140 expression on the platelet surface membrane increased up to 48 hpm. During this same time, TSP was not detected. Additional expression of surface activation markers could not be stimulated by incubating with

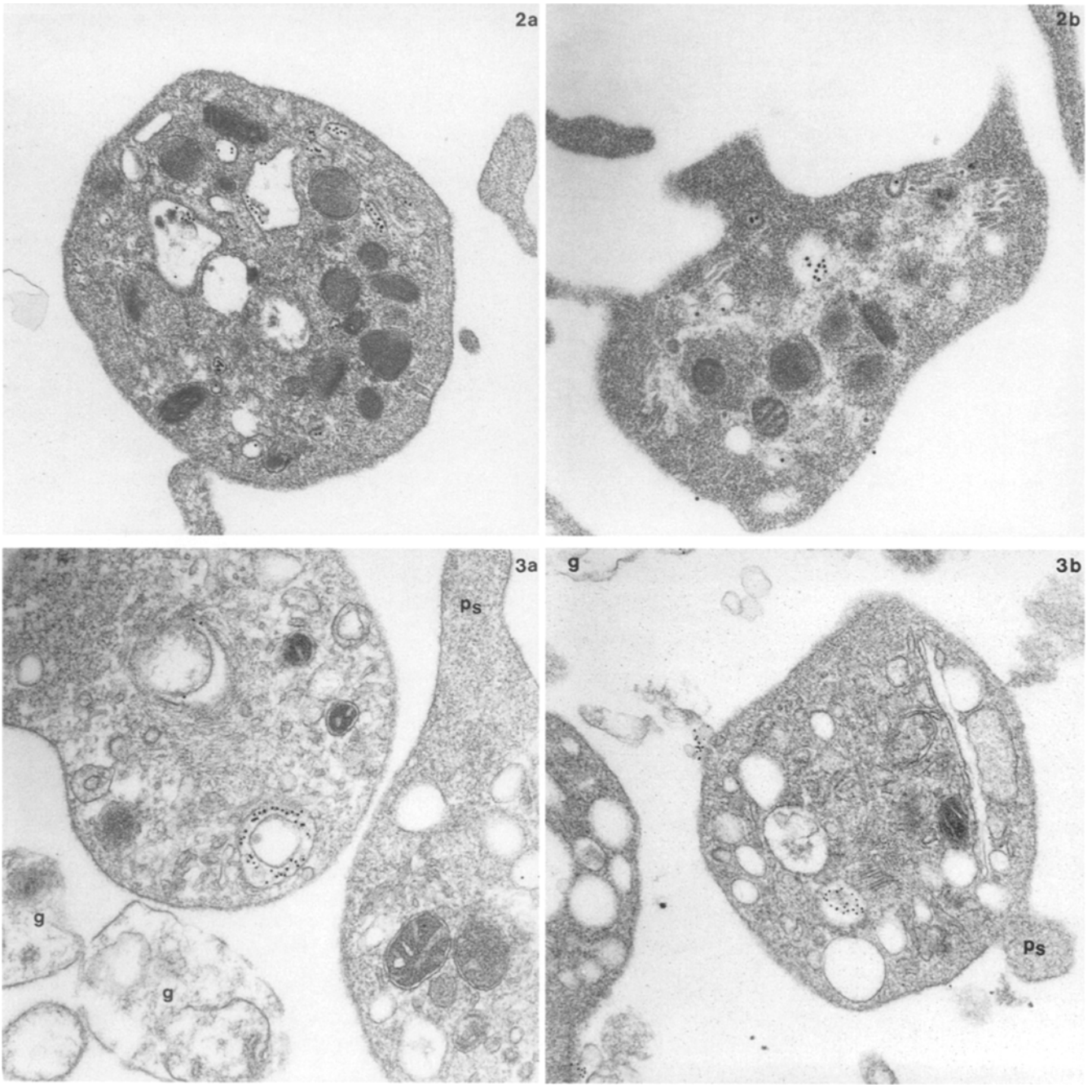

Fig. 2 Cadaver blood, late postmortem interval (a and b). The intragranular localization of the glycoproteins indicates that the platelets are still not activated (a) (anti-CD63, immunogold labeling x 36630). Unlike platelets in the early postmortem period, these platelets possess intragranular glycoproteins even after stimulation with thrombin (b) and thus are no longer capable of activation (CD63, immunogold labeling ×36630)

Fig. 3 Cadaver blood, late postmortem interval (a and b). After 24 hpm (a) and 48 hpm (b) the platelets show no signs of activation and could not be activated by thrombin. The glycoproteins are located intragranularly. (anti-CD63, immunogold labeling, a: ×57640, b: ×45155, g = "ghost". Ps = pseudopod)

thrombin. Although extracellular labeling increased with time postmortem due to progressive disintegration of the platelets ("ghosts", Figs. 3a, 3b), intracellular labeling continued to predominate (Figs. 3a, 3b).

Discussion

We developed a method for the localization of platelet activation markers in such heterogenous environments as thromboses, hematomas, and clotted cadaver blood. The nature of this material precluded the use of flow cytometry, which is suitable for quantitative analysis of suspended intravital platelets [30–32]. Immunoelectron mi-

croscopic demonstration of intragranular or surface activation markers is an established method [12, 13, 33, 34] that can also be applied to evaluate the activation status of platelets in blood clots and hematomas. We investigated a large number of sections (cf. Methods above) to determine the postmortem activation status of platelets.

Some platelets already express glycoproteins in circulating blood [35, 36], while others remain refractory to activation by thrombin [37, 38]. However, the number of intravitally activated or activation-refractive platelets is small: activated platelets are rapidly eliminated from the circulation [39]. Since platelets can be artifically activated during removal and subsequent handling [31, 40], processing steps were kept to a minimum. The platelets were studied in whole blood [7] or in whole blood suspended in Capture-P, which largely inhibits artificial activation [41, 42]. In order to study the samples in as native a state as possible, neither anticoagulants nor Ca^{++} were used. A high final concentration of 1 U/ml thrombin [21, 43–45] was applied to provoke any residual activation of platelets in the cadaver blood. The glycoproteins GMP140 and Gp53 – unlike TSP – were found to be insensitive to protease activation: they could be detected on the plasma membrane of activated platelets in a subarachnoidal hemorrhage even 8 days postmortem (Thomsen and Krisch, unpublished data).

Data on the number of glycoprotein epitopes vary: for Gp53, 12600 [13], 9000–11000 [12] and 6800 [30] epitopes have been reported per activated platelet, while for GMP 140 the numbers range from 9000 [30] to 13000 [17], up to 15000–20000 [14]. For TSP 2000 [46], 6400 [30], and even 35000 [47] epitopes have been counted. TSP-binding on the cell surface, however, is a transient phenomenon which, independently of protease activity, soon enters into a soluble phase [22, 48] and thus can no longer be detected on the plasma membrane. The explains why TSP antigen labeling was markedly lower than either Gp53 or GMP140 labeling.

Taking these facts into account, we were able to discriminate samples in which a vast majority of platelets were activated from those in which activation was largely lacking.

Activation status

In platelets from cadaver blood the antigens Gp53, GMP140 and TSP were located intragranularly. A re-internalisation of the glycoproteins from the cell surface to the storage granules by endocytosis could be ruled out [44, 49, 50], the surface labeling continued unabated once platelet activation had occurred – even after spontaneous fibrinolysis. Therefore, platelets in cadaver blood were not activated and hence death alone was not sufficient to induce platelet activation. If the cadaver blood we studied had undergone prior coagulation, then the platelets must have been activated since the thrombin concentration required to induce activation is much lower than that needed for hydrolysis of fibrinogen [15]. Therefore, thrombin-induced platelet activation must have preceded fibrin polymerization.

Stimulation studies

In the early postmortem interval, platelets in cadaver blood are just as capable of activation as platelets in blood from healthy donors. In the late postmortem interval clots can form spontaneously or be induced in vitro by thrombin. The blood then appears to be "coagulated" due to the formation of a fibrin mesh ("clot"). However, in these clots the adhesion-glycoproteins were labeled intragranularly and hence the platelets were incapable of activation. We attribute this phenomenon to the rapid onset of acidosis after death [52], since pH plays a decisive role in the functional integrity of platelets [53, 54]. With progressively sinking pH, platelets swell, leading eventually to the formation of "ghosts" and ultimately to thrombocytolysis [55]. The swelling of the platelets disturbs the complex interaction between cytoskeletal actin and Gp53, GMP140 and TSP [30, 56], which is necessary for the activation reaction.

Moreover, platelet activation depends to a large degree on the availability of energy-rich phosphates [57], which are rapidly consumed by thrombosthenin [57, 58]. The rapid postmortem disturbance of platelet function is also evident in the marked decline in the platelet adhesion index [2]. Unlike platelet adhesion, platelet aggregation and the release of granule contents require ongoing platelet metabolism and platelet stimulation by specific agonists [59]. These complex processes are especially vulnerable to changes in the cellular milieu.

Platelets in cadaver blood can be activated for only a brief period postmortem. After that, activation does not occur even if fibrin formation has been induced by thrombin. In forensic medicine, these findings can possibly be applied to distinguish intravital clotting from the postmortem coagulation phenomena and intravital hematomas from postmortem hematomas.

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