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Immunohistochemical alterations after muscle trauma

G. Fechner, Th. Bajanowski, and B. Brinkmann

Institut für Rechtsmedizin, Universität Münster, Von-Esmarch-Strasse 86, W-4400 Münster, Germany

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Summary. The proteins fibrin, fibrinogen, fibronectin and complement C5b-9 were investigated in mechanically damaged skeletal muscle. An accumulation of fibrin, fibrinogen and fibronectin could be observed immediately after intra-vital trauma in damaged fibre zones, later an accumulation at the torn edges of the fibres. The accumulation of complement C5b-9 began one hour after trauma. After post mortem trauma no positive reactions could be observed for any of the proteins. The degree of expression of these proteins can therefore be used to differentiate between vital and postmortem muscle damage as well as the estimation of wound age in the early antemortem time period.

Key words: Skeletal muscle – Mechanical trauma – Fibrin – Fibrinogen – Fibronectin – Complement

Zusammenfassung. Die Proteine Fibrin, Fibrinogen, Fibronektin und Komplement C5b-9 wurden immunhistochemisch mit der PAP-Methode an traumatisierter Skelettmuskulatur untersucht. Bei vitalen Traumen kommt es unmittelbar posttraumatisch zu einer zunächst diffusen Anreicherung in geschädigten Faserabschnitten, später zu einer herdförmigen Akkumulation an den Faserbruchrändern. Die Komplement C5b-9-Reaktion wird frühestens nach einer Stunde positiv. Bei postmortalen Traumen konnten positive Reaktionen nicht nachgewiesen werden. Das Auftreten und die Quantität dieser Proteine ist damit ein geeigneter Marker zur Differenzierung vitaler und postmortaler Muskelverletzungen und zur Abschätzung des Wundalters im früh-antemortalen Zeitintervall.

Schlüsselwörter: Skelettmuskulatur – Trauma – Fibrin – Fibrinogen – Fibronektin – Komplement

Introduction

The occurrence of vital reactions in skeletal muscles has often been investigated (Orsos 1935; Lindner 1967; Ojala and Lempinen 1968), most recently by Sigrist (1986). In previous studies, our group employed modern techniques such as electron microscopy (Fechner et al. 1990) and immunohistochemistry (Fechner et al. 1991). With the latter method the muscle proteins myosin, actin, desmin and myoglobin were investigated. Due to the reaction pattern it was possible to differentiate vital trauma to post mortem changes.

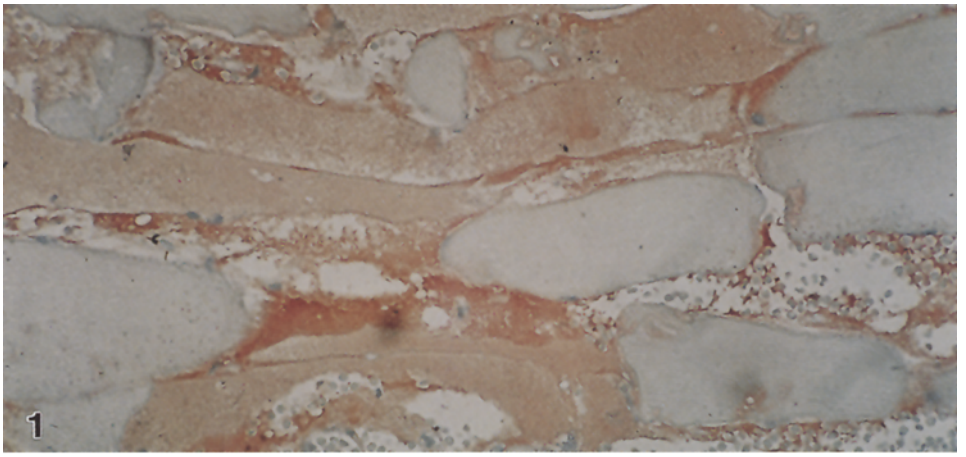
The proteins fibrin, fibrinogen, fibronectin and complement C5b-9 were used until now as markers at myocardial infarction. An accumulation of these proteins could be observed in the infarction area (Shekhonin et al. 1990; Thomsen et al. 1990). Immunohistochemical investigations of these proteins have not been carried out after muscle trauma.

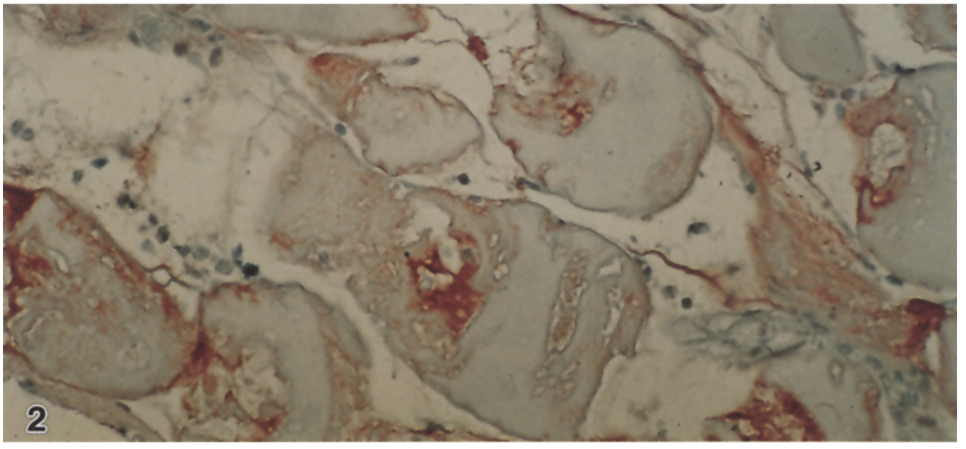
Correspondence to: G. Fechner

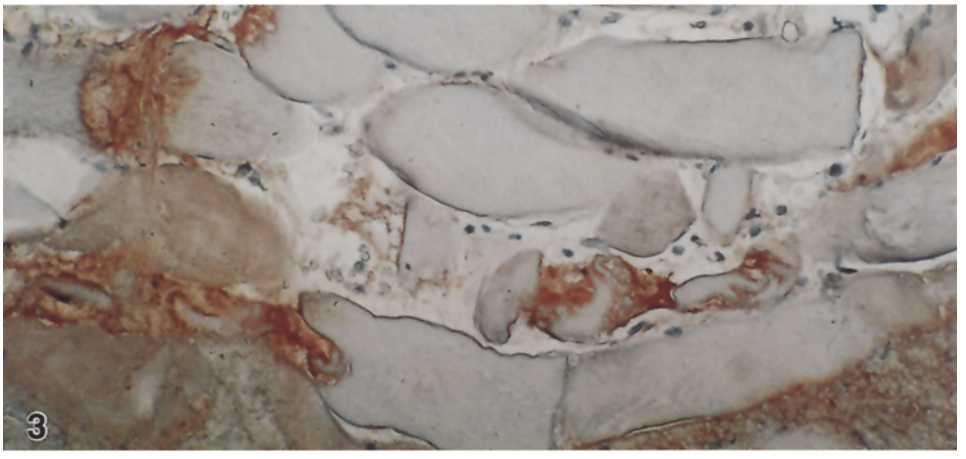
Materials and methods

1. Skeletal muscles (M. gastrocnemius) from 15 anaesthetized dogs and 15 mice were injured over a period 1 min to 17 h before death by pinching the muscle between anatomical clamps. The clamps were manually applied directly to the centre of the muscle through the skin and were strongly squeezed for a short (secs) period of time until damage could be seen macroscopically. No quantitative measurement of the intensity was made. These experiments were carried out in parallel with other clinical experiments after which the animals were sacrificed. Samples were removed by the method of Jerusalem and Bischhausen (6) immediately after death and after an interval of 24 h. Each sample was divided into 2 parts: one taken from the centre and the other from the periphery of the damaged muscle area. – Control muscle samples which had damaged in an identical fashion over a time period 15 min to 10 h postmortem were collected as previously described (6). – Undamaged muscle samples were taken as controls in parallel to the damaged samples from the same muscles.

2. Samples of human muscle tissue were obtained from 15 fatalities due to blunt mechanical trauma and with known survival periods. Muscle samples were taken from a variety of muscle tissues from victims of traffic accidents with varying degrees of trauma. The time period of the survival after injury ranged between 1 min and 24 h. The postmortem time interval of sampling ranged from 6 h to 72 h. The human and animal corpses were refrigerated at 4°C. The postmortem interval of 6 h is substantially greater than the intervals tested in the animal experiments, but no comparable human examples were available for control purposes. This fact should be borne in mind when comparing the results.







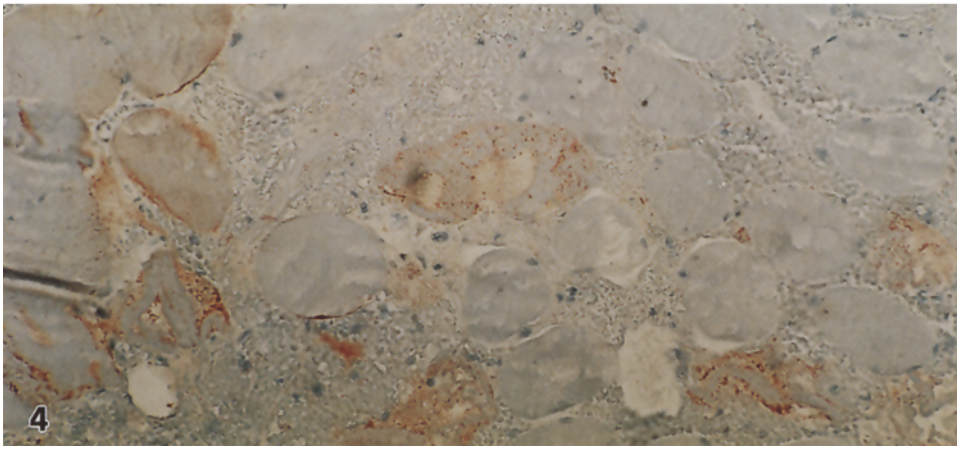


Fig. 1. Immunohistochemical detection of fibrinogen. Diffuse intensive reaction in the breakage areas of the fibres and in the sarcolemmal tubules and at the broken edges of the fibres. (Dog, muscle tissue, post traumatic interval 1 h, 250 ×)

Fig. 2. Immunohistochemical detection of fibrin. Accumulation of fibrin between, and at the edges of the broken fibres. Positive reaction also in the cavities of the discoid fibre destruction. (Mouse, muscle tissue, post traumatic interval ½ h, 160 ×)

Fig. 3. Immunohistochemical detection of fibronectin. Accumulation between the broken fibres and carpet-like covering of the ruptured edges of the fibrils. (Human muscle tissue, post traumatic interval 2 h, 160 ×)

Fig. 4. Immunohistochemical detection of Complement (C5b-9). Positive reaction in the centre and in the sarcolemmal area of the degenerated fibres. (Human muscle tissue, posttraumatic interval 2 h, 250 ×)

3. Specimens of histological investigation were selected from the trauma zones exhibiting a similar degree of bruising as observed in the animal experiments. All samples were fixed in 4% buffered formalin, embedded in paraffin and 5 µm sections were prepared. Using immunohistochemistry the following antigens were investigated:

Fibrin/Fibrinogen: primary antibody: anti-fibrinogen, polyclonal, dilution 1:60, incubation time 60 min at room temperature (Dako, Hamburg). *primary antibody:* anti-fibrin: monoclonal, dilution: 1:800, incubation time 60 min at room temperature (Dianova, Hamburg)

Fibronectin: primary antibody: anti-fibronectin, polyclonal, dilution 1:200, incubation time 30 min at room temperature, treatment with trypsin (Dako, Hamburg)

Complement C5-9: primary antibody: anti-complement C5b-9, polyclonal, dilution 1:10, incubation time 16 h at temperature of 4°C, treatment with pronase (0,1%, pH 7.5, 60 min at room temperature), (Calbiochem, Frankfurt/M). This antibody is human specific.

Secondary-antibody: antibody, polyclonal, "anti-rabbit Ig", dilution 1:1000 (Dako, Hamburg)

PAP-Complex: Dilution 1:100 (Dako, Hamburg)

AEC-Substrate dilution: 3-amino-9-ethylcarbazol 10–15 min (Sigma, St. Louis, USA)

4. Negative controls were carried out by omitting only the primary antibody from the procedures. In addition, undamaged muscle fibres served as negative controls and tissues other than muscles fibres served as positive controls. Sections were counterstained using haematoxylin. The evaluation of the stain reaction was made on a semi-quantitative basis as follows: (−) no visible reaction, (+) slight positive reaction, (++) clear positive reaction, (+++) strong positive reaction. Histological sections were also stained using the H & E and Masson trichrome staining methods.

Results

The muscle specimens taken for examination showed typical macroscopical changes such as haemorrhaging and tearing of fibres. The H & E and Masson trichrome stained preparations showed typical changes of trauma as described by Sigrist (1986), such as destruction of the fibre integrity, funnel-shaped edges of the broken surface of the fibre, disappearance of the cross-striations, and discoid and segmental disintegration of the fibres.

Undamaged muscle fibres did not exhibit the presence of the proteins studied in this investigation and were only detected in damaged fibres.

Fibrin/fibrinogen (Figs. 1,2) and fibronectin (Fig. 3) exhibited a nearly identical reaction pattern. Within vitally damaged fibres and fragmented fibres formed as a consequence of trauma, positive reactions corresponding to the following structures could be demonstrated: an accumulation could be observed within those muscle fibre segments/parts which usually appeared empty when viewed under light microscopy, and also between the segments of discoid fibre destruction and within microcystic cavities. In the early stages this reaction was faint and blurred but subsequently accumulated at the apparently broken edges of the fibres. Positive reactions for fibrinogen and fibrin were first seen within the first minute after injury, however the accumulation occurred more slowly with fibronectin.

Table 1. Immunohistochemical reactions dependent of the trauma interval (human)

	Post-mortem	Antemortem		
		0–30 min	30–60 min	>60 min
Fibrin/Fibrinogen	−(+)	+	+++	+++
Fibronectin	−	−	+	+++
Complement (C5b-9)	−	−	−	++

A positive reaction for the complement complex C5b-9 could only be demonstrated in human samples. Initially a positive reaction could be seen in the sarcolemma, and later on also in the fibre content (Fig. 4). The earliest complement reaction could be observed after one hour. The staining at the margin of the trauma zone was often more intensive than in the center.

The accumulation of fibronectin and complement C5b-9 was weaker in the center of extensive haemorrhages than in the periphery.

The time dependence of the immune reaction in the various species (dog, mouse, human) showed no discernable differences. In comparison to human tissues the immunohistochemical reaction in dog and mice tissues was relatively similar but the human tissues seemed to react slightly later (minutes).

Histological sections (H & E and Masson trichrome staining) exhibited alterations which paralleled the immunohistochemical reaction patterns described, but the information derived from the specific protein staining was more distinctive and gave a clearer picture especially in injuries caused before death.

All immunohistochemical reactions were reproducible and were not arranged by autolysis. Postmortem injuries did not show these positive reactions. Only fibrin/fibrinogen occasionally showed a slight accumulation at the outer surface of the sarcolemma where this was surrounded by haemorrhages and within the haemorrhages.

Discussion

Investigations on the accumulation of these proteins in traumatized skeletal muscles have not yet been documented previously. But time and again the importance of fibrin formation was discussed as a possible distinguishing feature of vitality of muscle injury.

But there is no doubt that postmortem haemorrhages also show fibrin formation (Mueller 1964; Berg 1975). Intra vitam, fibrin formation begins only seconds after trauma (Böhm and Tschomakow 1973; Schneider 1970). Hauser et al. (1990) demonstrated a distinctive topological organization of the fibrin network after vital bleeding. The influx and formation of fibrin/fibrinogen and fibronectin has been reported by Shekhonin et al. (1990) in damaged heart muscle fibres and they recognized the possibilities of early diagnosis of myocardial infarct. But this accumulation could only be recognized at the earliest after 3 hours. There is a close relationship between

fibrin formation and the occurrence of fibronectin, the latter showing covalent binding to the fibrin clot (Vaheri et al. 1985). The major component of fibronectin in plasma is produced by hepatocytes. Fibronectin plays a significant role in wound healing as has been demonstrated in a variety of organs (Grinell et al. 1981; Gulati et al. 1982; Lehto et al. 1985).

The investigations of Gauperaa and Seljedid (1986) showed that the influx of fibronectin into wounds is time-dependent. Following muscle trauma, rats were injected with plasma fibronectin labelled with J125. An accumulation of fibronectin was found in damaged areas starting 30 min after the trauma and reaching maximum concentrations after 6 h. After experimental inflammation this accumulation was chronologically similar but the reaction was much weaker.

Fibrin and fibronectin serve as substrates for adhesion and migration of the cells responsible for removal and repair. But cells such as macrophages, fibroblasts and neutrophils can also produce fibronectin (Hynes 1990). There are therefore two possible sources for the accumulation of fibronectin: transport of fibronectin by the circulation to the damaged area and subsequent influx through the damaged sarcolemma: this mechanism begins immediately after trauma. At a later phase fibronectin can be produced directly by the infiltrating cells within the granulation tissue. Both mechanisms of fibronectin enrichment require an intact circulation.

Degeneration as a consequence of inflammation, trauma and ischaemia, and also of other diseases, leads to stimulation of the complement cascade, the end product being the stabile C5b-9 complex (Sahashi et al. 1980; Schäfer et al. 1986). It has been recognized that the C5b-9 complex shows pronounced lipophilic properties and is incorporated as a circular structure within cellular membranes. This results in transmembraneous pore formation and subsequent cell damage (Bhakdi and Muhly 1983; Bhakdi and Tranum-Jensen 1987). The completion of the complement cascade obviously occurs over a longer time period. In this investigation the minimum time interval required to demonstrate positive reactions was at least one hour. – Thomsen et al. (1990) were able to immunohistochemically demonstrate positive reactions in case of sudden cardiac death, but this is not necessarily in contradiction to our results because the time period from the beginning of cellular injury to cardiac arrest is not known. – In addition our investigations also demonstrated the lack of relationship to autolysis. An accumulation of the investigated proteins occurred only after vital damage and could not be demonstrated after postmortem damage.

Clear differences were observed between the reaction pattern found in this investigation and the changes of muscle proteins such as actin, myosin, desmin and myoglobin (Fechner et al. 1991). In the early postmortem interval after trauma slight depletion on these proteins can occur in the direct zone of trauma. After vital trauma the depletion is more distinct and an accumulation between the ruptured muscle fibres occurs very rapidly. The time period of this reaction is comparable to that of fibrin/fibrinogen, but occurs later.

Investigations on the vitality and wound age of muscle damage have been carried out using enzyme histochemical methods (Smith 1965; Ojala 1968; Fechner 1991) and a reaction pattern dependent on the interval after trauma could also be observed. In both animal and human muscle tissues to phosphorylase activity was reduced immediately, the dehydrogenase activity disappeared after 1–4 hours and the non-specific esterase activity after 4–8 hours. After 2 days the total glycolytic and oxidative activity in the destroyed fibres had been lost. The disadvantages of these enzymatic histochemical methods are the variability of the postmortem stability of the enzymes and the difficulties in sample preparation (i.e. the requirement for frozen sections).

As a result of our investigations it is suggested that the influx and especially the accumulation of intracellular fibrin and fibronectin necessitates an active process which is dependent on the presence of a functioning circulation. Furthermore a time dependance of the reaction pattern could be demonstrated whereby the earliest reaction was found for fibrin/fibrinogen and fibronectin, and in a later phase the presence of complement C5-9 complex. The overall reaction pattern therefore allows an estimation of wound age.

Mechanisms leading to the influx and accumulation of the early phase proteins seem to be related to the type of injury. Ischaemic injury alone like in myocardial infarction necessitates hours while mechanical trauma, as for instance in our experiments, is nearly instantaneously and allows a very early positive immunohistochemical reaction.

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