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Immunohistochemical investigations to demonstrate vital direct traumatic damage of skeletal muscle

G. Fechner, R. Hauser, M. A. Sepulchre, and B. Brinkmann

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

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Summary. The muscle proteins actin, myosin, desmin and myoglobin were investigated in traumatically damaged human and animal skeletal muscle using an immunohistochemical PAP-method. A depletion of all the proteins investigated was observed in muscle fibres damaged in the antemortem period. The antigens could however also be demonstrated in the otherwise empty sarcolemma, the discoid disintegration zones of the fibres and between the fibres. The depletion begins immediately after the trauma and myoglobin is the first to be affected. No such changes could be observed after post mortem muscle damage. The antigens could be demonstrated until 72 hours post mortem. The demonstration of protein depletion is an important addition to the light microscopical findings in vital muscle alterations.

Key words: Skeletal muscle – Mechanical trauma – Muscle proteins – Immunohistochemistry

Zusammenfassung. An traumatisch geschädigter menschlicher und bei Tierexperimenten gewonnener Skelettmuskulatur wurden immunhistochemisch mit der PAP-Methode die Muskelproteine Aktin, Myosin, Desmin and Myoglobin dargestellt. Bei vital traumatisierter Muskulatur kommt es zu einer Depletion aller untersuchter Proteine aus der verletzten Muskelfaser. Die Antigene können aber in den sonst leeren Sarkolemm-Schläuchen, den diskoiden Faserzerfallszonen und auch außerhalb der Fasern nachgewiesen werden. Die Depletion beginnt Minuten nach dem Trauma, am frühesten beim Myoglobin. Bei postmortaler Traumatisierung waren die Veränderungen nicht nachweisbar. Die Antigene waren bis 72 Stunden postmortal stabil. Der Nachweis der Muskelprotein-Depletion stellt eine wichtige Ergänzung zu den lichtoptischen Kriterien vitaler Muskelfaseralterationen dar.

Schlüsselwörter: Skelettmuskulatur – Mechanisches Trauma – Muskelproteine – Immunhistochemie

Offprint requests to: B. Brinkmann

Introduction

Several published reports have dealt with the demonstration of an intra-vital reaction within skeletal muscles after injury [1, 12], the most recent by Sigrist [17]. In the present study the structural proteins myosin, actin and desmin and the functional protein myoglobin were investigated for their suitability as indicators of the intra-vital origin of the trauma and a PAP-technique [18] was used to specifically demonstrate these antigens.

Materials and methods

1. Skeletal muscles (Gastrocnemius) from 15 anaesthetized dogs 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 was made of the intensity of the pressure applied. These experiments were carried out in parallel with other clinical experiments after whose termination the animals were sacrificed. Samples were removed by the method of Jerusalem and Bischhausen [6] immediately after death. 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 been damaged in an identical fashion over a time period 15 min to 10 h post-mortem were collected as previously described [6]. Undamaged muscle samples were taken as controls in parallel to the damaged samples.

2. Furthermore, 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 fatalities from traffic accidents who had sustained varying degrees of muscle trauma. The time period between antemortem-traumatisation and death ranged between 1 min and 24 h. The post-mortem time interval of sampling ranged from 6 h to 72 h. The post mortem interval of 6 h is substantially greater than the intervals tested in the animal experiments, but no comparable human tissues were available for control purposes. Specimens for histological investigation were selected from the traumatised zones exhibiting similar degrees of haemorrhage as observed in the animal experiments.

3. All samples were fixed in 4% buffered formalin, embedded in paraffin and 5 µm sections were prepared. Using indirect immunohistochemistry the following antigens were investigated:

Myosin. Myosin is contained in the thick filaments of skeletal muscle and has a molecular weight of 480.000 d. Anti-myosin specifically binds to the A-bands (primary antibody: polyclonal, anti-myosin, dilution 1/20; ICN Biochemicals, W-3440 Eschwege). –

Actin. Actin is localised in striated muscle sites corresponding to the thin filaments and has a molecular weight of 42.000 d. Anti-actin specifically binds to the I-bands (primary antibody: polyclonal, anti-actin, dilution 1:10; ICN Biochemicals, W-3440 Eschwege). –

Desmin. Desmin belongs to the group of intermediate filament proteins and has a molecular weight of 53.000 d. Anti-desmin specifically binds to the Z-bands (primary antibody: polyclonal, anti-desmin, dilution 1/10; Sigma-Chemie, W-8024 Deisenhofen). –

Myoglobin. Myoglobin is a functional protein of oxygen transport (storage) in muscle and is distributed throughout the sarcoplasm (molecular weight 17.500 d). Anti-myoglobin specifically binds to myoglobin which is distributed diffusely over the entire cytoplasm (primary antibody: polyclonal, anti-myoglobin, dilution 1/50; Dako, W-2000 Hamburg).

Secondary antibody: polyclonal antibody anti-rabbit IgG, dilution 1/50, Dako, W-2000 Hamburg. PAP complex (Dako, W-2000 Hamburg).

4. Negative experimental controls were carried out by omitting only the primary antibody from the procedures. In addition, undamaged muscle fibres served as positive controls and tissues other than muscle fibres served as negative controls. Sections were counterstained using haematoxylin. The evaluation of the stain reaction was made on a semi-quantitative basis: (–) no visible reaction, (+) slight positive reaction, (++) definite positive reaction, (+++) strong positive reaction.

Sections were also stained using the H & E and Masson trichrome staining methods.

Results

The muscle specimens taken for examination showed typical macroscopic changes such as haemorrhage and tearing of fibres. After H & E and Masson trichrome staining preparations showed typical changes of trauma as described by Sigrist [17], such as destruction of the fibre integrity, funnel-shaped edges of the broken surface of the fibre, disappearance of the striations, and discoid and segmental disintegration of the fibres.

Sections from areas of undamaged muscle fibres served as positive controls, and showed a homogeneous distribution of the proteins and intensification of the cross striation with the exception of myoglobin which was homogeneously distributed.

After intra-vital trauma all 4 proteins exhibited an identical reaction pattern. A zone of direct mechanical injury could regularly be distinguished and this showed ruptures of fibres, haemorrhages and the other types of lesions. The zone of muscle adjacent exhibited less well-defined and probably secondary alterations to muscle fibres mainly caused by the sequelae of trauma. In the direct zone, the proteins showed a variegated distribution pattern: there was a loss of the normal staining pattern with a discontinuous alterations either in fibre segments or in single fibres; also small areas demonstrated one variety of altered reactivity alternating with areas showing a strikingly different type of reaction. Only small residual areas showed normal reactivity with or without loss of cross-striation. Other areas showed either a depletion (often combined with swelling) or an accumulation of the relevant protein. Such zones of accumulation were mainly associated with the following areas: the empty intra-sarcolemmal areas between ruptured and retracted fibre segments and especially on the surface of the contraction caps where a carpet-like intense accumulation was seen. In addition, fibre segments adjacent to ruptures showed cystic cavities with an internal accumulation of the protein, the configuration of these accumulations closely corresponded to these spaces. In addition, and especially in zones of fibre rupture, a positive reaction in the interstitial tissue between the fibres could frequently be found.

The zone of indirect reaction showed a more homogeneous type of alteration which consisted of loss of def-

Fig. 1. Immunohistochemical detection of myosin. *Darker fibres* = positive reaction, *lighter fibres* = depletion of myosin. Human muscle tissue post-traumatic interval 2 h (anti-myosin, PAP, 63 ×)

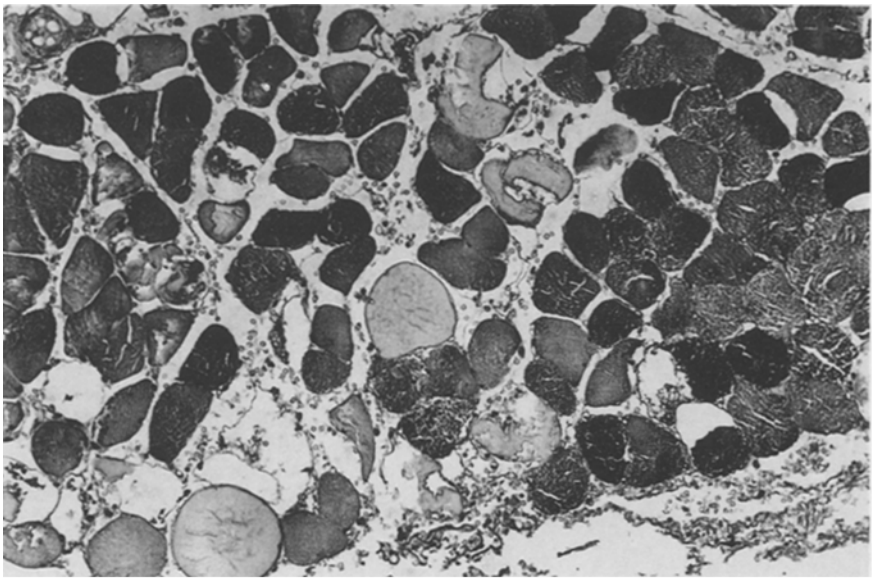

Fig. 2. Immunohistochemical detection of actin, transverse section of muscle; no immune reaction in the *lighter fibres*. Human muscle post-traumatic interval approx 15 min (anti-actin, PAP, 100 ×)

Fig. 3. Immunohistochemical detection of myoglobin. On the *right*, depletion of myoglobin in lighter fibres, on the *left* positive antigen reaction. More intensive positive reaction in the breakage areas of the fibres and in the sarcolemnal tubules. Dog, post-traumatic interval 45 min (anti-myoglobin, PAP, 250 ×)

inition or loss of cross-striation and as a consequence a homogeneous staining reaction, together with swelling of fibres and depletion of the proteins.

Areas in which the muscular damage was caused post-mortem showed a more uniform type of protein disintegration. The zone of direct damage showed homogeneous depletion of proteins with an increase of positive reaction inbetween the fibres. But no patchy or band-like accumulation of the protein, and no variegated type of protein distribution were seen as described for the similar zone of direct vital injury. The zone of protein depletion showed a continuous change in muscle fibres with normal morphological structure. In addition, only very weak haemorrhaging could be seen between muscle fibres in the direct trauma zone. In the indirect zone there was a complete absence of any alteration of fibre staining reaction similar to that of undamaged fibres and no change of the intramuscular protein reactivity.

The vital reaction pattern occurred within minutes after trauma, but there was a definite difference between the changes in the individual proteins. The earliest vital reaction pattern could be observed with myoglobin and this occurred immediately after trauma. The first visible alterations were seen with the 3 other proteins after a few minutes. Within the first 1 or 2 hours after trauma there was a definite increase in intensity of the described vital reaction pattern. The zone of indirect reaction also increased in size but with an obvious delay in comparison to the zone of direct injury.

Histological sections (H & E and Masson trichrome stains) exhibited alterations which paralleled the histochemical reaction patterns described, but the informa-

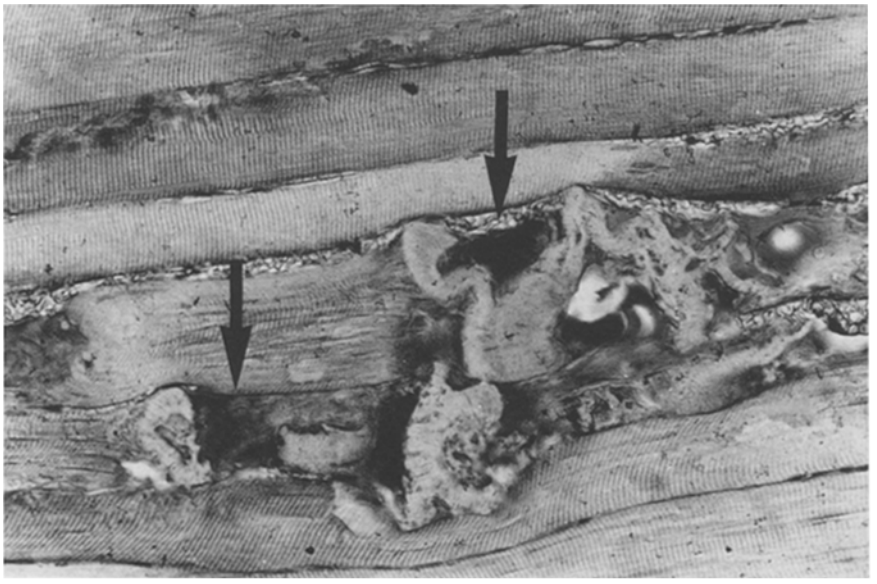

Fig. 4. Immunohistochemical detection of desmin. Loss of myofibrils in the breakage area of fibres and in the discoid disintegration zones with negative immunohistochemical reaction. Positive reaction *(arrows)* between the homogenous segments of fibres. Dog, post-traumatic interval 30 min (anti-desmin, PAP, 250 ×)

Fig. 5. Immunohistochemical detection of actin, homogeneous depletion of actin in the direct trauma zone *(arrows)*. The other proteins showed a similar reaction pattern. Dog, trauma 30 min post mortem (anti-actin, PAP, 160 ×)

tion derived from the specific protein staining was more distinctive and gave a clearer picture especially in injuries caused intravitally. In addition, zones that stained strongly in conventional histological methods frequently exhibited weak reactions in immunohistochemistry and vice versa.

No non-specific staining reactions were observed between the antibody reaction stages. This was also controlled by the omission of the primary antibodies from the reaction chain. The patterns described were obtained from animal experiments.

The specimens obtained from human pathology suffered the disadvantage that the time delay between damage and dissection differed from those in animals, but specimens with obviously identical conditions showed exactly the same reaction patterns as seen in the animals.

Discussion

Myoglobin is the oxygen carrier protein in cardiac and skeletal muscles and the release of myoglobin from heart muscle is one of the earliest signs of ischaemic injury [5, 9, 10]. The release of myoglobin from skeletal muscles has been found to be associated with a variety of types of rhabdomyolysis [16] and also with electrical injury [8].

Myosin is also released from cardiac muscle after myocardial infarct [10] and can be demonstrated in serum approximately 6 h later [7]. The release of myosin and actin after voluntary muscle injury has already been observed by Downey et al. [2] who demonstrated that myofibrillary proteins are extremely labile structures.

Desmin belongs to the group of intermediate filament proteins and is present in skeletal muscle [3]. Desmin is

an important marker protein made use of in the immunohistochemical diagnosis of the histogenesis of tumours of connective and supporting tissues [e.g. 11, 14]. This protein has a dynamic structure and can migrate to other sites, and there reorganize itself within a few minutes, its regulation being controlled by accompanying proteins [15, 19]. Desmin can, for example, be found in cytoplasmic bodies formed during muscle diseases [13].

In our investigations a zonal arrangement of lesions was found in mechanically traumatized muscle, i.e., a central zone of *direct injury* surrounded by a zone of *indirect damage*. The structural changes in the zone of direct lesion could be accounted for partly by the direct mechanical pressure but additional factors, such as contraction and contracture, must also occur as a consequence of intra vitam trauma, because the application of similar trauma post-mortem leads to distinctly different patterns. In the adjacent 'indirect damage' zone the main cause of the alterations would seem to be caused by chemical factors such as hypoxaemia induced by the primary lesion. – The influence that a variety of disturbances have on striated muscle fibres is well known: a release of myoglobin occurs even after clamping of blood vessels during operations [16] or after a rise in temperature [4]. Accompanying factors such as haemorrhage, oedema and the various stages of inflammation could therefore be contributing factors to the indirect lesions.

The time differences in the rate of depletion of the different muscle proteins might also partly be related to the relative molecular sizes of the individual structural proteins. Myoglobin has the smallest molecular weight and shows the quickest reaction, whilst the other proteins reacted more or less simultaneously, but more slowly.

Post-mortem influences must be excluded especially with regards to the depletion phenomenon. These possibilities have often been discussed [8, 16, 20]. According to Williams and Kent [20] the type of disturbance described is not to be expected even after up to 2 days autolysis time. In relevant cases this artefact would be easily excluded when microscopical findings are related to the zonal arrangement and to accompanying findings.

In combination with conventional staining methods, immunohistochemistry of muscle protein seems to provide an important additional tool to the diagnosis of vital injury of muscle. As the different proteins mainly show the same type of reaction, only two of these need to be tested in practice. The combined use of a "fast" reacting protein such as myoglobin and a "slow" reacting one such as myosin is therefore recommended.

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