Matrix Metalloproteinase-9 is Involved in the Fibrotic Process in Denervated Muscles after Sciatic Nerve Trauma and Recovery

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Abstract

Fibrosis of the injured muscles is a problem of recovery from trauma and denervation. The aim of the work was to investigate the interconnection of matrix metalloproteinase-9 (MMP-9) activity in denervated muscles with fibrosis and to estimate its role in nerve restoration by the epineurial suture, fibrin-based glue, and polyethylene glycol hydrogel. The activity of matrix metalloproteinases was estimated by gelatin zymography. Collagen density in muscles was determined histochemically. An increased level of the active MMP-9 is associated with the fibrous changes in the denervated skeletal muscles and after an epineurial suture. The use of fibrin glue and polyethylene glycol hydrogel resulted in a lower level of collagen and MMP-9 activity, which may be a therapeutic target in the treatment of neuromuscular lesions, and has value in fibrosis analysis following microsurgical intervention for peripheral nerve reconstruction.

Keywords
- MMP-9
- muscle atrophy
- sciatic nerve
- trauma
- fibrosis

Introduction

Muscle atrophy is a common problem after injury of the limb peripheral nerves. Nerve damage results in loss of innervation, metabolic disorders, and subsequent necrosis of muscle fibers and substitution of skeletal muscle by the connective tissue. Microsurgical restoration of the injured nerve does not always result in successful muscle reinnervation and progressive atrophy is accelerated by fibrous processes. Development of fibrous connective tissue is the central event underlying fibrosis processes, in which the extracellular matrix (ECM) enriched with collagen substitutes the muscle tissue. The state of ECM collagen is modulated by matrix metalloproteinases (MMPs), a heterogeneous family of Zn^{2+}-dependent endoproteases that are characterized by low tissue specificity and are involved in tissue damage and regeneration and contribute to the development of various diseases.[1] The MMP family includes 28 types of enzymes that differ in substrate specificity and are classified as collagenase, gelatinase, stromelysin, and membrane-type MMPs. The physiologic role of MMPs includes breakdown of ECM proteins and further tissue remodeling necessary for growth and morphogenesis of tissues and organs (for example, angiogenesis and vascular remodelling, skin regeneration in diabetic wounds, nerve growth, bone development and remodelling, proliferation, differentiation and regeneration of skeletal muscles, reparation of damaged tissues, remodeling of adipose tissue in obesity).[2–6]

In skeletal muscles, MMPs play an important role in homeostasis and maintaining of the functional integrity of myofibrils, ECM remodulation, and regulation of migration,

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differentiation, and regeneration of skeletal muscle cells.[7,8] Overexpression of MMPs in skeletal muscles occurs after injury or in the case of genetic diseases and Duchenne's muscular dystrophy (DMD).[9] However, significant increase in MMP levels aggravates tissue destruction in trauma, rheumatic arthritis, degenerative changes in intervertebral disks, cardiomyopathy, gastric ulcer, multiple sclerosis, and central and peripheral nervous system damage.[10–12]

The role of MMPs in neuromuscular regeneration remains controversial due to the involvement of these enzymes in various multidirectional tissue processes. Some MMPs at low levels constitutively work in peripheral nerves, for example, MMP-2, whereas MMP-9 is more specific for skeletal muscles. MMP-2 is activated during regeneration of muscle fibers, and MMP-9 expression increases due to the inflammatory response and activation of the myogenic satellite cells.[13] Both MMP-2 and MMP-9 are found in neuromuscular junctions, Schwann's cells, and perineurium of the denervated muscle fibers after sciatic nerve trauma.[14] They are capable of destroying myelin basic protein (MBP) involved in Wallerian degeneration, and remodeling ECM for angiogenesis.[15] Collectively, these data suggest that MMP-2 and MMP-9 are involved not only in inflammatory reactions but also in reconstruction of ECM during muscle regeneration after injury.

In previous studies, the potential antifibrotic effect of the fibrin glue and polyethylene glycol (PEG) hydrogel on nerve regeneration was shown on models of nerve injury and regeneration.[16] Hydrogels promote adhesion for regenerative peripheral nerves and limit fibrosis around a nerve gap.[17,18] An overall analytical review states that similar glue is easy to use and less traumatic than microsuture repair.[19] However, we have not found any articles assessing the action of fibrin glue and PEG hydrogel on muscle fibrosis in comparison with epineurial suturing techniques.

Thus, the aim of the present study was to evaluate reparative efficacy of two hydrogels applied after sciatic nerve transection and to assess their effects on MMP activity as a hallmark of skeletal muscle fibrosis.

Materials and Methods

The study was performed on 30 white male albino rats of the inbred strain (250 ± 25 g, 5–6 months of age) kept in the animal house of the State Institution "Romodanov Neurosurgery Institute" of the National Academy of Medical Sciences of Ukraine under standard laboratory conditions and fed with commercial rat feed and purified water ad libitum. Experiments were performed in accordance with all standards and principles of bioethics (EU Directive 2010/63/EU "on the protection of animals used for scientific purposes"), European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986), and Law of Ukraine of February 21, 2006, No. 3447-IV "About protection of animals against ill treatment." The protocol of the study was approved by the Bioethical Commission of Bogomolets National Medical University (protocol # 113).

Experimental rats were divided into six groups, as follows:

- Group 1: control (group C)—intact rats.
- Group 2: sham-operated (group SH)—a linear skin incision on the lateral surface of the femur was performed, and the left sciatic nerve was isolated and mobilized. This was followed by layer-by-layer restoration of soft-tissue integrity without nerve manipulation.
- Group 3: complete transection of the sciatic nerve (group CT)—the same procedures as in group 2 were performed with the additional complete transection of the sciatic nerve; the nerve stumps were not connected and remained free in the wound. This was followed by layer-by-layer restoration of soft-tissue integrity without nerve manipulation.
- Group 4: epineurial sutures (group ES)—the same procedures as in group 2 were performed with an additional complete transection of the sciatic nerve and its subsequent fixation by end-to-end epineurial neurorrhaphy with an atraumatic needle (4–6 epineurial sutures with a polyamide thread N° 10/0);
- Group 5: Tisseel (Baxter Healthcare Corporation, Westlake Village, CA, USA) fibrin glue (group TF)—the same procedures as in group 2 were performed with an additional complete transection of the sciatic nerve and its subsequent fixation with Tisseel fibrin glue and two "fixing sutures."
- Group 6: DuraSeal (Integra Life Sciences, Plainsboro, NJ, USA) hydrogel (group DH)—the same procedures as in group 2 were performed with an additional complete cross-section of the sciatic nerve and its subsequent fixation by means of DuraSeal, and two "fixing sutures."

The surgical procedures were conducted under general anesthesia (xylazine 15 mg/kg and ketamine 70 mg/kg, intraperitoneally), following the rules of asepsis and antiseptics. An access to the sciatic nerve in group 3 was performed as follows: an animal was placed in a standard physiologic position with its belly down; the skin in the area of the middle third of the lateral surface of the left thigh was shaved, treated with povidone-iodine solution, dissected along the line of the most superficial location of the external femur surface, and the area of attachment of both tendons of the biceps femoris to the femur was visualized; in this zone, a linear section along the bone was performed and the muscle was isolated in the middle. In the pocket, formed by the mobilized edge of the muscle and other muscles of the posterior group, the trunk of the sciatic nerve was displayed and opened at the interval from the exit from the pelvic cavity to the branching into the main branches. In animals of groups CT, ES, TF, and DH at the middle of this site, the nerve was transected with ophthalmologic scissors.

In group ES, traditional nodal epineurial neurorrhaphy was performed. An access was performed in the middle to upper third of the thigh with a projection line of the sciatic nerve on the left thigh. After the treatment of the operative field, soft tissues were dissected, and then the sciatic nerve was isolated by means of the "mosquito"-type clamp. The left

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sciatic nerve in the middle third of the hip was cut by means of ophthalmologic scissors. Microsurgical suturing was performed after sciatic nerve transection: microsurgical epineurial sutures were performed with 4–6 monofilament atraumatic thread No. 10.0 until the fascicles were matched satisfactorily.

In the animals from groups TF and DH, isolation and nerve transection were followed by two epineurial sutures made with an atraumatic thread No. 10.0 at 0 and 180 degrees of the nerve diameter.

In group TF, two fixation sutures were made. Then Tisseel was applied to the nerve stump connection site, using the Duo Syringe System. The system consists of solution of protein sealant (active ingredients: human fibrinogen, 91 mg/mL; synthetic aprotinin, 3,000 KIU/mL) and thrombin solution (active ingredients: human thrombin, 500 IU/mL; calcium chloride dihydrate, 40 µM/mL). Preparation of the system for use was performed according to the manufacturer's instructions. One drop of the prepared Tisseel solution was applied to each nerve; it was enough to cover the junction of the stumps of the transected nerve with a thin layer of glue.

In group DH, after two fixation sutures were applied, the DuraSeal solution (5-mL kit) was applied to the junction of the nerve stumps. DuraSeal consists of two solutions: polyethylene glycol ester (PGE) solution and trilysine amine solution. The kit for preparing 5 mL of the final solution consisted of the following components: a powder bottle (10-mL bottle, containing N-hydroxysuccinimide powder ester; PGE) and is combined with FD and C No. 1 blue dye as a visualization index), a syringe filled with solvent (2.5-mL sodium phosphate buffer), transparent precursor syringe (trilysine amine solution in 0.075-M sodium borate buffer, 2.5 mL), a syringe holder, a dual liquid applicator, a tip on a plunger, and tips to form spray. The system was prepared for use according to instructions from the manufacturer. After the preparation of the system, gel was applied in a thin layer to the connected nerve site.

After surgery and careful hemostasis in groups 2 to 6, layer-by-layer suturing of the postoperative wound was performed with an atraumatic needle with a monofilament polyamide thread 4–0. To prevent infectious complications, benzyl penicillin solution (1×10^6 U/kg body weight) was administered to the posterior cervical site. For anti-inflammatory and anti-edema therapy, dexamethasone solution of 6 mg/kg body weight was administered intraperitoneally.

Skeletal muscle sampling was performed 8 weeks after the surgery (gastrocnemius muscle) of the operated limb to determine the MMP activity and fibrosis. The level of collagenolytic activity of MMPs was determined by enzyme phoresis (gelatin zymography) according to the procedure described earlier.[20] Protein extraction was performed by homogenizing the muscles in 50 mM tris-HCl buffer (pH 7.4), followed by cell destruction by ultrasonic disintegration. Tissue residues and cell debris were separated from the liquid phase by centrifugation at 12,000 *g* for 45 minutes at 4° C. The protein concentration of the resulting extracts was determined by Lowry's method[21] using the Pierce Modified Lowry Protein Assay Kit (Thermo Scientific, United States). The application samples were mixed with Laemmli sample buffer and applied on the Polyacrylamide Gel Electrophoresis (PAGE) in the amount of 100 µg protein per well. Electrophoretic separation of protein samples in nonreducing conditions was performed using PAGE copolymer gel (8%) with gelatin (5 mg/mL) in tris-HCl glycine buffer (pH 8.6), containing 0.1% sodium dodecyl sulfate (SDS; current intensity: 100 mA; voltage: 20–35 V). After electrophoresis, the gel was removed and rinsed twice in cold 2.5% Triton X-100 solution for 30 minutes to remove SDS and renature proteins. The gel was then rinsed in five changes of cold bidistilled water. The collagenolytic activity was developed in the buffer containing 50 mM tris-HCl (pH 7.4), 20 mM NaCl, 0.02% of Tween 80, 5 mM $CaCl_2$, and 1 mM $ZnCl_2$. The gel has been incubated in the developing buffer for 18 hours at 37°C to allow gelatinases to cleave substrate. The gel was then stained in the PageBlue Protein Staining Solution (Thermo Scientific, United States) according to the manufacturer's instructions. The presence of collagenolytic enzymes was determined by the presence of light bands on a dark-blue background. The area of such sites was considered to be proportional to enzyme activity. Densitometric analysis is performed using TotalLab TL120 software (Nonlinear Inc., United States), the enzyme activity is presented in arbitrary units (a.u.). The results are presented as a median and interquartile Me interval [LQ – UQ], where Me is a median (50th percentile), LQ is the 25th percentile, and UQ is the 75th percentile.

To estimate the collagen density in the gastrocnemius muscle, muscle samples were fixed in 10% formalin solution (pH 7.4; phosphate buffered saline [PBS]). by means of the isopropanol paraffin method, they were poured into paraffin, and 8-µm slices were cut with Thermo Microm HM 360 microtome (Thermo Scientific, United States). Deparaffinized sections were held for 30 minutes at 25-degree picrosirius red (PSR) staining solution (0.5-g Direct Red 80; Magnacol Ltd, United Kingdom) in 500 mL of saturated picric acid,[22] dehydrated, and placed in the Canada Balsam (Merck KGaA, Darmstadt, Germany). The stereometric analysis was performed using the software ImageJ (Wayne Rasband, United States; algorithm: transformation of RGB image into 8-bit, threshold, correction by maximum PSR-positive index, measurements). Collagen density is expressed in percentage of the analyzed areas. The analyzed zones were presented as micro-image ($2{,}270 \times 1{,}700$ pixels, $1{,}120 \times 840$ µm); five images per cross-section slice of a muscle sample is approximately two-thirds of the muscle cross-section.

Statistical analysis was performed using Origin v.9.0 software. Statistical significance of the differences between the comparison groups was determined by one-way ANOVA, followed by the Bonferroni (post hoc) test. Differences between groups were considered statistically significant at $p < 0.05$.

Results

In the performed experiments, the association of the gastrocnemius muscle hypotrophy with the collagenolytic MMP

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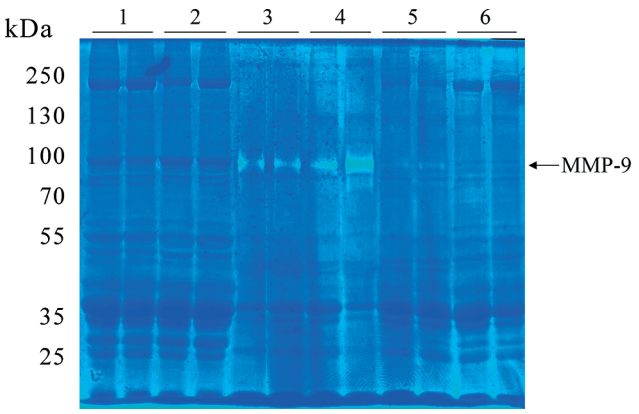

Fig. 1 Zymography of the gastrocnemius muscle of rats 2 months after trauma and sciatic nerve restoration ($n = 5$).

activity was investigated. The study has revealed a collagenolytic activity similar to the action of MMP-9 only in groups CT, ES, and TF ($p < 0.05$; ►**Figs. 1** and **2**).

The use of Tisseel resulted in a significant decrease of pro-MMP-9 metalloproteinase activity in comparison with the group CT (4.4 [2.4 – 7.8] vs. 26.0 [2.4 – 7.8] a.u., $p < 0.05$). At the same time, when DuraSeal was used (group DH), the activities of both pro-MMP and MMP-9 were possibly decreased in comparison with the CT and ES groups ($p < 0.05$). Moreover, as can be seen in ►**Fig. 1**, the protein profile in group DH is quite close to control indices, as opposed to groups CT, ES, and TF. These results indicate that MMP-9 is associated with the destruction and dystrophy of muscular fibers.

Morphologic studies revealed an increase in collagen of the gastrocnemius muscle in groups CT, ES, TF, and DH (►**Fig. 3**). An increase in collagen took place around fascicles, in the connective tissue of the perimysium and epimysium (PSR-positive areas are red). More collagen was observed in the areas with muscle fiber hypotrophy (diameter reduction and deformation).

According to the results of the morphometric (stereometric) analysis of the collagen density in the gastrocnemius muscle, in terms of the degree of PSR-positive staining, the density of collagen was significantly higher in groups CT, ES, TF, and DH, that is, in all the groups with muscle and sciatic nerve damage. After the use of epineurial suture, the density of collagen did not differ from group CT. At the same time, as can be seen in ►**Fig. 4**, in groups TF and DH, collagen density was lower: 7.8 (3.3–9.3) and 4.7% (2.2–7.4%) vs. 12.6% (10.8–14.7%), respectively; $p < 0.05$. The results indicate that less fibrosis occurs in the denervated gastrocnemius muscle, when using Tisseel glue and DuraSeal hydrogel after reconstruction of the injured sciatic nerve.

Thus, these results indicate that MMP-9 is associated with the development of fibrosis under hypotrophy in denervated muscles. The findings are consistent with those of other[23] researchers and give chance for understanding of the link between fibrosis and reconstruction of the injured peripheral nerve.

Discussion

The ECM plays an important role in regeneration of the damaged nerve and reinnervation of skeletal muscles. The success of neuromuscular restoration depends on the preservation of the ECM of the injured nerve, namely, endoneurium, and the basal membrane of muscle fibers that contain collagen and laminin. Growing axons adhere to collagen endoneural tubes in such a way that directed growth to denervated muscles takes place. But in the injured nerve and muscle, the native ECM degrades, and the sulfated proteoglycans and collagen, being formed in fibrosis, inhibit axon growth.[24] MMPs destroy ECMs, but the success of their involvement in restoration obviously depends on the time after the damage. Early studies[25] found a positive link between the tissue concentration of MMP, particularly MMP-1, in the skeletal muscle and the formation of new myofibrils, involving transplanted myoblasts. Among the mechanisms of this effect, breaking up of the fibrotic elements of the injured muscle, which blocks the migration of blast cells, was noted, as well as several other factors mediated by the products of the lytic activity of MMP.[25] In

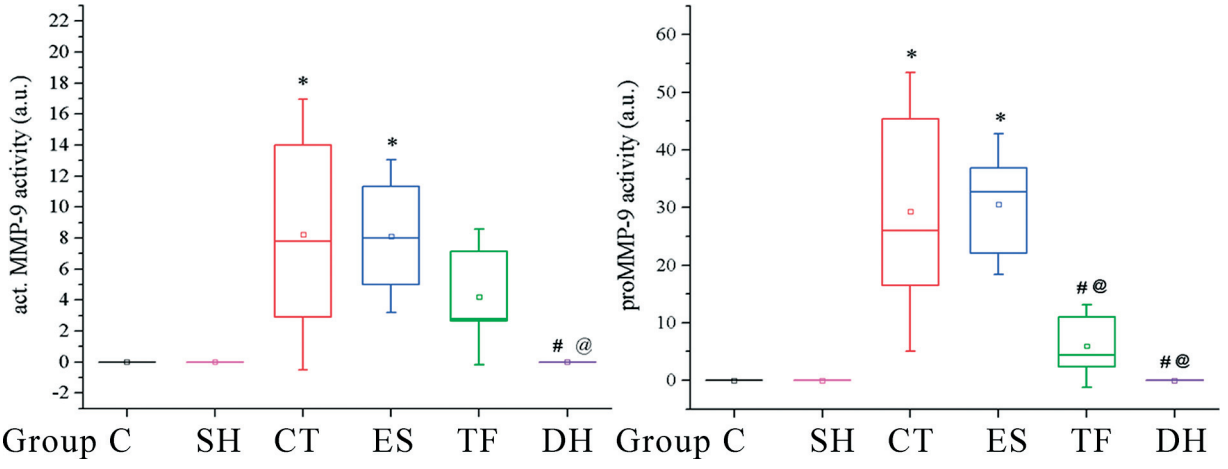

Fig. 2 Densitometric analysis of zymograms of the gastrocnemius muscle of rats 2 months after trauma and sciatic nerve restoration ($n = 5$). ($^{*}p < 0.05$ compared with control group (C) ; $\#p < 0.05$ compared with complete nerve transection group (CT); $@p < 0.05$ compared with epineurial suture group (ES).

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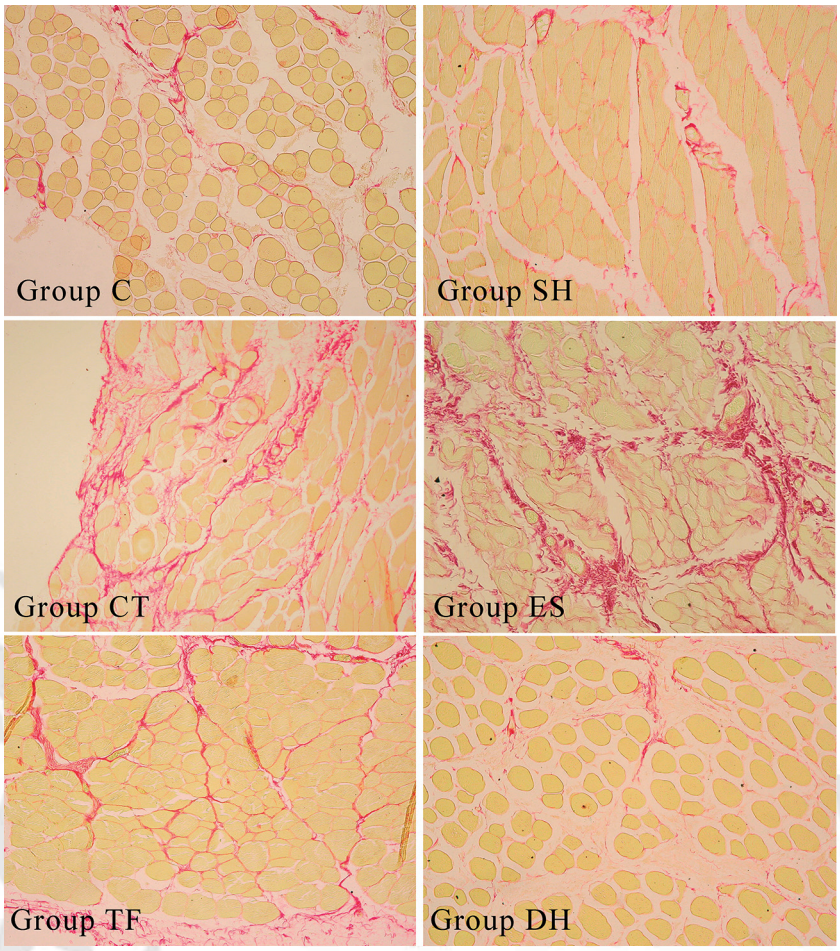

Fig. 3 Fibrosis in the denervated gastrocnemius muscle after trauma and sciatic nerve restoration ($n = 5$). PicroSirius Red staining, × 200.

particular, the role of hepatocyte growth factor (HGF), a known resident myogenic cell activator, whose production is directly dependent on the MMP activity, was discussed.[26]

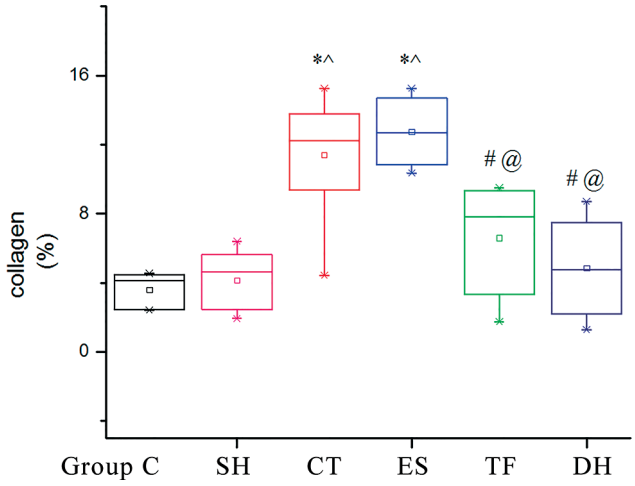

Fig. 4 Stereometric analysis of the collagen density of the gastrocnemius muscles of rats 2 months after the trauma and sciatic nerve restoration ($n = 5$). ($^{*}p < 0.05$ in comparison with control group (c) $^{\wedge}p < 0.05$ in comparison with sham-operated group (SH); $\#p < 0.05$ in comparison with complete nerve transection group (CT); $@p < 0.05$ compared with epineurial suture group (ES).

The factor inhibiting the myogenic activity and is in its turn offset by MMP is transforming growth factor-β1 (TGF-β1).[27] In addition, MMP, in particular MMP-2 and MMP-9, was considered one of the positive effects of relaxation (a member of the insulin-like growth factor [IGF] family on myogenic progenitors and angiogenesis in the context of muscle healing).[27] At the same time, there are also data on the negative role of MMP (in particular MMP-9) in regeneration of injured muscles[28] and in the model of Duchenne muscular dystrophy,[29] which is due at least to the negative indirect effect of MMP-9 on myoblast differentiation, probably due to degradation of ECM elements, particularly basal membranes of type IV muscle fibrils.[30]

After nerve and muscle injury, MMPs grow in different timescales: MMP-9 in 12 hours and the peak is observed in 48 hours, and the peak of active MMP-2 is on days 4 to 6.[12] In the early stages of MMP-2 and MMP-9, proteoglycans degrade. This promotes nerve regeneration and their levels slowly decrease.[31] But it is interesting that in the noncontralateral nerve (intact), a similar increase and decrease time course of MMP-2 was found. MMPs often function together and destroy gelatin, which is formed after degradation of type IV collagen of the basal membrane. MMP-9, satellite cells of muscle fibers and neutrophils,[32] are isolated. MMP-2 and MMP-9 are also found in neuromuscular junctions,

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Schwann's cells, and the epi- and perineurium of denervated muscle fibers of the sciatic nerve in the first 3 days after trauma.[14,33–35] MMP-2 promotes proliferation and migration of satellite cells at the site of the lesion; they merge with each other or with damaged myofibrils and provide muscle regeneration.[32] Their activation and formation of new myofibrils is one of the components of replacement, hypertrophy, or regeneration of the muscle.[36] First, the MMP-9 activity was associated with demyelination and myelin degradation in the injured nerve in addition to the remodeling of the ECM. Further, the regrowth of MMP activity in 28 days already indicates ECM remodeling, which is associated with necrosis and fibrosis.[37] It has also been found that changing of the environment in inflammation and obesity contributes to the accumulation of proinflammatory cytokines and growth factors that further stimulate MMP-2 synthesis and activation.[38]

The role of MMPs in neuromuscular restoration remains debatable, which is associated with the involvement of enzymes in various tissue processes that occur consistently. They are able to destroy the MBP involved in Wallerian degeneration, break down the matrix for angiogenesis, reduce neutrophil infiltration, and maintain the integrity of blood-tissue barriers.[11]

The use of hydroxamate inhibits MMP-2 and MMP-9 and reduces infiltration by macrophages and elimination of myelin degeneration products in the injured nerve.[12] High MMPs correlate with high dystrophic processes, but after the treatment or influence on the local inhibitors (tissue inhibitor of metalloproteinase [TIMPs]), the proteolytic activity of enzymes does not always regress to control values and may have a detrimental effect in the damaged tissue.[39]

Interestingly, the physical workouts alter the activity of MMPs in muscles. An increase of the MMP-2 activity was found after 12 weeks of training of young and old rats, indicating the role of MMPs in the connective tissue reconstruction around muscle fibers.[40] The expression and activity of MMP-2 increase in type I and II muscle fibers, that is, muscle fibers, rather than migratory phagocytes or connective tissue fibroblasts,[41] directly play a role in the change of the MMP-2 level after training. On the other hand, these muscle events accelerate age-related changes in skeletal muscle to enhance collagen I and III degradation.[42,43] The latter form a basal membrane of muscle fibers and their destruction reduces elasticity and endurance, which can impair the strength of the tendon connection to skeletal muscles.

The MMP subgroups localize in certain wound areas, their activation occurs during the different periods in the healing process, and the isoform spectrum and quantitative content depend on the cell filling of the wound. Taking into account the key role that MMPs play in ECM remodeling at the lesion locus, the activity of these enzymes serves as a molecular criterion for healing efficiency and fibrosis. In our own research, the MMP activity has been found to be associated with progressive collagenogenesis in the damaged and denervated muscle, which is due to muscle fiber atrophy and subsequent fibrosis of these sites. Collagenogenesis in denervated muscles is accompanied by increased MMP-9 activity, both after complete nerve transection without neurorrhaphy and after epineurial suture. The histologic studies revealed substitution of the muscle tissue by fibrous tissue after denervation. The denervated muscle is characterized by reduction of muscle fiber thickness and increase of collagen areas. These changes equally occur after both nerve injury and nerve suturing. However, progression of fibrous processes in muscle stromal elements from the site of the damage is not excluded.

Our studies have shown that the use of fibrin glue and PEG hydrogel is reflected in less collagenogenesis and limited fibrosis in denervated muscles, with little activity within 8 weeks after injury. MMP activity was hardly observed with the application of PEG hydrogel. The activity of MMP-9 and fibrosis after the use of PEG hydrogel was significantly lower compared with epineurial suture and fibrin glue. There was no difference in the density of collagen areas in muscle samples; however, the level of MMP-9 activity in the group with fibrin glue was higher than in the group with PEG hydrogel. These data give hope to consider hydrogels not only as an agent to improve the adhesion of the transected nerve stumps but also as a potential agent to prevent the progression of fibrosis.

Conclusion

The use of biotechnological fibrin glue and PEG hydrogel in neurorrhaphy of injured peripheral nerves reduces the damage to the paraneurial environment and thereby prevents the progressive development of connective tissue around the neuroma and in the denervated muscles. It is clear that surgical access to the nerve causes reactive changes in the connective tissue and an increase in the collagen content in ECM, but when gels are used, the role of the mechanical factor in nerve neurorrhaphy is decreased, which is reflected in less MMP-mediated remodelling of ECM. Our study on the role of MMP-9 in denervation-induced muscle atrophy can be continued in the direction of analyzing the role of resident myogenic cells under such experimental conditions.

Conflict of Interest
None declared.

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