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Mouse gastrocnemius muscle regeneration after mechanical or cardiotoxin injury

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Abstract: The goal of our study was to compare the skeletal muscle regeneration induced by two types of injury: either crushing, that causes muscle degeneration as a result of mechanical devastation of myofibers, or the injection of a cardiotoxin that is a myotoxic agent causing myolysis of myofibers leading to muscle degeneration. Regenerating muscles were analyzed at selected intervals, until the 14th day following the injury. We analyzed their weight and morphology. We also studied the expression of different myosin heavy chain isoforms as a molecular marker of the regeneration progress. Histological analysis revealed that inflammatory response and myotube formation in crushed muscles was delayed compared to cardiotoxin-injected ones. Moreover, the expression of myosin heavy chain isoforms was observed earlier in cardiotoxin-injured versus crushed muscles. We conclude that the dynamics of skeletal muscle regeneration depends on the method of injury. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 1, 144–153)*

Key words: mouse, skeletal muscle, regeneration, cardiotoxin, myosin heavy chain

Introduction

Muscle regeneration is essential for maintaining skeletal muscle functionality. Thus, a full understanding of this process is crucial not only for the elucidation of the mechanisms underlying muscle differentiation, but also for the studies devoted to the therapies of skeletal muscle diseases or severe muscle trauma. Skeletal muscle regeneration consists of two overlapping stages: muscle fiber degradation accompanied by an inflammatory response, and a reconstruction phase, which starts with the activation of muscle precursor cells, i.e. satellite cells, followed by their proliferation, and differentiation of satellite cells-derived myoblasts into muscle fibers. These are easy to distinguish by the presence of centrally positioned nuclei [1]. Myoblasts differentiation is associated with changes in the expression of various molecular markers, including myosin heavy chains (MyHCs). Among them are adult isoforms, i.e. slow (I) and fast (IIa, IIb, IId/x), and also fetal and neonatal ones. During embryonic myogenesis, MyHCs expression pattern changes according to the following scheme: fetal → neonatal, slow → fast (i.e. IIa, IIb, IId/x) isoform of MyHC [2, 3]. However, during regeneration, fast MyHC isoforms may be expressed before slow ones [4, 5].

To date, several models based on the method of injury, i.e. induction of myofiber degradation, have been used in various studies. Among them are: injection of myotoxins such as bupivacaine [6], snake venoms like cardiotoxin (CTX) [7] or notexin [8], physical trauma like freezing [9], whole muscle free graft [10], crushing [11], or denervation-devascularization [12]. All of these insults result in muscle injury, i.e. degeneration, followed by the induction of regeneration. However, the characteristics of regeneration has been shown to differ depending on the type of injury, making a comparison of the results obtained in various studies difficult. Moreover, only a few papers have included a detailed, side by side, comparison of the muscle regeneration induced using different methods, and these publications do not cover all the molecular changes occurring within the muscles [13–15].

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To fill this gap in knowledge, we aimed to carefully compare the dynamics of regeneration induced by two types of commonly used injury methods: crushing [11, 16–20] and CTX injection [7, 21–26]. Crushing, accompanied by denervation, causes muscle degeneration as a result of mechanical devastation of myofibers [11]. Cardiotoxin, on the other hand, is a myotoxic agent that leads to the myolysis of myofibers by inducing rapid plasma membrane depolarization [22]. In addition, internal structure of the muscle, i.e. basal lamina or microvasculature, is less affected after CTX injection than after crushing [27]. The major disadvantage of using crushing is connected to its invasiveness and the risk of infection. An advantage of using CTX relates to its relatively low harmfulness for an animal.

The molecular, cytological and histological analyses presented in this study allowed us to conclude that myoblast differentiation and fusion occur faster after CTX injury than after crushing. We compared the results of experiments in which skeletal muscles were injured either by crushing or by CTX injection.

Material and methods

Experimental procedures involving live animals were approved by the Local Ethical Committee No. 1, Warsaw, Poland. All animals were three month-old male BALB/c mice. Unless otherwise specified, all reagents were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Muscle injury. The right gastrocnemius muscle was injured either by mechanical crushing or by CTX injection. Before the procedure, the mice were anesthetized by injection of pentobarbital (30 μg/g of body mass). Muscles were either mechanically injured, i.e. crushed according to the previously described procedure [11] or injected with 50 μl of 10 mM CTX solution in 0.9% NaCl [28]. Briefly, in the case of mechanical injury, the muscles were exposed (from the achilles tendon to the knee), denervated by severing the motor nerve at the muscle surface area, and crushed along the entire length using forceps, resulting in damage to all the myofibers. Mechanically-injured and CTX-injured muscles were dissected on day 0 (i.e. 15 minutes after injury), and the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 14th days following. Intact muscles, i.e. those dissected from the right legs of non-injured mice, were used as a control. Immediately after isolation, the muscles were weighed, frozen in liquid nitrogen-cooled isopentane for sectioning, or in liquid nitrogen for mRNA isolation, and preserved at –80°C. In every experiment, at least three animals were analyzed for each time point following either crushing or cardiotoxin injury. The weights of at least five muscles for each time point were statistically analyzed using a *t*-test.

Reverse Transcriptase PCR. RNA was isolated from frozen muscles using PureLink Micro-to-Midi Total RNA Purification System (Invitrogen Ltd., Paisley, UK) and purified with Turbo DNA-free kit (Ambion Inc., Austin, TX, USA). Reverse Transcriptase PCR reactions were carried out with Titan One Tube RT-PCR System (Roche Diagnostics GmbH, Mannheim, Germany). Reaction conditions and primers specific for various MyHC isoforms, i.e. fetal, slow, fast IIa, fast IIb and fast IId/x, were used as described previously [29]. Primers and conditions for housekeeping gene glyceraldehyde 3-phosphate (GAPDH) were used as described previously [17]. Ten microliters of each reaction solution was supplemented with 2 μl of Blue-Juice Gel Loading Buffer (Invitrogen Ltd., Paisley, UK) and reaction products were separated by electrophoresis through 2% agarose gel containing ethidium bromide. Optical density of obtained bands was measured with GelDoc2000 (Bio-Rad, Hercules, CA, USA) using Quantity One software (Bio-Rad, Hercules, CA, USA) and standardized against GAPDH; median, minimum and maximum values of obtained data are presented. The procedure was repeated three times and each RNA sample was obtained from a separate muscle.

Histological analysis. Transverse cross-sections, each ten micrometers thick, of isolated muscles were obtained using a cryostat (Microm HM 500N, Microm International GmbH, Langenselbold, Germany), air-dried, and stored at 4°C. Sections were stained with hematoxylin for ten minutes (Merck KGaA, Darmstadt, Germany), washed in water, stained with eosin for one minute (The British Drug Houses Ltd, B.D.H. Laboratory Chemicals Division, Poole, UK), again washed in water, and mounted in aqueous mounting agent for microscopy (Aquatex, Merck KGaA, Darmstadt, Germany). Observations were made and pictures were taken using a Nikon TE200 microscope (Nikon Instruments, Tokyo, Japan) and NIS Elements software. The figures were assembled using CorelDRAW12. The procedure was repeated three times and three sections obtained from one muscle were analyzed for each experiment.

Immunolocalization. Muscle cross-sections were subjected to immunodetection of selected antigens. Briefly, they were washed in PBS (phosphate-buffered saline), fixed in 3% paraformaldehyde in PBS, washed in PBS, incubated in 0.05% Triton X100 in PBS, washed in PBS, incubated in 0.25% glycine (Merck KGaA, Darmstadt, Germany) solution in PBS, washed in PBS, and incubated in 3% bovine serum albumin (BSA) in PBS for one hour. Next, sections were incubated for four hours in primary antibody against laminin (rabbit L9393; diluted 1:100 in PBS containing 3% BSA), followed by overnight incubation in primary antibody against one of the analyzed isoforms of MyHC (mouse sc-53091, recognizing fetal, mouse sc-53090, recognizing slow, or mouse sc-32732, recognizing fast i.e. IIa, IIb, IId/x iso-

forms, Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:100 in PBS containing 3% BSA). Next, sections were incubated with appropriate secondary antibodies (goat anti-mouse IgG_1 conjugated with Alexa 594, A21125, and goat anti-rabbit IgG conjugated with Alexa 488, A11008; Molecular Probes, Invitrogen Ltd., Paisley, UK; diluted 1:200 in PBS containing 3% BSA). The cell nuclei were visualized with 5 μM DRAQ5 (BioStatus Ltd, Shepshed, UK). Sections were mounted with DakoCytomation Mounting Medium for fluorescence (Dako Denmark A/S, Glostrup, Denmark) and analyzed with a confocal scanning microscope Axiovert 100 M (Carl Zeiss Inc., Jena, Germany) equipped with LSM 510 software. The figures were assembled using CorelDRAW12. The procedure was repeated three times and three sections obtained from one muscle were analyzed for each experiment. To exclude the possibility of the unspecific binding of secondary antibodies, we performed a control staining, i.e. omitting the step of incubating the sections with primary antibodies.

Cell culture of primary myoblasts. Satellite cells were isolated from control intact muscles and from muscles on the 7th or 14th day of regeneration induced either by crushing or CTX injection. After excision, muscles were processed as described previously [30, 31]. Briefly, they were minced with scissors, washed in PBS, and digested with 0.15% pronase in Ham's F12 medium (Gibco, Invitrogen Ltd., Paisley, UK) buffered with 10 mM HEPES, and supplemented with 10% fetal calf serum (Gibco, Invitrogen Ltd., Paisley, UK). After digestion, the tissue was centrifuged in DMEM medium containing 4.5 g/l glucose (Gibco, Invitrogen Ltd., Paisley, UK). Obtained cells were counted and seeded at the concentration of $5 \times 10^3/cm^2$ on 3% gelatin-coated dishes in DMEM with 4.5 g/l glucose containing 10% fetal calf serum, 10% horse serum (Gibco, Invitrogen Ltd., Paisley, UK), and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; PenStrep, Gibco, Invitrogen Ltd., Paisley, UK). After 12 days of culture, the cells were washed with PBS and fixed with cold methanol (Merck KGaA, Darmstadt, Germany) for ten minutes at 4°C. Cells were stained with May-Grunwald and Giemsa (Merck KGaA, Darmstadt, Germany), and analyzed using a Nikon TE200 microscope (Nikon Instruments, Tokyo, Japan). Fusion index was calculated as the number of nuclei in myotubes per total number of nuclei visible multiplied by 100%. The procedure was repeated three times and one muscle was used for each primary culture. Figures were assembled using CorelDRAW12.

Results

Muscle weight and morphology

First, we analyzed the mean weight of gastrocnemius muscles dissected on day 0, and on the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 14th days after injury, as well as of control, i.e. intact, muscle. The mean weight of intact muscles analyzed was 0.12 g. On day 0, i.e. immediately after injury, the weight of CTX-injected muscles increased dramatically ($p < 0.0001$), reaching a mean value of 0.19 g, while the weight of crushed muscles was unaltered (Figure 1A). Next, regardless of the type of injury, muscle weight increased, reaching a peak on the 2nd day after injury. This change in muscle weight, at the time of degeneration and the beginning of regeneration, can be explained by developing inflammation. Nevertheless, the increase in the weight was less profound in muscles regenerating after crushing, reaching a mean value of 0.14 g, than after CTX injection, reaching a mean value of 0.24 g. Starting from the 3rd day of regeneration, the weight of analyzed muscles started to decrease. On approximately the 7th day after injury, the weight had reached the mean value of 0.11 g which was similar to the one characteristic for intact muscles. However, after 14 days of regeneration, the mean weight of crushed muscles was significantly lower (0.05 g) than that of intact ones ($p < 0.0001$). CTX-injured muscle weight (0.12 g) 14 days after injury was slightly increased compared to that observed on the 7th day of regeneration, but was similar to that of the intact muscle (Figure 1A). The significant difference between the weight of the muscles analyzed on the 14th day after crushing and after CTX injection ($p < 0.0001$) shows that either the regeneration of mechanically-injured muscles proceeds more slowly, or that it leads to the atrophy of myofibers.

Our previous analyses of crush-injured muscles showed that subsequent stages of regeneration can be characterized by specific morphological changes [17]. Just after injury, an inflammatory response takes place, followed by phagocytosis of damaged myofibers and the activation of satellite cells. Next, satellite cells start to proliferate, and fuse into myotubes that finally differentiate into myofibers [17, 18]. In the current study, histological analyses were performed on intact (Figure 1B) and regenerating muscles on the 1st, 4th, 6th, and 14th days after mechanical or CTX injury (Figure 1C). On the 1st day after CTX injection, the degeneration of myofibers was more extensive than in crush-injured muscles. Moreover, the number of single-nucleated cells, i.e. inflammatory cells or proliferating myoblasts, was higher (Figure 1C). Also, the process of regeneration was clearly accelerated in CTX-injured muscles. The first reconstructed myotubes, with centrally positioned nuclei, were observed on the 4th day after CTX injection, and the 6th day after crushing (Figure 1C). Also, on the 14th day after injury, the regeneration of CTX-

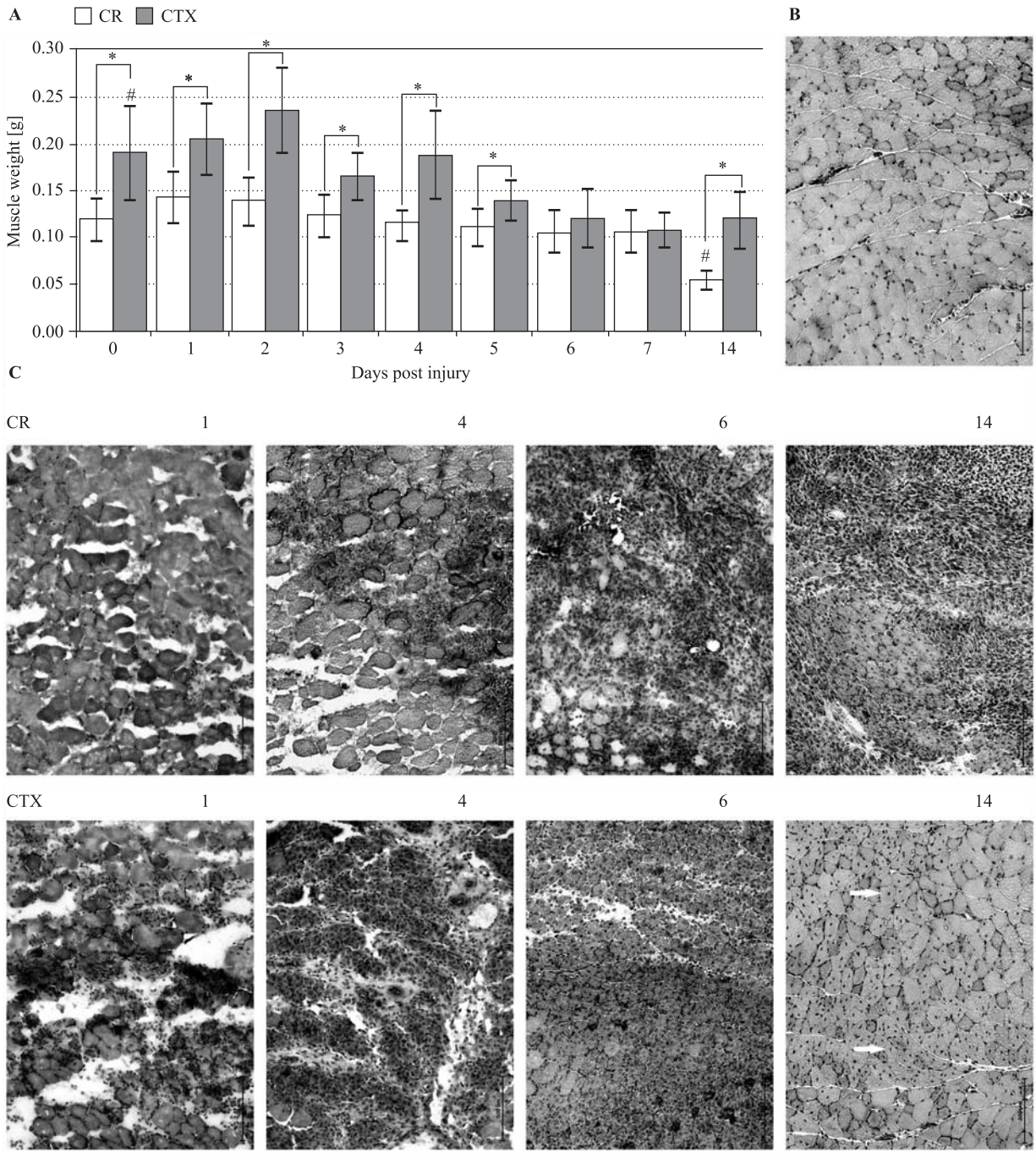

Figure 1. Muscle weight and morphology of crush- and CTX-injured muscles. **A.** Changes of muscle weight. Muscles were analyzed on the day of injury and on the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 14th days after injury. Each bar represents a mean value with line indicating standard deviation. *difference in muscle weight between CR and CTX was statistically significant at $p < 0.02$; #difference in muscle weight compared to weight of an intact muscle was significant at $p < 0.0001$. CR (white) — crushed muscle; CTX (gray) — cardiotoxin-injured muscle. **B.** Histology of intact muscle. Bar represents 500 μm. **C.** Histology of muscles injured either by crushing or CTX injection analyzed on the 1st, 4th, 6th, and 14th days after injury (1, 4, 6, 14). Bar represents 500 μm. Arrows indicate newly formed myofibers (with centrally positioned nuclei). CR — crushed muscles; CTX — cardiotoxin-injured muscles

-injured muscles was more advanced than that of crushed ones. This was indicated by the higher number of single-nucleated cells and undifferentiated myotubes in mechanically-injured muscles, and the higher number of newly formed myofibers in CTX--injected ones (Figure 1C). Taken together, analyses of the changes in muscle weight and histology revealed that the inflammatory response is more abundant af-

ter CTX injection than after crushing. It also showed that the reconstruction of myofibers starts earlier in CTX-injured muscles.

Changes in the expression of MyHC mRNAs

Dynamics of the expression of mRNA encoding various MyHC isoforms, i.e. neonatal, slow, fast IIa, fast IIb, and fast IId/x, served as a marker of the regeneration progress. According to already published data, the first isoforms to be expressed in differentiating myofibers are fetal, then neonatal, then adult ones (slow and fast) [32, 33]. In control, i.e. intact, muscles, transcripts encoding slow, fast IIa, and fast IIb MyHC isoforms were detected. The level of MyHC slow isoform was the lowest (2%, calculated as the proportion of GAPDH, compared to 15% and 16% for MyHC IIa and IIb; respectively, Figure 2). In crushed muscles, the level of mRNA encoding neonatal, slow, and fast IIa isoforms of MyHC increased starting from the 5th day of regeneration (11%, 16% and 36%, respectively, Figures 2A–C). A rise in the level of mRNA encoding MyHC fast IIb was clearly observed on the 14th day after mechanical injury (38%; Figure 2D). In CTX-injured muscles, an increase in the level of mRNA encoding slow and fast IIa isoforms of MyHC (38% and 81%, respectively) was observed as early as the 3rd day of regeneration (Figures 2B, C). An increase in the level of mRNA encoding neonatal MyHC isoform was not prominent until the 5th day of regeneration induced by CTX injection (5%; Figure 2A). At the 14th day after injury, an increase in the level of mRNA encoding fast IIb MyHC isoform was observed, both in CTX and crush-injured muscles (55%; 38% respectively, Figure 2D). The expression of mRNA encoding fast IId/x MyHC isoforms was not detected in any investigated muscle group (data not shown). Analysis of the expression of mRNAs encoding various forms of MyHC revealed a delay in the expression of slow and fast IIa isoforms after crushing compared to muscles injured by CTX.

Localization of different isoforms of MyHC

To verify if the changes in the expression of fetal, slow and fast (IIa, IIb, and IId/x) MyHC isoforms analyzed at the protein level correlated with changes in the level of the transcripts analyzed, we immunolocalized selected MyHCs. We analyzed intact and injured muscles on the 3rd, 4th, 5th, and 6th days of regeneration. Control staining did not show non-specific binding of secondary antibodies. Localization of laminin helped us to delineate the borders of developing myofibers within the regenerating muscles. The fetal form of

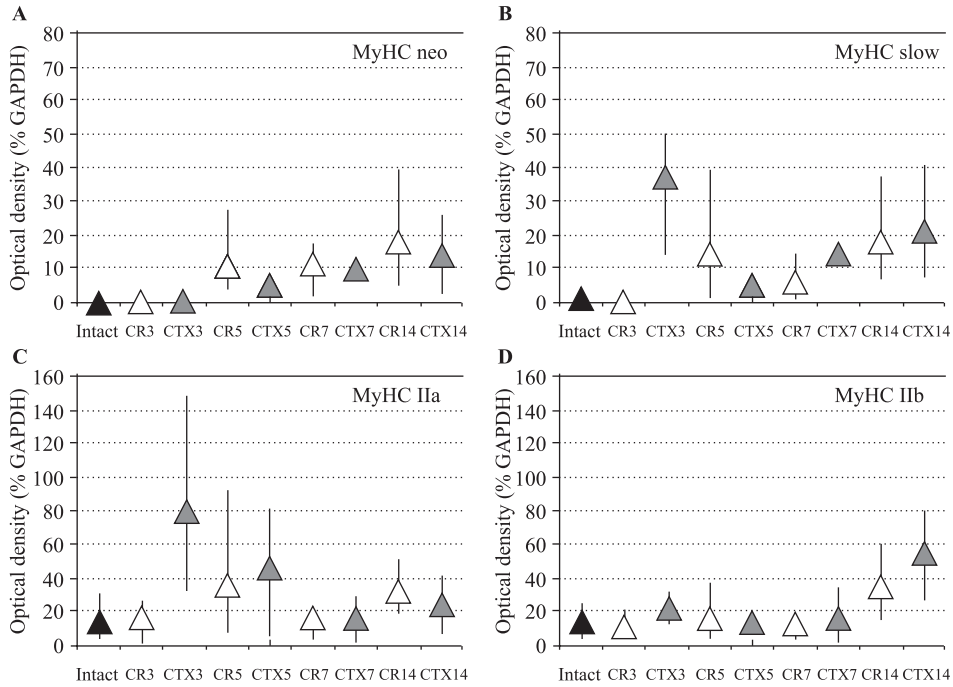

Figure 2. Expression of MyHC isoforms in crush- and CTX-injured muscles. Expression levels of mRNAs encoding different MyHC isoforms (A — neonatal, B — slow, C — fast IIa, and D — fast IIb) revealed by RT-PCR analysis on the 3rd, 5th, 7th, and 14th days after injury. Graphs show lines indicating minimum and maximum values and triangles indicating median values (optical density of bands) standardized against GAPDH. Intact (black) — control, intact muscle; CR (white) — crushed muscles; CTX (gray) — cardiotoxin-injured muscles

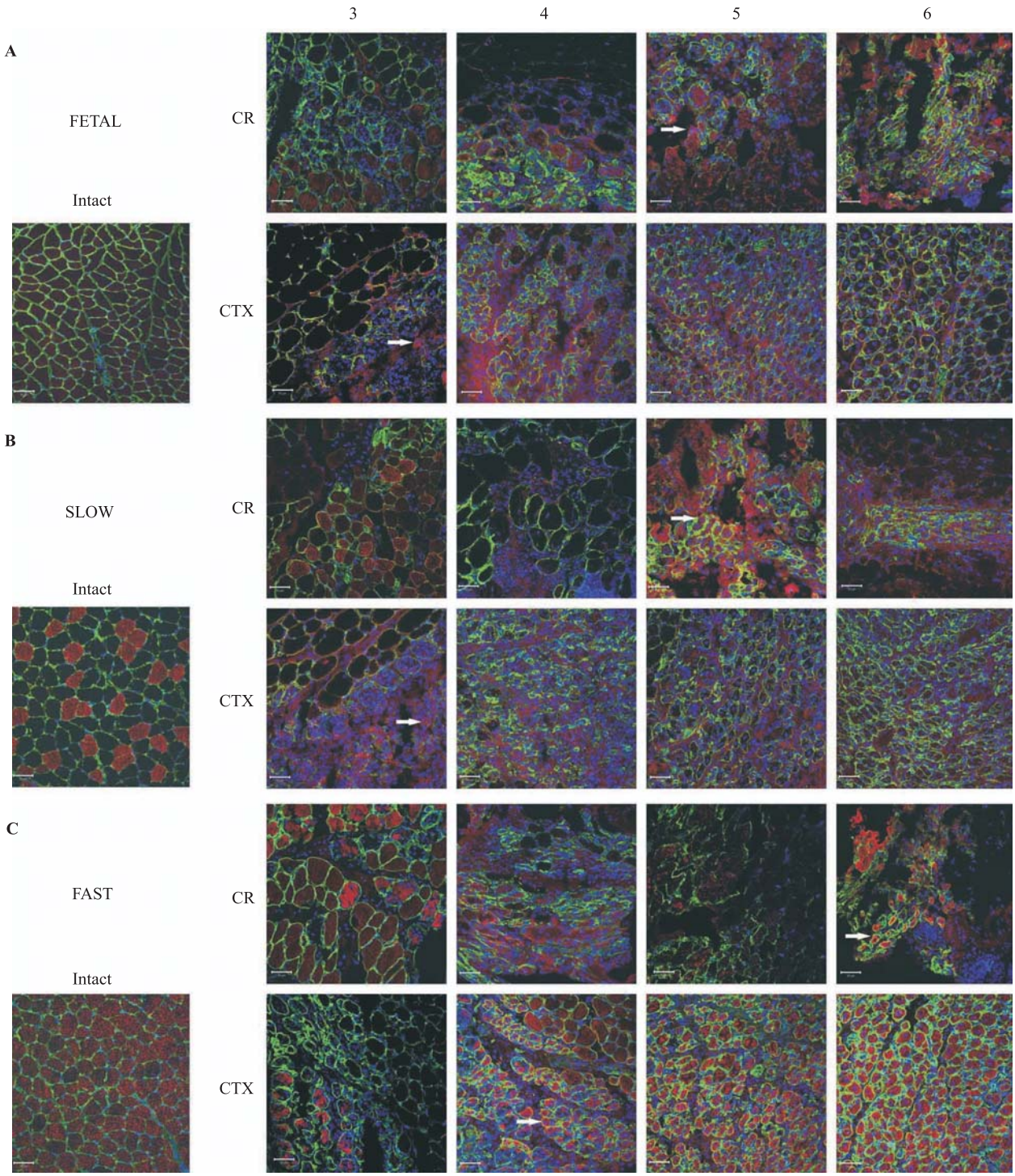

Figure 3. Localization of MyHC isoforms in crush- and CTX-injured muscles. Immunolocalization of MyHC isoforms, i.e. fetal (**A**), slow (**B**), fast (**C**), (IIa, IIb and IId/x) (red) and laminin (green) in intact muscles and in muscles on the 3rd, 4th, 5th, and 6th days of regeneration induced either by crushing or CTX injection. Nuclei were visualized with DRAQ5 (blue). Arrows indicate representative myotubes expressing MyHC. Intact — control, intact muscle; CR — crushed muscles; CTX — cardiotoxin-injured muscles. Bar represents 50 μm

MyHC was not detected in control muscles (Figure 3A). Although the gastrocnemius muscle is known to be composed of both types of myofibers, i.e. fast- and slow-twitch, in intact muscles slow MyHC isoform was localized only within a few myofibers (Figure 3B). In control muscles, fast, i.e. IIa, IIb, and IId/x, isoforms predominated and were present in almost all analyzed myofibers (Figure 3C).

During the first days after both types of injury, slow and fast forms of MyHC were observed only in de-

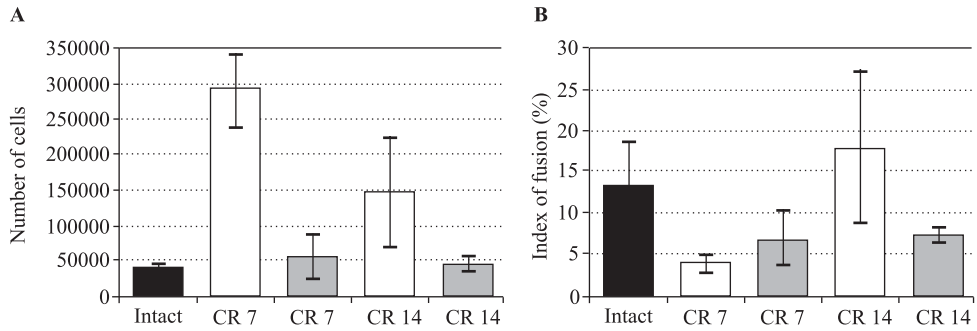

Figure 4. Myogenic potential of cells isolated from crush- and CTX-injured muscles. Cells were isolated from an intact muscle and from muscles on the 7th and 14th days of regeneration induced by crushing (CR 7 and CR 14) or CTX injection (CTX 7 and CTX 14). **A.** Mean number of cells isolated from all types of muscles. **B.** Mean index of fusion of cells isolated from intact muscle and regenerating muscles after 12 days in culture. Each bar represents mean value with line indicating standard deviation. Intact (black) — control, intact muscle; CR (white) — crushed muscle; CTX (gray) — cardiotoxin-injured muscle

generating myofibers (data not shown). In CTX-injured muscles, fetal and slow MyHC isoforms became apparent around cell nuclei that were not surrounded by laminin, i.e. did not yet form myofibers, as early as the 3rd day (Figures 3A, B), and in crushed ones on the 5th day after injury (Figures 3A, B). Also newly formed myofibers containing fast isoforms appeared earlier in CTX-injected than in crushed muscles, i.e. the 4th versus the 6th day of regeneration (Figure 3C). At the same time points, regardless of the type of injury, fast MyHC isoforms were observed in myotubes surrounded by laminin.

Analysis of myogenic potential of the cells isolated from CTX- and crush-injured muscles

One of the tests revealing the efficiency of regeneration relies on the analysis of the number and myogenic potential of cells that can be found either within regenerating or already regenerated muscle. In order to investigate this aspect, we analyzed the mean number and differentiation potential of cells isolated from control, i.e. intact, muscle, and muscles on the 7th and 14th days of regeneration induced either by crushing or by CTX.

Crushed muscles contained more single-nucleated cells than CTX-injured ones, both on the 7th and the 14th day after injury (Figure 4A). To analyze the myogenic potential of these cells, assuming that a significant proportion of them consisted of satellite cells, we plated them and *in vitro* cultured for 12 days, sufficient to differentiate and form myotubes. Next, we calculated the fusion index, i.e. the number of nuclei in myotubes per total number of nuclei visible, multiplied by 100%. The mean value of fusion index of cells isolated from intact, non-injured muscles and cultured for 12 days was determined as 13.3% (Figure 4B). Cells isolated on the 7th day after crushing or CTX injury were characterized by mean fusion indices of 4% and 6.8%, respectively (Figure 4B). Cells isolated on the 14th day after crushing or CTX injury were characterized by mean fusion indices of 17.9% and 7.2%, respectively (Figure 4B). Thus, the mean fusion index of cells isolated from CTX-injured muscles was similar regardless of the advancement of regeneration (6.8% on the 7th day versus 7.2% on the 14th day). However, in the case of crush-induced injury, the mean fusion index differed significantly (4% on the 7th day versus 17.9% on the 14th day). Such an observation suggests that cells isolated from crushed muscles differentiate with certain delay, compared to the cells obtained from CTX-injured muscles of which the fusion index did not change during the *in vitro* culture. It was only slightly higher than the mean fusion index of cells isolated from muscles on the 7th day after crushing (4%, Figure 4B). Analysis of the mean number of nuclei per myofiber showed, however, that only myotubes formed from cells isolated on the 7th day of regeneration, regardless of the type of injury, contained ten or more nuclei per myotube (data not shown). These results show that CTX regeneration was more advanced and that more myoblasts were already included into forming myofibers, or that crush-injured muscles contained more inflammatory cells.

Discussion

Method of muscle injury influences the process of muscle regeneration

Crushing as a method of inducing regeneration was first described by Bassaglia and Gautron [11], and has since been used in many research studies by us

[17–20] and by others [11, 16]. One of the important steps of this procedure is denervation, which not only ensures reproducibility of the process, but also makes the initial steps of regeneration less painful for the animal. However, to fully regenerate, muscle needs to be innervated. Also, other elements of skeletal muscle internal architecture affected by crushing need to be restored. Among them are basal lamina surrounding skeletal muscle fibers and microvasculature securing blood supply [11, 14]. Because of the strongly invasive nature of crushing, i.e. denervation and the need for muscle exposure, methods relying on myotoxic agents have gained popularity over time. Among the toxins tested have been snake venoms such as cardiotoxin [7] or notexin [8]. Cardiotoxin leads to the myolysis of myofibers by inducing plasma membrane depolarization [22], and is also postulated to be neurotoxic as its injection destroys neuromuscular plates [21]. Moreover, CTX does not influence muscle architecture like basal lamina or microvasculature, making the regeneration process less complicated [27]. For example, proper blood supply might result in a decrease of fibrosis [34]. Another postulated advantage of using CTX is its relatively low harmfulness for the animal [7].

All of the above factors have caused methods involving myotoxins to be favoured when muscle regeneration has been studied. However, the progress of muscle reconstruction might not be the same in a case of myotoxin and other injury methods used. Moreover, differences in the process of regeneration may be caused not only by different methods of injury [13––15], but might also be associated with the type of regenerating muscle. Slow-twitch muscle regeneration has been shown to be impaired compared to fast-twitch muscle [19, 20]. Furthermore, cells isolated from different muscles have been shown to express different myogenic potential [11, 35]. Another variable is associated with the choice of mouse strain. For example, myotubes of regenerating tibialis anterior muscle were bigger in muscles of SJL/J than in those of BALB/c mice [36]. In order to clarify one of the points raised above, i.e. the influence of the method used on the progress of muscle reconstruction, we compared regeneration induced by invasive crushing and by a 'milder' method i.e. CTX injection. Our results documented that the regeneration of crushed and CTX-injured muscles differs significantly.

Morphological changes accompanying regeneration after mechanical injury and CTX injection

The progress of muscle regeneration can be followed by analyses of muscle weight and histological analyses of muscle cross-sections. Previous studies have documented that one month after injury caused by freezing, murine BALB/c soleus muscle weight was significantly lower than the weight of uninjured muscle [37], which suggests that freezing injury might result in muscle atrophy. Our results also show that 14 days after crushing, the weight of regenerating muscle was significantly lower than that of intact one, suggesting that this method also has a negative effect on muscle regeneration. On the other hand, CTX-induced regeneration can, as has been shown in analyses of regenerating rabbit tibialis anterior, result in a significant increase in muscle weight compared to a control [38]. This observation, explained by significant hypertrophy of myofibers within the regenerated muscle, is consistent with our findings which also show an increase in the weight of CTX-injured muscles analyzed from the 7th to the 14th day of regeneration. However, toxin-induced regeneration does not always result in an increase of muscle weight, as has been shown by Fink et al.: notexin injured rette soleus muscles analyzed from the 4th to the 8th day of regeneration were characterized by significantly lower weight then control, i.e. non-injured, ones [13]. Discrepancies between our results and those presented by Fink may suggest a difference between the properties of notexin and cardiotoxin. Accelerated reconstruction of CTX-injured muscles, observed in our study, may prove the chemotactic properties of this agent resulting in enhancement of macrophage invasion and satellite cells activation after CTX injection, as has been postulated [27]. As a result, we observed extensive degeneration of myofibers and an earlier start of the reconstruction phase of CTX-injured muscles compared to crushed muscles. Faster myofiber reconstruction after CTX injection may be also explained by the presence of basal lamina which is not destroyed by CTX and may serve as scaffold for new myofiber formation [9].

Molecular changes accompanying regeneration after mechanical and CTX injury

In order to follow the progress of muscle regeneration, we investigated changes in the mRNA expression level of neonatal, slow, fast IIa, fast IIb, and fast IId/x MyHC isoforms and also the localization of fetal, slow and fast MyHC isoforms. During muscle regeneration, as during embryonic myogenesis, the first synthesized MyHC isoforms are fetal and neonatal, and their expression is followed by synthesis of adult MyHC isoform, i.e. slow and then fast [32, 33]. According to that description, the reappearance of MyHC fetal and neonatal isoforms and the elevation

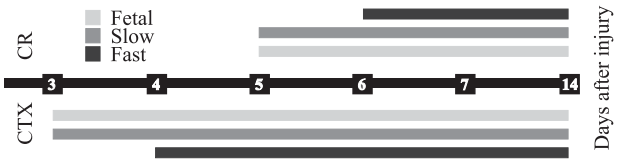

Figure 5. Timing of MyHC isoforms expression in crush- and CTX-injured muscles. Scheme based on the results obtained from immunolocalization of fetal, slow, and fast MyHC isoforms on the 3rd, 4th, 5th, and 6th days presented in Figure 3, as well as the 7th and 14th days after injury (data not included). CR — crushed muscle; CTX — cardiotoxin-injured muscle

of slow and fast MyHC isoforms can indicate the regeneration progress. Also, during *in vitro* studies, MyHCs detected by an antibody recognizing all MyHC isoforms, was firstly expressed in a myoblast that initiated the fusion process [26]. In our study, after both types of injury, muscle regeneration was accompanied by the subsequent reappearance of fetal, slow and fast MyHC isoforms in myotubes differentiating into myofibers. Immunolocalization assays showed also a time delay in the regeneration caused by crushing compared to the regeneration after CTX injury (Figure 5). Also RT-PCR analyses showed that at least elevation of slow and fast IIa MyHC isoforms expression, when compared to uninjured muscles, started earlier after CTX injection than after crushing. Interestingly, the initial increase in the expression of slow and IIa MyHC, observed on the 3rd and 5th days of regeneration, was most probably caused by myoblast specification and fusion. Their expression was then decreased after myoblasts fusion, and then again upregulated during maturation of myotubes. In regeneration caused by crushing, these changes were delayed by two days.

We did not notice any difference in the start of the neonatal and fast IIb isoforms expression between muscles injured using the two methods analyzed. But it is possible that due to the limited time points of our analyses we missed the exact moment when the expression of these MyHCs was initiated. Altogether, our histological and molecular analyses indicate an accelerated start of reconstruction, i.e. myoblast fusion phase, after injury caused by cardiotoxin.

Experiments studying the myogenic potential of cells isolated from muscles on the 7th day of regeneration have shown that cells from CTX-injected muscles start to fuse earlier than cells from crushed ones. This was manifested by the fact that they formed myotubes containing more nuclei. It suggests that cells isolated from CTX-injured muscles start proliferation earlier than cells isolated from crushed ones. Moreover, the relatively high number of cells isolated from crushed muscles, in combination with their lower myogenic potential, indicates that myogenic precursors were isolated with a higher number of non-muscle cells. Among these cells were probably inflammatory ones, as our technique of satellite cells isolation does not exclude non-myogenic cells. The presence of such cells, of which not all were capable of myogenic differentiation, has been demonstrated by others using the same isolation technique [35].

Altogether, our analysis of *in vitro* differentiation of cells isolated from crushed or CTX-injured muscles confirms that when muscle degradation is induced by CTX, the reconstruction phase starts earlier.

In conclusion, we showed that both types of skeletal muscle injury lead to regeneration following the typical, previously described scheme (reviewed in [1]). However, the dynamics of this process depends on the injury method used. Cardiotoxin injection resulted in faster and more extensive muscle degeneration, and an earlier start of the reconstruction phase, than crushing.

Thus, the selection of injury method greatly influences the dynamics of regeneration manifested not only at the histological level but also at the level of expression of crucial structural proteins such as MyHC. One has to be careful when comparing results obtained using different injury methods. However, the presented results provide us with information that can be used to choose an appropriate injury method when planning an experiment.

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