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# Regenerating Skeletal Muscle: A Study in Experimentally Injured and *mdx* Muscles

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**Matrix metalloproteinases (MMPs) cooperatively degrade all components of the extracellular matrix (ECM). Remodeling of ECM during skeletal muscle degeneration and regeneration suggests a tight regulation of matrix-degrading activity during muscle regeneration. In this study, we investigated the expression of MMP-2 and MMP-9, in normal muscles and their regulation during regeneration process. We further investigated their secretion by C2C12 myogenic cell line. Two models of muscle degeneration–regeneration were used: (1) normal muscles in which necrosis was experimentally induced by cardiotoxin injection; (2) *mdx* muscles which exhibit recurrent signs of focal myofiber necrosis followed by successful regeneration. MMPs were studied by zymography; their free activity was quantified using $^{3}$H-labeled gelatin substrate and mRNA expression was followed by Northern hybridization. Muscle degeneration–regeneration was analyzed by conventional morphological methods and *in situ* hybridization was performed on muscle sections to identify the cells expressing these MMPs. Results show that MMP-2, but not MMP-9 expression, is constitutive in normal muscles. Upon injury, the active form of MMP-2 is transiently increased, whereas MMP-9 is induced within 24 h and remains present for several days. Quantitative assays of free gelatinolytic activity show a progressive and steady increase that culminates at 7 days postinjury and slowly returns to normal levels. In adult *mdx* mice, both pro and active forms of MMP-2 and MMP-9 are expressed. Northern blot results support these findings. Zymography of C2C12-conditioned medium shows that myogenic cells produce MMP-2. By *in situ* hybridization we localized MMP-9 mRNA in inflammatory cells and putative activated satellite cells in injured muscles. Our data allow the correlation of the differential expression of pro and/or active forms of MMP-2 and MMP-9 with different stages of the degeneration–regeneration process: MMP-9 expression is related to the inflammatory response and probably to the activation of satellite cells, whereas MMP-2 activation is concomitant with the regeneration of new myofibers.** 

## INTRODUCTION

The extracellular matrix (ECM) plays a central role in maintaining the structural integrity of primitive multicellular organisms as well as that of highly complex mammals. Degradation and synthesis of several macromolecular components of ECM have been demonstrated after injury to adult myofibers (Gulati *et al.*, 1983). These multinucleated syncitia arise from the fusion of mononucleated precursors, the myoblasts, which continue to fuse with adjacent myofibers during postnatal growth, and persist in mature muscle as "satellite cells" (Mauro, 1961) providing a source of cells for new muscle formation during muscle regeneration. Injury to a muscle induces the necrosis of affected myofibers and their subsequent regeneration which results from the activation, proliferation, and fusion of the myogenic mononucleated stem cells into centrally multinucleated myotubes that mature into myofibers (Carlson and Faulkner, 1983; Plaghki, 1985). This degeneration–

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regeneration process occurs in several genetically inherited myopathies such as Duchenne muscular dystrophy (DMD) and its murine homologue, *mdx* mice (Bulfield, 1984), characterized by recurrent signs of degeneration–regeneration, and results from the absence of dystrophin, a subsarcolemmal cytoskeletal protein that contributes to myofiber integrity. It can also be experimentally induced in normal muscles by several myotoxic agents (Duchen *et al.*, 1974; Harris, 1975; Plaghki, 1985). Regeneration clearly requires migration of the myoblasts to the fusion site (Grounds and Davies, 1996; Schultz *et al.*, 1985) and has been shown to occur *in vivo* (Hauschka, 1994). On the other hand, there is evidence of extensive remodeling of ECM molecules during myoblast fusion (Chen, 1977), synapse elimination in neonatal development (Hantaï *et al.*, 1989), and muscle degeneration–regeneration under experimental conditions (Gulati *et al.*, 1982, 1983). Previous work has demonstrated the ability of myoblasts to secrete ECM-degrading enzymes (Guérin and Holland, 1995; Strittmatter and Couch, 1986; Strittmatter *et al.*, 1982), but little is known about their ability to participate directly in the remodeling of the ECM.

Among the enzymes involved in matrix degradation is a group of zinc metalloproteinases known as matrix metalloproteinases (MMPs). These enzymes share similar characteristics including a common mode of activation, conserved amino acid sequences in the putative metal binding active site region, and inhibition by specific proteinase inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs) (Edwards *et al.*, 1996). The final *in vivo* level of degradative activity is thought to result from the interactions of many factors controlling expression and activation of latent enzymes and interaction of active enzymes with inhibitors as well as their inactivation and degradation. Their balance determines the extent of matrix degradation. The majority of MMPs are secreted as latent proenzymes and can be activated by several factors as other proteinases or organomercurials such as amino phenyl mercuric acetate (APMA). This activation is characterized by a loss of molecular weight of approximately 10 kDa (Birkedal-Hansen, 1995; Woessner, 1991).

The aim of our work was to study the expression of MMP-2 (72-kDa type IV collagenase; gelatinase A, EC 3.4.24.24) and MMP-9 (92-kDa type IV collagenase; gelatinase B, EC 3.4.24.35), in normal skeletal muscles and their eventual induction and/or activation during the degeneration–regeneration process. We also investigated their secretion by C2C12 myogenic cell line. MMP-2 is secreted by a wide range of cell types from connective tissues and tumors and has the ability to degrade many connective tissue components including denatured fibrillar type I (Aimes and Quigley, 1995), II, and III collagens, native type IV and V collagen (Birkedal-Hansen, 1995). Additionally, 72-kDa gelatinase is able to degrade elastin (Okada *et al.*, 1990) and has low activity toward proteoglycan and fibronectin. MMP-9 degrades the same substrates and is produced mainly by inflammatory cells including polymorphonuclear leukocytes, macrophages, eosinophils (Stahle-Backdahl *et al.*, 1994), and lymphocytes (Montgomery *et al.*, 1993) by various tumor cells such as fibrosarcoma HT1080 or leukemic cell HL-60 (Ries *et al.*, 1994) and by normal cells such as placental cytotrophoblasts, keratinocytes, osteoclasts (Tezuka *et al.*, 1994; Wucherpfenning *et al.*, 1994), and amniotic epithelial cells (Lehtovirta and Vartio, 1994). Human myogenic cells have been shown to produce and secrete MMPs (Guérin and Holland, 1995) but their expression *in vivo* has not been documented. In injured skeletal muscles, MMPs may act by proteolytically releasing growth factors from ECM during the regenerative process. These growth factors have been shown to stimulate and activate myogenic cells *in vitro* (Allen *et al.*, 1995; Bischoff, 1997; Clegg *et al.*, 1987) and *in vivo* (Tatsumi *et al.*, 1998).

The expression and regulation of MMP-2 and MMP-9 in normal and remodeling skeletal muscles was carried out using two models of degeneration–regeneration: (1) experimental injury induced in normal mice muscles by cardiotoxin (CTX) injection (Duchen *et al.*, 1974) and (2) spontaneous regeneration in *mdx* mice that share genetic, biochemical, and histopathological features with DMD (McArdle *et al.*, 1995) and exhibit myofiber necrosis followed by regeneration.

## MATERIALS AND METHODS

### *Animals*

The mice used were 3-month-old C57Bl10/*mdx*, the murine model for DMD, and C57Bl10ScSn control strain in which the mutant *mdx* originally arose. The latter were used for experimental induction of muscle injury. Both were maintained as a breeding colony from stock kindly provided by Dr. T. Partridge (Department of Histopathology, Charing Cross & Westminster Medical School, London, UK). The animals were kept at room temperature with a natural night–day cycle and fed with pellets and water *ad libitum*.

### *Experimental Injury of Muscle*

Injury of normal muscles was performed by injecting cardiotoxin (CTX) (Latoxan, France) (0.06 $\mu$g/$\mu$l) along the longitudinal axis of the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of the C57Bl10 ScSn mice. CTX was released while pulling out the needle to deliver myotoxin along the muscle mass. The experiments were performed in right hindlimb muscles and contralateral intact muscles were used as controls. Morphological and biochemical examinations were performed at 1, 3, 7, 10, 14, and 30 days after injection.

### *Histological Assessments*

At selected times, EDL–TA muscles of the C57Bl10ScSn and 3-month-old *mdx* mice were removed after cervical dislocation. They were carefully dissected, mounted with tragacanth gum on a piece of cork, and frozen in isopentane chilled by liquid nitrogen. Blocks were stored at −80°C prior to sectioning. Transverse cryostat sections (10 $\mu$m thick) were stained with hematoxylin–eosin (H&E).

### Tissue Extract Preparation

The *mdx* and CTX-treated muscles were immediately frozen in liquid nitrogen (−165°C) and preserved at −80°C. Muscles were weighed and homogenized (1:10, w/v) in extraction buffer 100 mM Tris–HCl, pH 7.6, 200 mM NaCl, 100 mM $CaCl_2$ and 1% Triton X-100 at 4°C. After centrifugation (15,000*g*), the supernatant was divided into aliquots, and protein concentration was determined against an albumin standard curve using the Micro BCA kit (Pierce). Equal amounts of total protein were loaded for zymography (60 $\mu$g/lane) and Western blotting (100 $\mu$g/lane). For the $^3$H-labeled gelatin assay, three tissue samples per time point were pooled and the assay was performed in triplicate.

### Cell Culture

Frozen C2C12 myogenic cells were thawed and cultured in DMEM medium (GIBCO) in presence of 20% fetal calf serum (GIBCO) and 0.5 mg/ml gentamycin (GIBCO). Cells ($10^6$) were plated in 36-mm culture dish and allowed to attach for 16–17 h in complete medium. They were rinsed in 1× PBS (phosphate-buffered saline) and fresh DMEM with no serum added. C2C12-conditioned medium was collected after 48 h of incubation and 20 $\mu$l was analyzed by gelatin zymography.

### Zymography

Zymography of muscle extracts and conditioned medium of C2C12 cell line was carried out according to Heussen and Dowdle [Heussen, 1980]. Zymogram gels consisted of 7.5% polyacrylamide impregnated with gelatin at 1 mg/ml. After electrophoresis, the gels were washed twice for 30 min in a 2.5% Triton X-100 solution at room temperature and incubated for 24 h in a substrate buffer (50 mM Tris–HCl buffer, pH 8.0, 5 mM $CaCl_2$, 0.02% $NaN_3$) at 37°C. Metalloproteinases are secreted in a latent form and require cleavage of a peptide from their $NH_2$ terminus for activation. However, exposure of proenzymes of the tissue extracts to SDS during gel separation procedure leads to activation without proteolytic cleavage (Talhouk *et al.*, 1991). The gels were then stained in Coomassie blue R250 for 1 h and destained in water overnight. Gelatin-degrading enzymes were visualized as clear bands, indicating proteolysis of the substrate protein.

### Gelatinase Assay on Radiolabeled Gelatin

Free gelatinase activity was assayed using radiolabeled gelatin as substrate. Gelatin (Sigma) was radiolabeled with $^3$H-labeled acetic anhydride (Lafuma *et al.*, 1994). Specific activity was 880 kBq/mg. To measure the free form of gelatinase, aliquots of muscle extracts were incubated in the presence of 50 $\mu$g of acetylated $^3$H-labeled gelatin. The proteolytic reaction was allowed to proceed for 48 h at 37°C, pH 7.4, in the presence of toluene to prevent bacterial contamination. Gelatin was precipitated at 4°C with a mixture of trichloroacetic and tannic acid to a final concentration of 4 and 0.8%, respectively. The reaction media were centrifuged at 6000*g* for 10 min at 4°C. Aliquots of the resulting supernatants were mixed with equal volumes of scintillation liquid (optiphase "Hisafe 3") and counted for radioactivity in a 1209 Rackbeta LKB counter instrument. Results are expressed as micrograms of gelatin hydrolyzed per 48 h per muscle.

### Characterization of Metalloproteinases

To characterize the MMP activity, specific enzyme inhibitors or APMA (Sigma), a known MMP activator, were added to incubation media. In one series of experiments, the serine protease inhibitor AEBSF (Calbiochem) (1 mM), cysteine protease E64 (Calbiochem) (10 $\mu$M), and metalloproteinases inhibitor EDTA (Sigma) (10 mM) were used for activity assay on $^3$H-labeled gelatin, whereas EDTA (20 mM) and PMSF (Sigma) (1 mM) were added to the incubation buffer after gel electrophoresis. In a second set of experiments, APMA (1 mM) was mixed with the samples and incubated for 1 h at room temperature before being analyzed by zymography.

### Western Blot Procedures

Tissue extracts were submitted to SDS–PAGE using 7.5% polyacrylamide gels (Laemmli, 1970) under nonreducing conditions. The proteins were transferred onto a nitrocellulose membrane (Towbin *et al.*, 1979), and stained with ponceau S (Sigma) in 0.1% acetic acid. The membrane was blocked with 10% nonfat milk (Gloria, Nestlé) in PBS and incubated overnight at room temperature with polyclonal rabbit antibodies against human MMP-2 and MMP-9 (Valbiotech, France), respectively, diluted at 1:500 and 1:1000 in PBS +3% milk. The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Dako, Denmark) at 1:2000 for 1 h at room temperature. After a thorough wash, the membrane was processed with an enhanced chemiluminescence Western blot analysis system (ECL kit, Amersham). The immunoreaction was visualized by exposure of the membrane to X-OMAT films (Kodak).

### Northern Blot

Total RNA was extracted and purified from freshly isolated muscle tissue according to the procedure of Chomczynski and Sacchi (1987). Ten micrograms of total RNA was resolved in 1.1% agarose–formaldehyde gels under denaturing conditions and transferred onto a nylon membrane Hybond $N^+$ (Amersham) by a 24-h capillary transfer in 20× SSC. The gels were photographed on a UV transilluminator to record RNA integrity. RNA was cross-linked to the membrane by use of the Stratalinker-2400 (Stratagene, La Jolla, CA). Filters were prehybridized at 45°C for 3 h in 50% formamide (Fluka), 20× SSC, 50× Denhardt's, 10% SDS, 0.5 M Pipes (Sigma), pH 6.4, 0.5 M EDTA (Promega), and 5 mg/ml denatured salmon sperm DNA (GENBLOC). Hybridizations were carried out under high stringency conditions in the same solution at 45°C overnight. Blots were then washed at medium stringency in 1× SSC, 1% SDS (3 times, 15 min) at 55°C and then at 60°C (1 time, 15 min) before exposure to autoradiography on Kodak X-OMAT films. Blots were sequentially hybridized with a 2.8-kb *Eco*RI insert in pBR322 plasmid of mouse 72-kDa gelatinase (MMP-2) and a 1.8-kb *Eco*RI–*Hin*dIII insert in pBlueScript II plasmid of mouse 92-kDa gelatinase (MMP-9). The recombinant plasmids were kindly provided by Dr. K. Tryggvason (Department of Medical Biochemistry and Biophysics, Karolinska Institute, Sweden) (Reponen *et al.*, 1992, 1994). To normalize signal intensity, blots were later stripped and rehybridized with an 18S oligoprobe $^{32}$P-radiolabeled by nick translation. The cDNA probes were labeled with [$^{32}$P]dCTP by random priming using the Klenow enzyme (Megaprime DNA labeling system kit, Amersham). Unincorporated radioactivity was removed using Nick Spin columns (Pharmacia). Quantification of the labeled bands was performed with a $\beta$-imager (Biospace Mesure, France) using the Biovision computer program.

### *Radioactive in Situ Hybridization*

*In situ* hybridization was performed to detect the cellular source of MMP-2 and MMP-9 expression. [$^{35}$S]UTP-labeled RNA probes were synthesized using Ampliscribe Sp6 and T7 high yield transcription kit (TEBU). For MMP-9 probe preparation, pSP65 and pSp64 plasmid containing the M-92 KD-2 clone were respectively linearized with *Bam*HI (antisense) and *Eco*RI (sense) and then transcribed *in vitro* with Sp6 polymerase to obtain a 323-pb RNA (Reponen *et al.*, 1994). [$^{35}$S]UTP-labeled pGEM single-stranded RNA and sense MMP-9 probes were used as negative controls. For MMP-2 probe preparation, pGEM-3Z plasmid containing a mouse 72-kDa type IV collagenase cDNA clone was linearized with *Eco*RI (antisense) or *Bam*HI (sense) and transcribed *in vitro* using Sp6 or T7 polymerase, respectively; this resulted in a 331-pb RNA (Reponen *et al.*, 1992). MMP-2 and MMP-9 cDNA clones were kindly provided by Dr. K. Tryggvason (Department of Medical Biochemistry and Biophysics, Karolinska Institute, Sweden).

At selected times, normal and injured EDL–TA skeletal muscles were removed under RNase-free conditions, mounted with tragacanth gum, and immediately frozen in liquid nitrogen; and they were stored at −80°C until use. Transverse cryostat sections (7 $\mu$m) were performed, placed on silanized slides, and postfixed in a freshly prepared solution of formaldehyde 4% in 1× PBS buffer for 15 min. Slides carrying sections were then rinsed in PBS for 15 min and quickly dehydrated through a series of ethanols (60, 70, 95, and 100%). Slides were then treated with a fresh solution of 7 $\mu$g/$\mu$l proteinase K (Sigma) for 15 min at 37°C in 20 mM Tris–HCl and 2 mM $CaCl_2$, pH 7.4. Slides were then rinsed in sterile distilled water and acetylated in a 0.1 M solution of triethanolamine (Fluka) with acetic anhydride, 0.25% (Fluka) for 10 min. Slides were rinsed with 2× SSC for 5 min (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) and allowed to dry at least 1 h prior to hybridization. Probe was applied directly to tissue sections at a final adjusted concentration of $2 \times 10^6$ cpm/section in hybridization buffer containing: 50% deionized formamide (Sigma), Tris–EDTA, NaCl, 1× Denhardt's, 10% Dextran sulfate (Pharmacia), 1 mg/ml total yeast RNA (Sigma), 0.5 mg/ml salmon sperm DNA (GENBLOC), and sections were covered with a siliconized coverslip (14 mm, diameter). Hybridization was carried out at 50°C for approximately 16 h in a humidified chamber. Coverslips were gently floated off in 50% formamide, 4× SSC at room temperature and the tissue was then subjected to two 15-min washes at 55°C in 50% formamide, 2× SSC, 20 mM $\beta$-mercaptoethanol. Slides were then rinsed in 2× SSC for 10 min at room temperature and treated with RNase A (Sigma) (100 $\mu$g/$\mu$l, 15 min at 37°C) in washing buffer. Following one wash in 2× SSC for 30 min at 37°C and two 5-min washes at 20°C, slides were rapidly dehydrated in a series of 70, 90, and 100% ethanols. Slides were processed for standard autoradiography using LM-1 Kodak emulsion and exposed for 2 months in light-tight boxes with dessicant at 4°C. Photographic development was carried out in full-strength Kodak D-19. Slides were fixed (Ilford) and lightly stained with H&E. Analysis was carried out using light-field optics on a Leica microscope.

## RESULTS

### *Histological Analysis of the Necrotic–Regeneration Process*

In contrast to the normal control (Fig. 1a), cross sections of 3-month-old *mdx* muscles exhibited the typical appearance of regenerated muscles with centrally nucleated muscle fibers of variable size (Fig. 1b). They presented little if any necrosis and infiltration by invasive cells. CTX-injured muscles appeared edematous within the first 3 days by gross morphological observations. At day 1, muscles were already infiltrated by polymorphonuclear leukocytes (PMN), predominant within the enlarged intercellular space separating the pale necrotic myofibers (Fig. 1c). By day 3, the necrotic fibers were largely invaded by PMN and macrophages and spindle-shaped mononucleated cells began to appear (Fig. 1d). At day 7 after injury, the majority of injured fibers regenerated into groups of centrally nucleated myotubes (Fig. 1e), whereas a few necrotic fibers infiltrated by macrophages could occasionally still be observed. At later stages (Figs. 1f–1h, days 10, 14, and 30), regenerated myofibers exhibited increase of their cross-sectional area and further matured into myofibers with central nuclei.

### *Zymography*

Muscle extracts from the different experimental groups exhibited different zymographic profiles under nonreducing conditions (Fig. 2). In normal muscles, a major 60-kDa gelatinase activity was detected in addition to two barely detectable bands at 66 and 55 kDa. By contrast, in muscle extracts of *mdx* mice, two zones of lysis bands one at 100 kDa and the other at 66, 60, and 55 kDa were detected (Fig. 2, *mdx*). These bands suggested the presence of latent and/or active forms of MMP-9 (100 kDa) and MMP-2 (66, 60, and 55 kDa). In normal muscles injured by CTX, MMP-9 expression was induced within 24 h after injury and represented the major gelatinase identified. At more advanced stages (days 3 and 7) this activity progressively decreased until it completely disappeared by the beginning of the second week. At the same time, the active form of MMP-2, present at low levels in the normal muscle, gradually increased reaching a maximum 7 days after injury and concomitant overexpression of 66- and 60-kDa species was observed. Between days 14 and 30, the pattern and level of gelatinolytic activity returned to normal. In C2C12-conditioned medium, a major 60-kDa gelatinase activity was detected in addition to two bands at 66 and 55 kDa (Fig. 2, CM).

To determine the exact nature of these gelatin-degrading proteases, we examined the effect of APMA, a known activator of metalloproteinases, and two protease inhibitors by zymography. Treatment of extracts from normal muscles in the presence of 1 mM APMA at 37°C for 1 or 3 h resulted in an increase of the 55-kDa species (Fig. 3A) which we interpreted as a conversion of a latent to an active form. Additionally, the gelatinolytic proteases from *mdx* muscle extracts were completely inhibited in presence of EDTA (20 mM), whereas PMSF (1 mM), a serine protease inhibitor, had no effect on their ability to degrade gelatin (Fig. 3B), confirming that they belong to the MMP family.

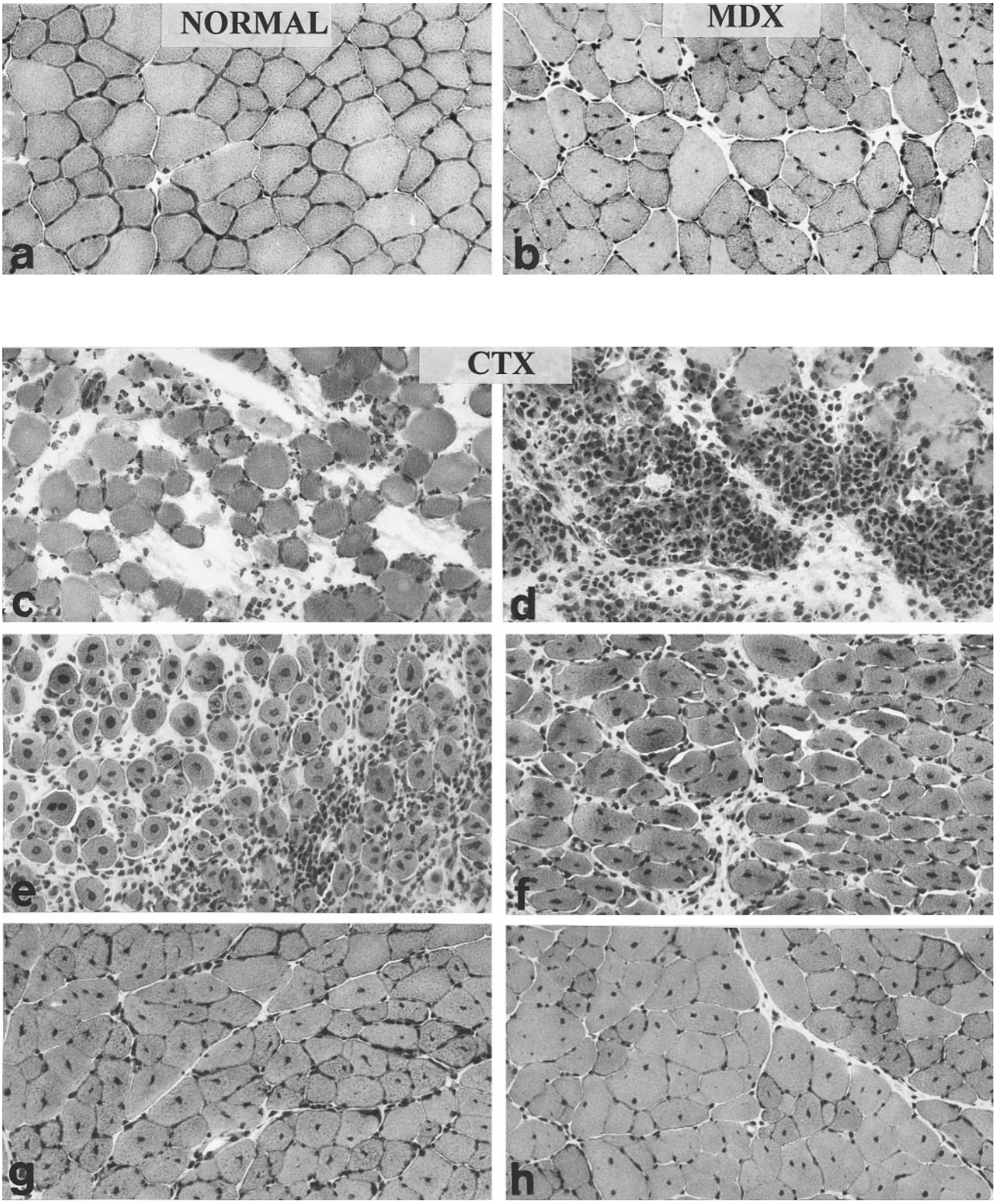


**FIG. 1.** Pattern of necrosis-regeneration of 3-month-old CTX-injured and *mdx* TA muscles. (a) Normal, (b) *mdx*, (c–h) CTX-injured muscles. Inflammatory cells have invaded the enlarged extracellular space of the edematous muscle at 1 day postinjury (c) and have completely infiltrated the necrotic myofibers by day 3 (d). Numerous young myotubes have regenerated at day 7 and inflammation has decreased (e). Maturation of myotubes into multinucleated myofibers progressively occurred (f, 10 days; g, 14 days; and h, 30 days). Cross sections stained with H&E. Magnification, ×100.

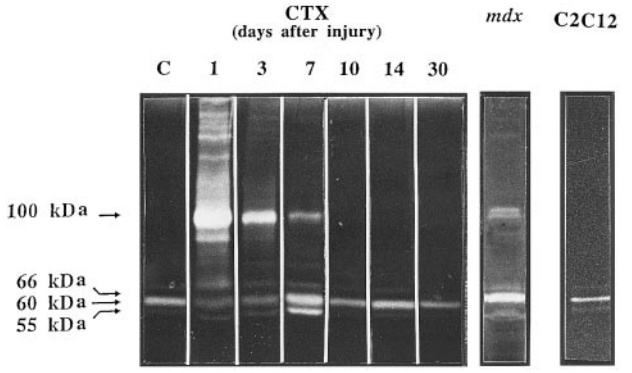


**FIG. 2.** Zymographic analysis of normal, CTX-injured, and *mdx* EDL–TA muscles and conditioned medium of C2C12 myogenic cell line. Gelatin at 1 mg/ml was copolymerized in 7.5% SDS–PAGE and equal amounts of protein (60 $\mu$g/lane) were loaded. After electrophoresis of muscle extracts, the gelatin substrate gels were washed twice with Triton X-100 to remove SDS and incubated overnight in a 100 mM Tris–HCl incubation buffer with $CaCl_2$ at 37°C. Gels were then stained with Coomassie blue and the lysis areas appear as clear bands. C, 3-month-old normal muscle. 1, 3, 7, 10, 14, and 30 correspond to CTX-injured muscles at 1, 3, 7, 10, 14, and 30 days after injury. *mdx*, 3-month-old *mdx* muscle; C2C12, conditioned medium of C2C12 cell line. Molecular weights are on the left.

### *Gelatinase Assays on Radiolabeled Gelatin*

To investigate the modulation of free gelatinolytic activity during the time course of necrosis–regeneration and determine the precise contribution of gelatinases to the total degradative activity in CTX-treated muscles, we performed a quantitative gelatinase assay using $^3$H-labeled gelatin as a substrate in the presence of specific protease inhibitors. This assay allowed the detection of only free gelatinolytic activity not inhibited by TIMPs of both MMP-2 and MMP-9 since gelatin is a common substrate for both enzymes. In tissue extracts from normal muscles, free gelatinolytic activity was very low and could be attributed to serine proteases on the basis of inhibition profiles. In CTX injured muscles, free gelatinolytic activity was progressively increased from day 1 postinjury until day 7, when it reached its maximum, before returning to the normal level. The increase measured between days 1 and 10 postinjury was mainly due to metalloproteinases (60–90%) with some serine and cysteine proteinase activities. At days 14 and 30, the level of free gelatinolytic activity returned to the normal values and was associated with serine protease activity (Fig. 4).

### *Western Blot Analysis*

Western blot analysis under nonreducing conditions of CTX and *mdx* muscle extracts with polyclonal antibody against human MMP-9 revealed the absence of band in normal muscles and the presence of one band at 100 kDa in CTX-treated muscles during the first week after injury and in all examined *mdx* muscles (Fig. 5). This band corresponds to mouse MMP-9 known to migrate slightly higher than the human protein (Unemori *et al.*, 1991). Western blots of muscle extracts using polyclonal antibodies against human MMP-2 did not allow the detection of mouse MMP-2.

### *Northern Blot Analysis*

Northern blot analysis using cDNA probes for mouse MMP-9 and MMP-2 was performed on 10 $\mu$g of total cytoplasmic RNA extracted from normal, CTX-treated, and *mdx* muscles to determine whether enzyme expression of these two MMPs was controlled at the transcriptional level (Fig. 6A). The Northern blot of normal muscles showed the absence of MMP-9 transcript(s) which is in accordance with the absence of MMP-9 protein and its corresponding activity. In CTX-injured muscles, MMP-9 transcripts were induced as early as the first day after injury and two transcripts of 3 and 3.8 kb were detected. The relative amount of MMP-9 transcripts was maximal at day 1 and progressively decreased before disappearing completely at the beginning of the second week (Fig. 6B). In *mdx* muscles, we could not detect MMP-9 transcripts in 3-month-old mice. MMP-9 transcripts were also undetectable in younger mice (1 month old) undergoing active necrosis and regeneration even when higher amounts (30 $\mu$g) of total RNA were loaded. On the other hand, MMP-2 transcripts were detected in the normal and *mdx* muscles at 3.1 kb, hence reflecting the constitutive expression of MMP-2. In normal muscles after CTX injury, the level of MMP-2 transcript increased progressively. The threefold increase observed at day 7 corresponded to the higher levels detected during degeneration–regeneration. A slow decrease was observed thereafter (Fig. 6B).

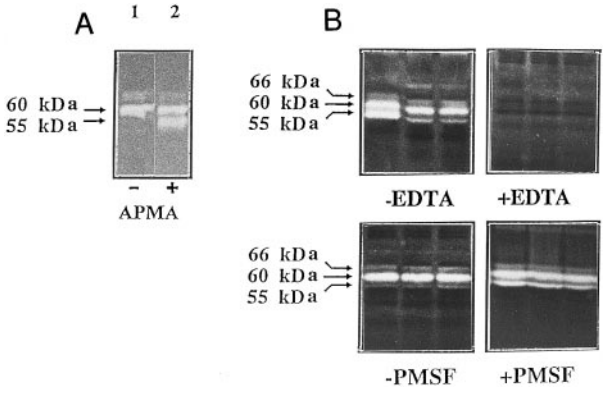


**FIG. 3.** Characterization of metalloproteinases secreted by skeletal muscle. (A) APMA treatment was performed on normal extracts. Lane 1, control; Lane 2, APMA (1 mM), 1 h, 37°C. (B) Inhibition was performed on *mdx* muscle extracts. After electrophoresis, the gels were incubated in a 100 mM Tris–HCl incubation buffer containing either EDTA (20 mM) or PMSF (1 mM) overnight. Both gelatinases were inhibited by EDTA, but not by PMSF.

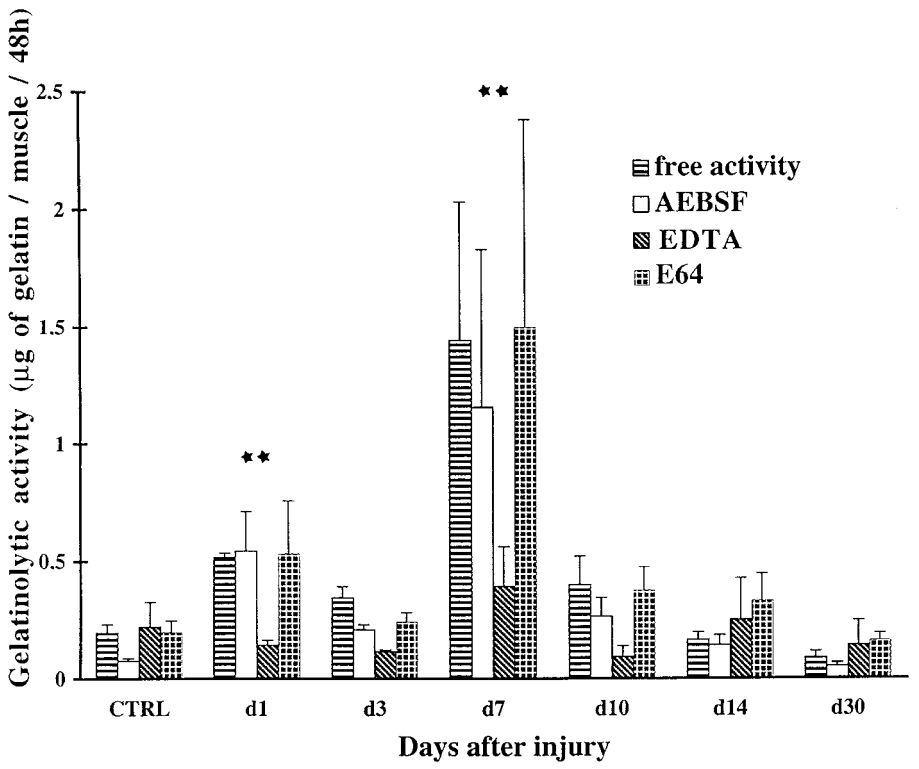


**FIG. 4.** Gelatinase assay on $^{3}$H-labeled gelatin of normal and CTX-injured muscles. Extracts from normal (CTRL) and CTX-injured muscles were incubated with specific protease inhibitors AEBSF, EDTA, and E64 to determine the relative contribution of MMP activity. Results are the means of triplicate determinations and expressed as $\mu$g of gelatin hydrolyzed per muscle per 48 h. Bars represent means ± SEM (**$P \leq 0.05$).

### *Localization of Gelatinases Expression in Skeletal Muscle by in Situ Hybridization*

*In situ* hybridization performed on CTX-injured muscles early after injury with antisense MMP-9 probe revealed the presence of a large number of positive cells in the injured tissue. Silver grain accumulation was detected in aggregates of inflammatory cells (Figs. 7A and 7B) identified as polymorphonuclear leukocytes and macrophages (data not shown) and in mononucleated cells localized at the periphery of injured myofibers (Fig. 7C). Adjacent control sections hybridized with sense MMP-9 and pGEM control probes were negative (Fig. 7D′); sense MMP-9 gave high background (not shown). No expression of MMP-9 was observed in normal muscles (not shown).

*In situ* hybridization performed with MMP-2-riboprobe on muscle sections did not allow the localization or the identification of the cellular source of MMP-2 mRNA. This could be due to a low number of transcripts per cell.

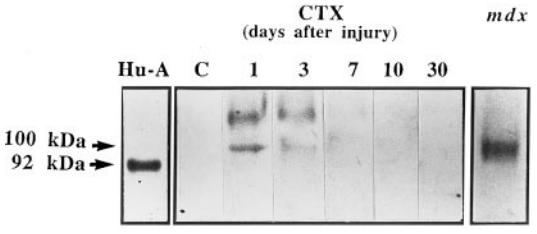


**FIG. 5.** Western blot analysis. Protein extracts of CTX-injured and *mdx* muscles were analyzed by immunoblotting with anti-human MMP-9. Equal amounts of protein (100 $\mu$g) were loaded per lane and immunoblotted under nonreducing conditions with rabbit polyclonal antiserum against 92-kDa human gelatinase diluted at 1:500. Hu-A, human MMP-9 antigen (100 ng). C, 3-month-old normal muscle. 1, 3, 7, 10, and 30 correspond to CTX-injured muscles at 1, 3, 7, 10, and 30 days after injury. *mdx*, 3-month-old *mdx* muscle. Molecular weights are on the left.

## DISCUSSION

The aim of our work was to investigate the involvement of MMP-2 and MMP-9 gelatinases in tissue remodeling which occurs during skeletal muscle degeneration–regeneration. Two situations were chosen to carry out the research: (i) the experimentally induced regeneration by cardiotoxin injection which induces injury in normal

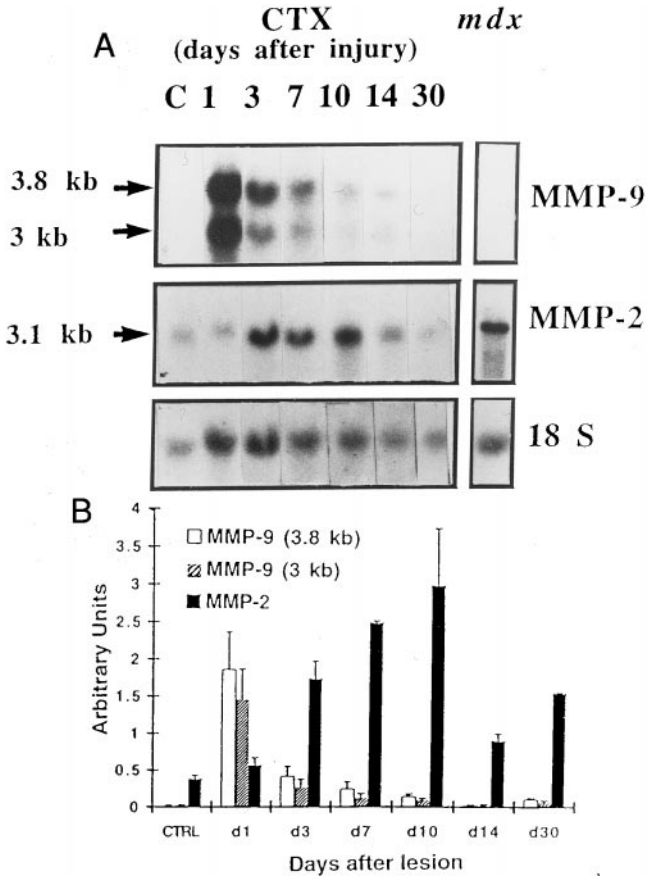


**FIG. 6.** Northern blot analysis and evaluation of transcriptional activity of normal, *mdx*, and CTX-injured muscles. (A) 10 $\mu$g of total RNA extracted from normal, CTX-injured, and *mdx* muscles was resolved in 1.1% agarose gel under denaturing conditions and transferred onto a nylon membrane Hybond $N^+$. the membrane was sequentially hybridized with mouse MMP-2 and MMP-9 cDNA probes labeled with [$^{32}$P]dCTP by random priming and later with 18S oligoprobe $^{32}$P-labeled by nick translation as standard. C, 3-month-old normal muscle. 1, 3, 7, 10, 14, and 30 correspond to CTX-injured muscles at 1, 3, 7, 10, 14, and 30 days after injury. *mdx*, 3-month-old *mdx* muscle. Molecular sizes are on the left. (B) Radioactivity of MMP-2- and MMP-9-labeled bands was quantified using a $\beta$-Imager system, normalized as the ratio between samples versus 18S standard activity and expressed in arbitrary units.

muscles; (ii) the spontaneous muscle regeneration which takes place in mutant *mdx* mice, the murine model of Duchenne muscular dystrophy. The capacity of myogenic cells to produce these gelatinases was also investigated in conditioned medium C2C12 cell line.

Our results clearly show that skeletal muscle can produce both MMP-2 and MMP-9 gelatinases as latent and/or active forms, but with differential patterns related to the time course of muscle necrosis and regeneration. Several findings indicate that these enzymes are members of the matrix metalloproteinase family: (i) they were produced as constitutive 60-kDa MMP-2 and inducible 100-kDa MMP-9 proenzymes that could be activated by APMA; (ii) their activity was inhibited by metallochelators such as EDTA, but not by phenylmethylsulfonyl fluoride; (iii) MMP-9 protein was recognized by a specific antibody; (iv) their free gelatinolytic activity was characterized against a specific gelatin substrate in the presence of serine and cysteine inhibitors; and (v) mRNAs were identified by use of specific mouse molecular probes. Conditioned medium of C2C12 myogenic cells exhibits the presence of MMP-2 latent and active forms. *In situ* hybridization revealed that MMP-9 mRNA are produced by inflammatory cells predominantly polymorphonuclear leukocytes and by putative activated satellite cells.

### *Constitutive Expression of MMP-2 and Not MMP-9 by Normal Skeletal Muscle*

Our zymography results demonstrate that, under basal conditions, skeletal muscles of adult mice constitutively express MMP-2 as 66-, 60-, and 55-kDa forms. Such differences of MMP-2 molecular masses between mouse and human proteins have already been reported (Talhouk *et al.*, 1992). In mouse, the major 60-kDa form could represent a latent unglycosylated or an intermediary form of MMP-2. Nonetheless, the steady-state level of MMP-2 mRNA was detectable by Northern blotting. In contrast, MMP-9 was not detected either at the protein or at the mRNA levels.

The scarcely detectable level of free gelatinolytic activity evaluated against $^3$H-labeled gelatin is in accordance with the low level of MMP-2 active form evaluated by zymography. This strongly suggests that the gelatinase/antigelatinase balance *in vivo* is maintained in a healthy physiological condition.

*In situ* hybridization did not allow the identification of the cellular source of MMP-2 possibly because of low RNA copy per cell. In normal skeletal muscle, endothelial and fibroblast cells are able to produce MMP-2. Nevertheless, by analysis of C2C12-conditioned medium we show that myogenic cells constitutively produce MMP-2, confirming the results of previously reported data showing that human satellite cells express MMP-2 (Guérin and Holland, 1995).

### *Modulation of MMP-2 and MMP-9 Expression in Cardiotoxin-Injured Skeletal Muscle*

The injection of cardiotoxin, a depolarizing fragment from snake venom *Naja nigricollis*, into the muscle (Duchen *et al.*, 1974) rapidly induced muscle damage and the inflammatory pattern was evidenced within a few hours by our morphological study. The damaged muscles produce soluble chemoattractants thus promoting a rapid migration of PMNs from the vasculature to the injury site (Grounds and Davies, 1996). PMNs are the first infiltrating cells to invade the necrotic muscles and are followed by macrophages which become the dominant infiltrating cells by 3 days after injury (Lefaucheur and Sebille, 1995a). In turn,

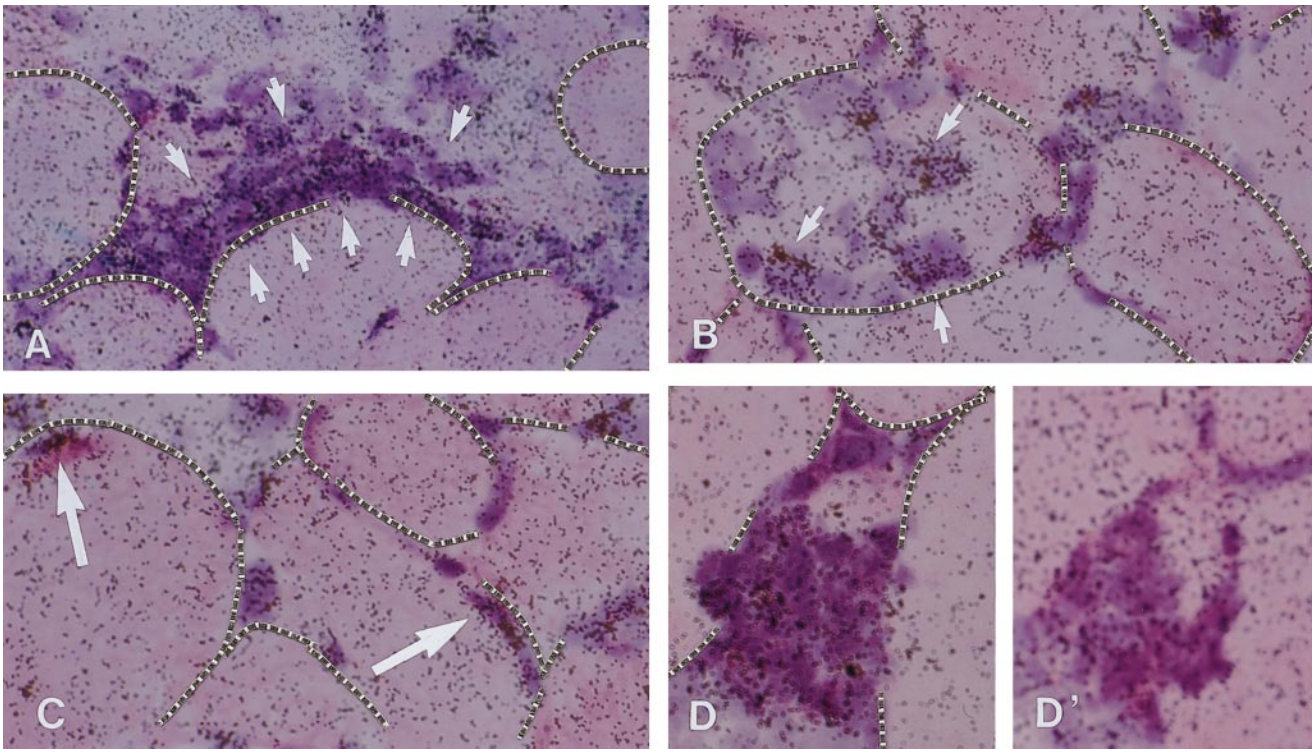


**FIG. 7.** *In situ* hybridization of day 1 CTX-injured EDL–TA muscle with MMP-9 RNA probe. Transverse cryostat sections counterstained with a light H&E show several positive cells at the site of injury. (A) A dense accumulation of silver grains was observed in aggregates of infiltrated inflammatory cells (identified as PMN and macrophages), and in B necrotic fibers are seen to be completely infiltrated by the latter cell types. (C) Dense silver grains are found over mononucleated cells at the periphery of injured fibers, suggesting that activated satelitte cells express MMP-9 transcripts. (D) Adjacent control sections hybridized with MMP-9 antisense and (D′) pGEM control probes show a differential labeling of inflammatory cells. The periphery of necrotic fibers is delineated by lightly dotted black lines. Magnification, ×670.

activated exsudate macrophages attract muscle precursor cells to the site of tissue repair by producing various enzymes, ECM products, and growth factors (Grounds and Davies, 1996).

Our zymography, Western blot, and Northern blot data indicate that mouse MMP-9 is extensively up-regulated during the first 3 days following cardiotoxin injury, i.e., during the acute inflammatory stage that also correlates with active proliferation of myogenic cells. Two MMP-9 mRNA types of 3 and 3.8 kb were identified in muscle extracts due to the presence of two poly(A) sites (Reponen *et al.*, 1994; Tanaka *et al.*, 1993). Between 3 and 7 days following cardiotoxin injury the amount of MMP-9 mRNA and protein begins to decay; both disappear by the beginning of the second week. Inflammatory response in cardiotoxin-injured muscle is acute and numerous activated PMNs and macrophages are present. *In situ* hybridization revealed that by day 1 after injury, these cells express MMP-9 mRNA that was also localized to mononucleated cells at the periphery of injured muscle fibers. These cells may be putative activated satellite cells since immunofluorescence with MyoD antibody exhibited a similar staining pattern (data not shown). Our observations related to MMP-9 expression by muscle precursor cells during early normal muscle development (unpublished results) or by cultured human satellite cells after TPA treatment (Guérin and Holland, 1995) indicate that it is conceivable that MMP-9 expression may be induced in myoblasts. Satellite cells are activated early after injury (Grounds *et al.*, 1992) and present cytoplasmic processes that pass through interruptions of parent basal lamina (Maltin *et al.*, 1983). These observations and others (Bischoff, 1979; Hughes and Blau, 1990) suggest that myogenic cells have migratory capacities. Similar regulation of MMP-9 was also observed during dedifferentiation/regeneration of axolotl appendages (Yang and Bryant, 1994).

Our zymography data clearly show that mouse MMP-2 is up-regulated and activated between 3 and 10 days postinjury, with maximal activation at day 7. This is evidenced by the gradual increase in free metallogelatinolytic activity with a maximal response at day 7 postinjury. This time interval corresponds, in our study, to myogenic cell prolif-

eration, migration, and fusion. By day 10, when myoblast fusion has been completed, the latent and active forms of MMP-2, the free metallogelatinolytic activity, and mRNA return to baseline levels. The role played by MMP-2 during myoblast fusion could be to degrade the type IV collagen as well as other basement membrane macromolecular components such as entactine (Sires *et al.*, 1993). Our overall results strongly suggest that MMP-2 up-regulation at mRNA, protein, and activity levels correlates with the regeneration.

### *Expression of MMP-2 and MMP-9 by Skeletal Muscle of mdx Mice*

Skeletal muscles from *mdx* mice exhibit recurrent signs of degeneration and necrosis followed by successful regeneration (Louboutin *et al.*, 1993; Torres and Duchen, 1987). Foci of degenerating and regenerating myofibers exist concomitantly within the same muscle.

When compared to normal muscles, our zymography results demonstrate that *mdx* muscles exhibited: (i) a weak but constant expression of MMP-9 as a double band at approximately 100 kDa and (ii) up-regulation of MMP-2 as a major 60-kDa form and a significant level of the 55-kDa active form. MMP-9 was identified by Western blot analysis. The constitutive production of mouse MMP-2 was corroborated by Northern blot analysis. On the basis of our results obtained in cardiotoxin-injured muscles, it is possible to hypothesize that a constant MMP-2 activation may intervene during muscle regeneration. Moreover, the presence of a significant level of 55-kDa active form suggests that an imbalance between MMP-2 and its specific inhibitor, TIMP-2, may develop in the microenvironment of necrotic fibers as a result of inflammation-related factors. In *mdx* muscles, the absence of dystrophin directly or indirectly results in the inability of *mdx* fibers to maintain a low intracellular concentration of calcium (McArdle *et al.*, 1995; Turner *et al.*, 1988). This inability of cells to regulate the calcium flux may favor activation of proteases as has been described in dystrophic muscles (Kar and Pearson, 1978). Since MMPs require proteolytic cleavage for their activation and since calcium ions are required to promote catalytic site liberation, calcium increase in *mdx* muscles may be one of the physiological pathways explaining the constant MMP-2 activation. Several *mdx* muscles (gastrocnemius, soleus, diaphragm, and heart) have shown similar expression and activation patterns (unpublished data). Nevertheless, in addition to the possible involvement of increased calcium flux in MMP-2 activation, other MMP-2 activation mechanisms may intervene, implying matrilysin (Crabbe *et al.*, 1994) or MT-MMP1, a membrane-type matrix metalloproteinase (Sato *et al.*, 1994; Yamamoto *et al.*, 1996).

Mouse MMP-9 is known to migrate at 105 kDa (Tanaka *et al.*, 1993) and the two closely related forms of MMP-9 identified by zymography might be different glycosylated proforms. The blurred MMP-9 band identified by Western blot supports this hypothesis. However, no MMP-9 mRNA was detectable by Northern blot analysis, either when the total RNA loaded on gels was increased threefold or when total RNA was isolated from highly necrotized muscles of 1-month-old mdx mice. In fact, skeletal muscles of 1-month-old *mdx* mice suffer chronic necrosis–regeneration spatially limited to small foci. From this it could be inferred that few inflammatory cells are present at a given time. This may explain the difficulties in detecting MMP-9 transcripts within the Northern blot detection limits, presuming that it is produced by both inflammatory and activated satellite cells. Numerous histological studies have identified the presence of PMNs, macrophages (Lefaucheur and Sebille, 1995a), and mast cells (Gorospe *et al.*, 1994) in injured muscles. These cell types are known to store (Delclaux *et al.*, 1996) and produce MMP-9 in response to different stimuli from necrotic tissue, and represent one important source of MMP-9.

It is not known what factors up-regulate MMP-9 or MMP-2 expression during the degeneration–regeneration process. Several studies showed that MMP expression is greatly modulated by cytokines and growth factors and involves the gene products of Fos and Jun families of oncogenes (Mauviel, 1993). Although they have a similar substrate specificity, expression of the MMP-2 and MMP-9 appears to be differentially regulated (Yokoo and Kitamura, 1996) by various cytokines and chemokines produced by degenerating muscles and activated macrophages (Robertson *et al.*, 1993). MMP-9 promoter gene is characterized by the presence of NF-$\kappa$B and SP-1 binding sites in synergistic cooperation with TRE sequence (TPA responsive element) which are absent in the MMP-2 gene. For example, TNF-$\alpha$ and IL-1$\beta$ secreted by activated macrophages up-regulate MMP-9 (Brenner *et al.*, 1989) but do not affect MMP-2 expression (Unemori *et al.*, 1994; Yao *et al.*, 1996). These data may explain the preferential MMP-9 up-regulation during acute inflammation of cardiotoxin-injured muscles. Because MMP-2 and MMP-9 are activated during the regeneration process, it is highly probable that these enzymes play an important role in myogenesis, partly in response to chemokines. After injury to skeletal muscles, satellite cells are activated, they proliferate, migrate and differentiate to regenerate new muscle fibers. Numerous studies suggest that signals produced locally by the damaged muscle itself are responsible for these events (Bischoff, 1997; Grounds and Davies, 1996). Several growth factors such as FGFs (Johnson and Allen, 1993), IGF, and TGF-$\beta$1 and -2 (Lefaucheur and Sebille, 1995b) have the ability to modulate the proliferative activity of cultured satellite cells (Grounds and Davies, 1996). Recently, it has been demonstrated that HGF/SF has the ability to activate quiescent satellite cells *in vivo* (Tatsumi *et al.*, 1998) to stimulate satellite cell migration (Bischoff, 1997) and exerts mitogenic activity on cultured satellite cells (Allen *et al.*, 1995). In this respect, one of the more mechanistically relevant aspects of MMPs implication in the regenerative process is the possibility that either one or both type IV gelatinases could be involved

in releasing and/or cleaving matrix-associated growth factors, so that they become free to stimulate satellite cell proliferation during the regeneration process (Anderson *et al.*, 1991; Clegg *et al.*, 1987). In addition, early after muscle injury, the gelatinase activity could be necessary to degrade the type IV collagen thus promoting the detachment of satellite cells from the basement membrane, and their proliferation, migration, and subsequent differentiation. It is noteworthy that proteases such as metalloendoprotease (Couch and Strittmatter, 1983) and calpain (Kumar *et al.*, 1992) are involved in myoblast fusion which is blocked by the addition of specific inhibitors *in vitro*. In this context, it would be interesting to analyze the consequences of the inhibition of either or both metalloproteinases *in vivo* to demonstrate more precisely the involvement of MMP-2 and MMP-9 in skeletal muscle degeneration and regeneration.

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