Skeletal muscle apoptosis after burns is associated with activation of proapoptotic signals

SHINGO YASUHARA,1 MARY-ELLEN PEREZ,1 EMI KANAKUBO,1 YOKO YASUHARA,1 YONG-SUP SHIN,1 MASAO KANEKI,1 TOSHIRO FUJITA,2 AND J. A. JEEVENDRA MARTYN1

1Department of Anesthesiology and Critical Care, Massachusetts General Hospital, Boston 02114, Shriners Burns Hospital, Boston 02114, and Harvard Medical School, Boston, Massachusetts 02115; and 2Department of Internal Medicine, University of Tokyo, 112-0015 Tokyo, Japan

Received 4 February 2000; accepted in final form 30 May 2000

Yasuhara, Shingo, Mary-Ellen Perez, Emi Kanakubo, Yoko Yasuhara, Yong-Sup Shin, Masao Kaneki, Toshiro Fujita, and J. A. Jeevendra Martyn.

Skeletal muscle apoptosis after burns is associated with activation of proapoptotic signals. Am J Physiol Endocrinol Metab 279: E1114–E1121, 2000.—Critical illness is associated with muscle wasting and muscle weakness. Using burn injury as a model of local and systemic inflammatory response, we tested the hypothesis that thermal injury causes apoptosis in muscle. After a 40% body surface area burn to rats, abdominal muscles beneath the burn and limb muscles distant from the burn were examined for apoptosis at varying times after burn. Ladder assay, ELISA, and histological methods showed evidence of apoptosis in the abdominal muscles within 4–12 h with peak changes occurring at 3–7 days. Maximal apoptosis was also evident at distant limb muscles at 3–7 days. Investigation of proapoptotic pathways indicated mitochondrial membrane potential to be altered by 1 h after burn. Starting at 15 min after burn, cytochrome c was released from the mitochondria into the cytosol, followed by increased activity of caspase-3, starting at 6 h after burn. These studies suggest that mitochondria and caspase-mediated apoptotic pathways may be an additional mechanism of muscle weight loss in burns and may be potential therapeutic targets for prevention of muscle wasting.

Any form of critical illness, including burn injury, with or without sepsis, is associated with many functional, metabolic, and pharmacological aberrations (2, 3, 29, 32). The important functional change in skeletal muscle during and after critical illness is weakness or decreased tension-generating capacity, resulting in hypoventilation, difficulty in weaning off respirators, decreased mobilization, and/or muscle contractures (3, 13, 17). The characteristic morphological alteration occurring in muscle is the loss of muscle mass. The muscle wasting that occurs in catabolic states such as burns, sepsis, lipopolysaccharide injection (8), and/or denervation syndromes results largely from an accelerated breakdown of muscle protein (5, 31, 32). In these conditions, the protein synthesis mechanism is unable to compensate for the markedly activated catabolic state. One pathway responsible for accelerated muscle protein breakdown has been identified as the ubiquitin-proteosome system. Proteolysis, especially degradation of myofibrillar protein, increases within 1 h after critical illness, apparently due to release of cytokines, which activate ATP-dependent proteolysis and increase ubiquitin mRNA in the muscles (31, 37).

Apoptosis might be an alternative mechanism by which loss of parenchymal tissue occurs and can be initiated by perturbations such as ischemia, direct injury, heat shock, growth factor withdrawal, or toxins (38, 43). Thus some of the conditions that cause apoptosis can also cause necrosis. In contrast to necrosis, however, apoptosis is a process that is energy dependent and follows a sequence of genetically programmed events. Fragmentation of chromosomal DNA is the biological hallmark of apoptosis and can be detected by a ladder formation pattern on gel electrophoresis by ELISA and/or by in situ end-labeling of formalin-fixed tissues (7). Cellular events preceding apoptosis are the change in membrane potential of the mitochondria and the release of cytochrome c from the mitochondria to the cytoplasm (26, 35). The release of cytochrome c to the cytoplasm is followed by activation of death effector caspases (18). Thus mitochondria are now thought important, not only in energy production but also in control of apoptosis.

Apoptosis has been observed in vivo in both parenchymal and nonparenchymal tissues after systemic inflammatory disorders, including sepsis (22). Apoptosis in skeletal muscle has been observed in vivo during development and after denervation injury (14, 42). The role of apoptosis in skeletal muscle wasting after critical illness has not been defined. Apoptosis can be detected by utilization of various methods, such as DNA ladder assay, cell death ELISA and in situ terminal deoxyribonucleotidyl (dUTP) transferase nick end labeling (TUNEL). The present study in rats, with

Kaneki, Toshiro Fujita, and J. A. Jeevendra Martyn.

Address for reprint requests and other correspondence: J. A. Jeevendra Martyn, Dept. of Anesthesia & Critical Care, Massachusetts General Hospital, 55 Fruit St., Boston, MA 02114 (E-mail: martyn@etherdome.mgh.harvard.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
the use of major thermal injury as a paradigm of critical illness with local and systemic inflammatory responses, examined whether apoptosis occurs in skeletal muscle at sites local to and distant from the burn. Some of the pathways causing apoptosis and the potential role of mitochondria, previously documented only in in vitro systems, were also examined. We demonstrated that apoptosis occurs in muscle at local and distant sites from burn and is associated with changes in mitochondrial membrane potential, release of cytochrome c from subsarcolemmal mitochondria to cytosol, and activation of the caspase pathway.

METHODS

Burn model and timing of experiments. The protocol for the studies was approved by the Institutional Animal Care Committee. Adult male Sprague-Dawley rats of 200 g body wt were purchased from Taconic Farms (Germantown, NY). The rats were anesthetized with pentobarbital sodium (50 mg/kg body wt) administered intraperitoneally. After clipping of the hair, a full thickness, third degree thermal injury of 40% of total body surface area was administered to the animals by immersing the back of the trunk for 15 s and the abdomen for 8 s in 80 °C water. This procedure, based on previous experience and histology, has been shown to cause direct heat damage only to the skin and not to deeper tissues (47). This model has been used extensively in burn research to induce metabolic changes (11, 23, 50). A weight- and time-matched sham-burn group (controls) was treated in the same manner as the trauma group, except that they were immersed in lukewarm water. The burned or sham-burned area was dressed with 1% silver sulfadiazine cream (Silvadene), and all of the animals were fluid resuscitated with 10 ml of normal saline and kept warm with a heat blanket until full recovery from anesthesia.

Animals were then euthanized, and muscle tissues were dissected out at the following time points: 15 and 30 min, 1, 2, 4, 6, and 12 h, and 1, 3, and 7 days after burn or sham injury. All biochemical experiments were performed in the rectus abdominis (mixed type) muscle immediately beneath the burn or in the limb muscles distant from the burn, most consistently the tibialis anterior (fast twitch). The nutritional status of the animals was checked daily by measuring body weight and food intake. The amount of food intake was decreased in both burn and sham-burn rats during the first 24 h, possibly because of the anesthetic. After that period, the food intake of both groups was similar, although weight gain was confirmed by addition of specific inhibitor Ac-DEVD-}

**DNA ladder and ELISA for apoptosis.** DNA ladder assay was performed according to the method previously described (34). In brief, 0.2 g tissue was minced in 5 ml of lysis buffer [Triton X-100 0.1%, Tris-HCl (pH 8.0) 5 mM, EGTA 20 mM, EDTA 20 mM] and homogenized with Polytron homogenizer (Brinkmann Instruments, Westbury, NY). DNA and protein concentration of this initial homogenate were measured. Protein concentration was adjusted, and polyethylene glycol 8000 and NaCl were added to a final concentration of 2.5% and 1 M, respectively. Samples were centrifuged at 16,000 × g for 10 min at 4 °C. Part of the sample was taken for ELISA. For ladder assay, DNA was extracted by the phenol-chloroform extraction method. DNA was harvested by ethanol precipitation, treated with RNase A, and electrophoresed on 1.2% agarose gel. Cell death ELISA analysis was done according to manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN) after appropriate dilution.

**In situ TUNEL analysis.** In situ TUNEL staining was done according to the manufacturer’s instructions (Oncor, Gaithersburg, MD). Briefly, frozen tissues were cut to 10-μm thickness with cryosection and were fixed in 4% paraformaldehyde for 10 min at room temperature. After being washed with PBS, specimens were postfixed in ethanolic-acetic acid (2:1) for 5 min at −20°C. Samples were then treated with terminal deoxynucleotide transferase. After the reaction was stopped, the fragmented DNA was visualized with fluoresceine-conjugated antibody. Nuclei were counterstained with 4,6-diamidino-2-phenylindole. Muscle tissue was counterstained with anti-caveolin-3 antibody, which is specific for muscle, and Texas Red-conjugated secondary antibody.

**Analysis of mitochondria.** All of the procedures related to mitochondria analysis were performed without freezing and were conducted at 4 °C. Muscle mitochondria were prepared as described previously (6). In brief, 1.0 g of the tissue was minced thoroughly in 10 ml of homogenizing buffer [sucrose 250 mM, HEPES-KOH (pH 7.5) 20 mM, KCl 10 mM, EDTA-Na, pH 8.0, 2.5 mM] and homogenized for 30 s with a Polytron homogenizer at 50% power. The homogenate was centrifuged at 800 g for 10 min to separate the subsarcolemmal mitochondria from myofibrils (myofibrillar pellet). The supernatant was filtered through cheesecloth, and the filtrate was centrifuged at 10,000 g to pellet down the mitochondria. This final pellet was washed once with homogenizing buffer.

**Mitochondrial membrane potential and Western blotting.** For measurement of mitochondrial membrane potential, freshly prepared mitochondria samples were subjected to fluorescence-activated cell sorting (FACS) analysis within 4 h after tissue collection. Mitochondria were stained with membrane potential-dependent dye as described (51). The dye, 3,3-dihexyloxacarboncyanide iodide (DiOC6), was obtained from Molecular Probes (Eugene, OR). DiOC6 is known to stain mitochondria in a potential-dependent fashion. FACS analyzed the intensity of staining, and the x-axis stands for the staining intensity, which corresponds to the membrane potential of the purified mitochondria. Western blotting was done according to a standard method to document changes in cytochrome c with time. The cytochrome c antibody for Western blotting was obtained from Pharmigen (San Diego, CA).

**Caspase assay.** Caspase-3 substrate, Asp-Glu-Val-Asp (DEVD)-7-amido-4-methylcoumarin (AMC), was purchased from Calbiochem (La Jolla, CA). Caspase-3 activity was examined by measurement of the rate of cleavage of fluorescence-conjugated substrate DEVD-AMC. After 200 μg of cytosolic fraction, prepared as described above, were placed onto a 96-well plate, DEVD-AMC was added in the final reaction volume of 150 μl in reaction buffer [HEPES (pH 7.4) 100 mM, glycerol 20%, phenylmethylsulfonyl fluoride 0.5 mM, aprotinin 5 μg/ml, leupeptin 10 μg/ml, pepstatin 5 μg/ml], and the concentration of substrate was adjusted to 50 μM. The mixture was incubated at 37 °C for 10 min in the dark. The activity was measured every 10 min up to 30 min with a fluoroplate reader (Molecular Devices) and divided by time. The specificity of the assay was confirmed by addition of specific inhibitor Ac-DEVD-
aldehyde (DEVD-CHO) in the reaction mixture at a concentration of 50 μM during the incubation.

Statistical analysis. Where appropriate, Student’s t-test was used for testing significance at $P < 0.05$. The data reported in these instances consisted of three muscle samples per group per time period.

RESULTS

Burn injury causes apoptosis in muscle beneath the site of injury. Burn injury resulted in degradation of DNA in rectus abdominis muscle, as demonstrated by DNA ladder pattern on gel electrophoresis (Fig. 1A), and by ELISA, which detected fragmented DNA (Fig. 1B). The degradation of DNA, as assessed by ladder formation, begins at ~4–6 h after burn and is prominent by 12 h to 7 days after burn injury. Evidence for apoptosis was also confirmed by morphological in situ end labeling (TUNEL). ELISA and TUNEL (Fig. 2) assays detected apoptosis as early as 4 h after burn injury. The number of apoptotic nuclei reached a maximum at days 3–7 and then decreased at days 14–28 (data not shown). In situ TUNEL analyses indicated that most of the apoptotic nuclei were derived from muscle cells (Fig. 2); that is, the apoptotic nuclei were contained within the area stained positive by caveolin-3, a specific marker for skeletal muscle protein.

![Fig. 1. Evidence of apoptosis by DNA ladder or ELISA assay in rectus abdominis muscle. A: DNA was extracted from abdominal muscles of rats with and without thermal injury by NaCl-polyethylene glycol extraction method. DNA was loaded on 1.2% agarose gel to detect ladder formation, and the gel was stained with ethidium bromide. Ladder formation was evident starting at 4–6 h and became prominent with time up to 7 days after burn (B). No changes were observed in the time-matched control sham rats (S). B: extracted DNA was diluted, and ELISA was used for detection of fragmented DNA. Data indicate spectrophotometric absorption units at 405-nm wavelength with reference to 490 nm. ELISA demonstrates significant increase of DNA fragmentation in muscles from burned animals compared with controls as early as 4 h that peaked at 3–7 days after burn. OD, optical density. * and **Significance at $P < 0.05$ and $P < 0.01$, respectively; $n = 3$ per group per time period.](http://ajpendo.physiology.org/doi/shortcut)
Fig. 2. In situ terminal deoxyribonucleotidyl transferase nick end labeling (TUNEL) assay of rectus abdominis muscle from burned animals. Nuclei are stained blue with 4',6-diamidino-2-phenylindole, and apoptotic nuclei are stained green with fluoresceine-conjugated antibody. Apoptosis was absent immediately after burn (0 min, A-D; 2 h, E-H). With increasing time after burn, the apoptosis, evidenced as green staining, appeared in muscle (4 h, I-L; 1 day, M-P) and was most prominent at 3 days after burn (Q-T). It was still detectable at day 7 (U-X). Anti-caveolin-3 staining (red) shows that muscle structure was unaltered at 0–2 h (A-H) but is gradually disorganized with increasing time until almost complete disorganization of structure at 3–7 days (Q-X). Green (C, G, K, O, S, W) and red images (A, E, I, M, Q, U) are superimposed (D, H, L, P, T, X) to show that most of the apoptotic nuclei are within muscle cells and not outside.
Thus the apoptosis observed was not due to infiltrates from outside the muscle. Mitochondria membrane potential depolarization precedes caspase activation. Mitochondria have been implicated to be involved in various types of apoptosis (30, 36, 40). Thus mitochondrial membrane potential, as a reflector of mitochondrial function, was examined next in the rectus abdominis muscle. Mitochondria were purified from nonfrozen samples in rectus abdominis muscle and stained with DiOC6. As shown in Fig. 3, mitochondrial membrane potential was disturbed within 1 h after burn.

Cytochrome c is released from mitochondria into cytosol after burn. Cytochrome c, also known as apoptotic protease activating factor-2 (Apaf-2), together with Apaf-1, caspase-9, and dATP/ATP has recently been reported to cause caspase-3 activation in vitro. The activation of the latter is the final common or distal effector pathway for many apoptotic processes. Normally, cytochrome c is located between inner and outer mitochondrial membranes and is released into the cytosol upon stimulation to bind Apaf-1 to activate caspase-3. The release of cytochrome c from mitochondria into cytosol was, therefore, examined at varying times in rectus abdominis muscle after burn. Within 15 min of administration of burn injury, concentrations of cytochrome c decreased in the mitochondria and increased in the cytosol (Fig. 4). In fact, the cytochrome c depletion in mitochondrial fraction seemed proportional to the accumulation in the cytosol (Fig. 4). Very low levels of cytochrome c were present in the cytosol at time 0 in both burn and sham burn, but this may have been due to the effect of homogenization of the tissue. In contrast to burned rats, time-matched sham-burn controls showed no change in cytochrome c content in cytosolic or mitochondrial fraction.

Caspase-3 activity is increased in rectus abdominis muscle after burn. Caspase-3 is recognized as the final step in the activation of apoptotic cascade. With the use of DEVD-AMC as substrate, caspase-3 was found to be activated in rectus abdominis muscle at 6 h and peaking at 3–7 days after burn (Fig. 5). The specificity of the activation was confirmed by the addition of specific inhibitor DEVD-CHO, which inhibited the caspase-3 activity. In the presence of DEVD-CHO, caspase-3 activity was completely inhibited, suggesting that the reaction was specific. Time-matched sham controls (sham-burns) did not show any change in caspase-3-like activity.

Burn injury also causes distant muscle apoptosis. We next examined whether burn injury induces apoptosis in muscles distant from burn. Thus muscles examined from lower limb were the tibialis anterior. DNA fragmentation was observed in the tibialis anterior (Fig. 6). Time-matched controls did not show any apoptotic change in the muscle studied. Statistical comparison between burn and sham groups showed significant differences in the numbers of apoptotic nuclei at days 1, 3, and 7. Other hindlimb muscles such as gastrocnemius and soleus also showed an increase in DNA fragmentation after burn (data not shown).
DISCUSSION

All forms of critical illness, including burns and sepsis, induce several protein degradation mechanisms. These include muscle protein breakdown for enhanced gluconeogenesis by the liver and myofibrillar degeneration after activation of the ubiquitin-proteosome pathway (5, 12, 31, 32, 37). These two pathways have been recognized as important factors contributing to muscle wasting in burns and other critical illnesses. Because of the muscle protein loss, critically ill patients are at high risk for neuromuscular complications that increase hospital stay and mortality (2, 3, 13). Relative to burns, especially when burn size exceeds 30% of total body surface area, a variety of functionally debilitating skeletal muscle changes occur. These functional deficits can be due to nerve- or muscle-related factors and can be observed at local and distant sites suggestive of local and systemic effects of burn (17, 39). Factors contributing to this neuromuscular dysfunction and muscle weakness have yet to be investigated in detail.

This study provides evidence for an additional mechanism for the loss of muscle mass after burn injury. In the present study, we show by ladder assay, which is evidence of apoptosis, the fragmentation of DNA in skeletal muscle due to apoptosis. Involvement of nuclei within the muscle was confirmed by TUNEL staining, which showed that muscle cell itself, and not infiltrating cells outside the muscle, underwent apoptosis. The quantitation of DNA fragmentation was performed by ELISA. All three independent methods were in good concordance, indicating the credibility of the assay for muscle apoptosis. The apoptosis in rat skeletal muscle was evident beginning at least at 4 h and continuing for up to 7 days after thermal injury to skin. Beginning at 3 days after burn injury, this apoptosis was quite pronounced in these muscles at sites both distant from and local to burn. These findings may therefore partly explain the functional deficits, including muscle weakness and muscle atrophy, observed in other studies during these periods after burn (44).

Apoptosis in skeletal muscle has been observed in neonatal and not adult muscle after injection of local anesthetic bupivacaine (14). Despite that fact, however, anesthetics did not play a role in apoptosis in our system, because the controls receiving the same anesthetic did not demonstrate this. The apoptosis, particularly within the first 24 h, was not due to decreased food intake, because the food intake was similar in both groups at this period. We attribute this weight loss and decreased food intake at 24 h in both burned and control rats to the residual effects of the anesthetic. Even in the later period, food intake was similar in burned and control animals. Thus inappetence is not the cause of apoptosis after burn. Despite the same or higher food intake, burned subjects lost weight relative to controls because of the hypercatabolic state (9–12, 50).

Our data suggest that burn injury causes apoptotic DNA fragmentation also at sites distant from burn. Consistent with this finding, there have been other reports of apoptosis after burn injury in various tissues, including cardiac muscle (28) and gut epithelium (48). These findings suggest that the effect of burn injury can be systemic. After burn injury, a systemic shock response with release of cytokines or hormones, including tumor necrosis factor-α, interleukin-1β, and...
interleukin-6, has been reported (4, 10). These endogenous substances may have played a role in the affected muscle. Alternatively, the lack of anabolic hormone levels or signaling from growth factors could have induced these changes (see below).

Whether this effect is specific to burn or to other accompanying factors, such as the inflammatory response, needs to be clarified in the future. For example, systemic apoptosis in parenchymal or nonparenchymal tissues has been observed after cecal ligation and puncture model of sepsis (20) and endotoxin injection (15), both conditions associated with loss of muscle mass (22). Another factor that may be involved in burn-induced muscle change is compromised cardiac function (21, 41). In chronic heart failure, both heart (33) and skeletal muscles (27, 46) show apoptotic changes. If cardiac function was compromised after burn injury due to systemic shock response, this could lead to cardiogenic shock-related apoptosis. Mortality in our study was very low (<3%). Thus this possibility also seems unlikely.

Recently, muscle fiber loss due to apoptosis has been observed after denervation or immobilization (1, 42). The apoptosis observed here was unlikely due to immobilization or burn-induced denervation, because these animals were freely mobile and were breathing spontaneously. However, a biochemical feature common to denervation, burns, sepsis, and other forms of critical illness associated with muscle wasting is insulin resistance or decreased anabolic effect in muscle in response to insulin (25, 32, 45). In a recent report (23), we have confirmed the insulin resistance after burns and have documented that the postreceptor insulin signaling mechanism was impaired. Of note was the observation that phosphatidylinositol 3-kinase (PI 3K) activation was impaired. PI 3K, via its activation of a further downstream molecule, Akt, is a key anti-apoptotic factor. It is conceivable, therefore, that the apoptosis observed in denervation, sepsis, or burns is related to impaired growth factor (insulin) signaling. Correction of this impaired signaling pathway may attenuate apoptosis and muscle wasting.

Mitochondria are now thought to be an important factor not only in energy transfer but also in the control of apoptosis. There are several pathways involving mitochondria that control apoptosis (35, 49, 51). Cytochrome c has been shown to switch on caspase-3 in the presence of dATP/ATP and caspase-9 and Apaf-1 in vitro systems. Changes in mitochondrial membrane potential are also reported to activate caspase-3 by releasing apoptosis-inducing factor, an pathway independent of the cytochrome c-Apaf-1 pathway. Although cytochrome c release and mitochondrial membrane potential change are both considered to be important in the initiation of caspase cascade leading to apoptosis, most of these studies thus far, as indicated above, have been on cell line systems or biochemically reconstituted systems. Our study also provides early evidence of mitochondrial dysfunction preceding muscle apoptosis in vivo. Within 15 min of administration of burn injury, cytochrome c is released from mitochondria into cytosol; within 1 h after burn, mitochondrial membrane potential is altered. Our present in vivo report thus confirms that these initiators of apoptosis may be clinically important. The time course of the changes observed suggests that cytochrome c release precedes mitochondrial membrane potential change, which is consistent with the other in vitro studies (26, 49).

Skeletal muscle wasting is a ubiquitous finding after major thermal injury. Enhanced gluconeogenesis with muscle protein beakdown and myofibrillar degeneration due to induction of the ubiquitin-proteosome pathway have been attributed to this loss of muscle mass. The finding of apoptosis in skeletal muscle after burns raises another fundamental question about the pathophysiology of this phenomenon. The process of apoptosis in a single cell runs its course in a short time, lasting only several hours; thus quantifying the contribution of apoptosis to muscle wasting has been difficult. However, once the upstream and downstream signaling pathways are identified, specific inhibitors could be applied to control at least the component of apoptosis-induced muscle wasting. Previous studies have shown that, after stress such as burn, sepsis, or ischemic shock, the tissue and/or circulating insulin-like growth factor I (IGF-I) levels are decreased (9, 16, 19, 24). Additionally, IGF-I works as a potent antiapoptotic factor in some types of cells and tissues; therefore, administration of IGF-I, for example, may be considered as one of the antiapoptotic therapies after burn injury.

We would like to thank Drs. T. Okamoto, C. Ibebunjo and L. Van de Water for critical advice and R. Khiroya and C. Mani for assistance.

This work was supported in part by R01 Grants, GM-31569–17, GM-55081–4, and GM-61411–01 (to J. A. Jeevendra Martyn) from the National Institute of General Medical Sciences.

REFERENCES


