Experimental Induction of Apoptosis and Necrosis in Neonatal Rat Skeletal Muscle

Anna M. Kamińska and Anna Fidziańska

Department of Neurology, Medical Academy, Warsaw, Poland and Neuromuscular Unit, Medical Research Center, Polish Academy of Sciences, Warsaw, Poland

Abstract

An electron microscopic study of neonatal muscle cell degeneration induced by different non-physiological agents such as bupivacaine, notexin and cold was performed. All those injurious stimuli caused apoptosis in younger rats and necrosis in rats older than 7 days of age. Muscle dying by the mechanism of apoptosis bore the same morphological characteristics as apoptotic cells in other tissues. The ability of neonatal muscle to undergo apoptosis or necrosis in response to injury is modulated by the stage of its maturity.

Key words: apoptosis, necrosis, immature rat skeletal muscle.

BAM 6 (4): 251-256, 1996

Apoptosis takes place during embryogenesis, in the course of normal tissue turnover, and after withdrawal of a trophic hormone from its target tissue [6, 9, 26, 28]. It can also be provoked in susceptible tissues by various pathological stimuli. It has been recently suggested [5] that "true" apoptosis or programmed cell death must be genetically mediated. Many morphological features of apoptosis, however, can be induced without engaging a genetic mechanism [1, 2, 4, 11, 21].

Recent observations imply that the mode of the cell death is modulated by the state of the cell development and differentiation [27, 29, 31]. Several genes associated with cell proliferation play a role in the control of apoptosis [12, 13, 18]. Histological and kinetic data [7] suggest an enhanced level of cell death at the early stages of tumor development.

Apoptosis in embryonic muscle has long been known [15, 32, 33]. As in many other tissues it is governed by the developmental program and is considered a process whereby excessive immature cells are eliminated.

Thus in the present experiments the skeletal muscles of neonatal and developing rats were chosen for study because they are immature and undifferentiated at birth. During the brief period of postnatal development, they reach their morphologic and physiologic maturity.

Our previous results [16] indicate that myotoxic substance Bupivacaine (BPVC) is able to induce apoptosis in immature rat skeletal muscle and suggest that the effect of BPVC on muscle cell is influenced by the stage of muscle maturity. Thus, the main purpose of this study was to investigate the age - related factors influencing the mode of muscle cell death - either via apoptosis or necrosis.

Additionally we decided to compare the ability of divergent noxious stimuli such as BPVC, notexin and cold to induce cell death in immature rat skeletal muscle.

Material and Methods

A total of 48 rats at 1, 3, 5 and 7 days of age were used in this study. The following experimental procedures were performed:

a) injection of BPVC into calf muscles (0.75 % in Saline)
b) injection of notexin into calf muscles (10 µg/ml solution)
c) applying a dry ice on the surface of exposed calf muscles.

The rats were operated in hypothermia. Rats 1- and 3-day-old were injected with 0.02 ml and rats 5- and 7-day-old with 0.04 ml of the solution. Each group consisted of 4 rats. Left, unlesioned leg, served as a control. Muscles were removed at 24 hours after injury and prepared for electron microscopy (EM).

In brief, the muscle was fixed in 6% glutaraldehyde in 0.1 µ phosphate buffer at pH 7.4, postfixed in 1% osmium tetroxide in the same buffer, dehydrated and embedded in Spurr resin. Semithin sections were stained with toluidine blue for light microscopy. Ultrathin sections double stained with uranyl acetate and lead citrate were examined on a JEM 1200 EX2 Electron Microscope.

Results

One-day-old rats

In control muscle light microscopy revealed muscle fibers at different stages of development, loosely arranged. In electron microscopy muscle cells were classified into several groups:

1) presumptive myoblasts - numerous cells without myofibrils, with hyperchromatic nucleus, abundant ribosomes, scant mitochondria and well developed Golgi apparatus;
2) myotubes - elongated multinucleated cells with centrally placed nucleus surrounded by sarcoplasm. Early myotubes had small bundles of myofilaments whereas more mature myotubes had well developed myofibrils with a hexagonal array in the A band in cross section. The amount of myotubes was 47% of all cells with myofibrils;
3) immature muscle fibers - cluster of several cells at different stage maturity under a common basal lamina. These clusters constituted 49% of all cells with myofibrils;
4) mature muscle fibers with nuclei located at the periphery and with sarcoplasm tightly filled with myofibrils. The quantity of such cells was only 4% of all cells with myofilaments.

Twenty four hours after injury degenerating cells bearing characteristics of apoptotic cells were observed in all three experimental groups (after BPVC and notexin injection and application of cold). Such cells scattered throughout the muscle tissue were markedly contracted and stained darkly with toluidine blue in light microscopy. EM revealed a highly characteristic morphology of those cells with aggregation and margination of nuclear chromatin (Fig. 1), condensation of sarcoplasm with maintenance of cell integrity (Fig. 2) and formation of surface protuberances resulting in separation of membrane-bound fragments. Such fragments were eventually ingested by neighbouring muscle cells (Fig. 3). Cells at different stage of apoptosis coexisted in tissue sections.

Three-day-old rats

In control muscle the number of myotubes decreased. Immature muscle fibers were very often encountered. Similarly to the first group, degenerating cells were observed after three experimental procedures. Beginning stages of apoptosis showing irregular surface blebbing resulting in separation of membrane - bound fragments were seen in longitudinal sections. Apoptotic bodies containing remants of cell organelles such as nuclei, myofibrils and mitochondria were often encountered. Such bodies were located freely in the tissue or were ingested by neighbouring immature muscle cells (Fig. 4, 5) and by macrophages (Fig. 6). No necrotic muscle cells were seen in this experimental group.

Five-day-old rats

Control muscle contained 8% of myotubes, 28% immature muscle fibers and 64% of mature muscle fibers. In the muscles of experimental animals treated with BPVC, notexin and cold fewer apoptotic cells were found. Late apoptotic bodies presented as a dense, round structures containing very often unidentified cellular remnants were observed. In the cell clusters consisted of muscle cells at different stage of development, apoptosis was usually observed in the youngest cell of muscle cell cluster (Fig. 7). Interestingly enough, necrotic and apoptotic cells lying in the close proximity were also observed (Fig. 8). Necrotic muscle cells were pale, swollen, characterized by a rup-

Figure 1. One-day-old rat. 24 hours after Notexin injection. Early nuclear changes with condensation of chromatin at the periphery observed in undifferentiated cell, probably presumptive myoblast. EM x 10 000.

Figure 2. One-day-old rat. 24 hours after Marcaine injection. Apoptotic cell with condensed cytoplasm containing myofibrils. EM x 7000.

Figure 3. One-day-old rat. 24 hours after Marcaine injection. Early apoptotic body with well preserved myofibrils within immature muscle cell. EM x 10 000.

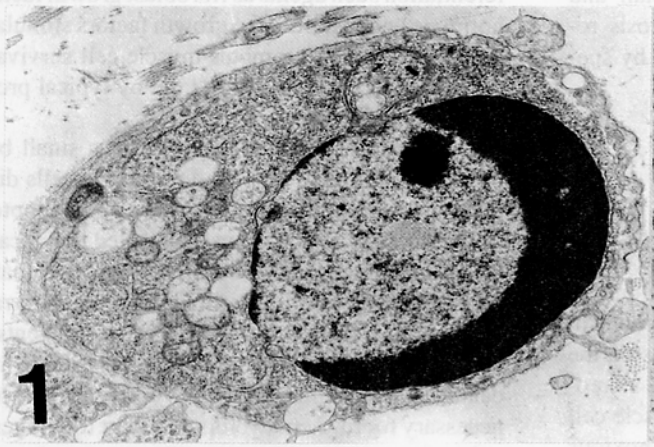
Figure 4. Three-day-old rat. 24 hours after Notexin injection. Apoptotic bodies at different stages - early nuclear and late cytoplasmic - engulfed by immature muscle cells. EM x 7000.

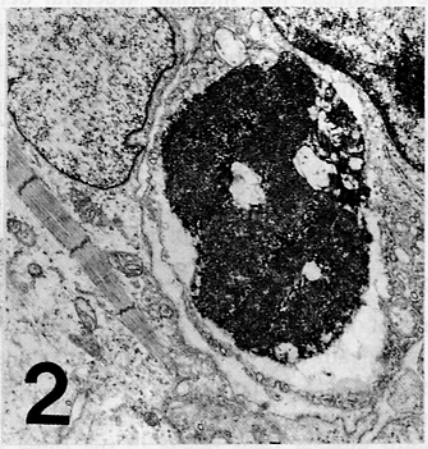
Figure 5. Three-day-old rat. 24 hours after Marcaine injection. Two late apoptotic bodies within immature muscle cell. EM x 5600.

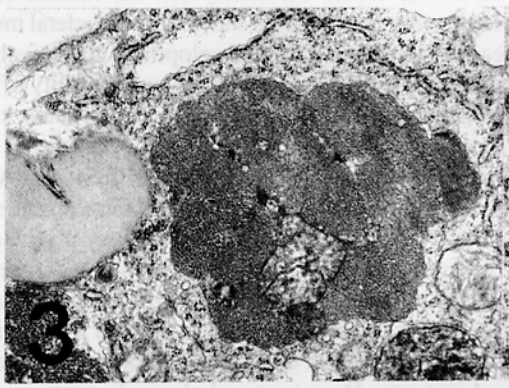
Figure 6. Three-day-old rat. 24 hours after cold injury. Apoptotic bodies containing fragments of nucleus phagocyted by macrophage. EM x 12 000.

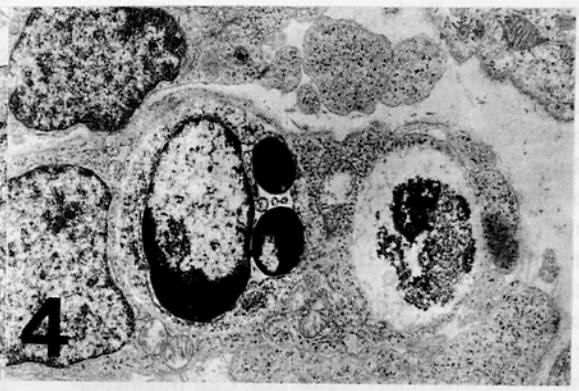
Figure 7. Five-day-old rat. 24 hours after Notexin injection. Cluster of muscle cells at different stage of maturation under common basement membrane with one dark cell undergoing apoptosis. EM x 2800.

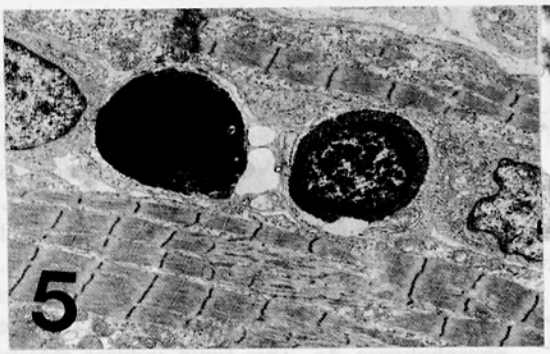
Figure 8. Five-day-old rat. 24 hours after Marcaine injection. Apoptotic (dark, shrunken) and necrotic (pole, swollen) cells lying in the close proximity. EM x 11 000.

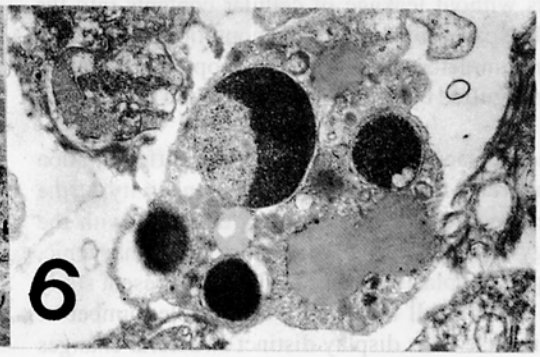

1

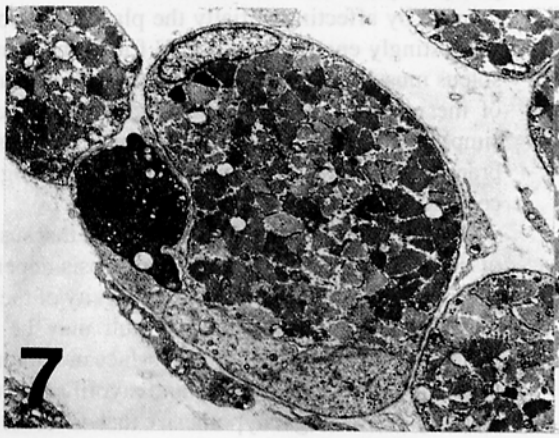

2

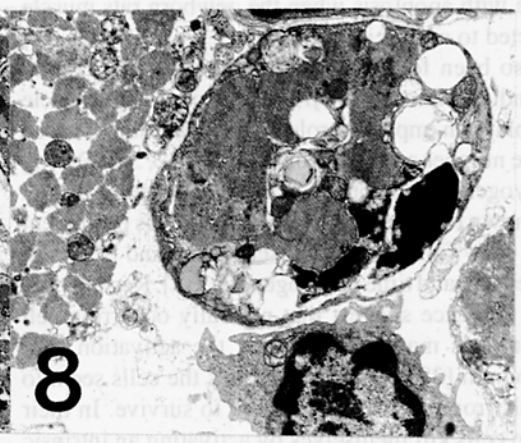

3


4


5


6


7


8

tured nuclear and plasma membranes. Their mitochondria contained often flocculent or granular matrix densities. Contractile apparatus lost internal structural detail and appeared homogenized. Progression of the necrosis resulted in ingestion and degradation of cell debris by specialized phagocytic cells.

Seven-day-old rats

Numerous muscle cells bearing characteristics of necrosis were encountered in all three experimental groups. Apoptotic cells were not observed in 7-day old rats.

Discussion

The results of our experiments show that the mechanism of muscle cell death in response to injury depends on the state of cell maturity. It seems that apoptosis is a stereotyped cellular response to injury of immature muscle cell whereas adult muscle fiber reacts to the same noxious stimuli with necrosis. Both apoptosis and necrosis forms of cell death are clearly defined by morphological criteria. In the absence of a definitive biochemical or molecular marker the electron-microscopic examination of the tissue remains the best way of confirming apoptosis. The major distinguishing feature of apoptosis is the highly characteristic morphology. The first stage of apoptosis is condensation of nuclear chromatin and the breakdown of cell - cell interaction. This is followed by cell shrinkage, condensation of cytoplasm and budding away of membrane - bound bodies. Such apoptotic bodies contain nuclear fragments consisting largely of condensed chromatin as well as cytoplasmic fragments with intact organelles. Cell fragmentation occurs without leakage of cellular contents into the extracellular space, and removal of apoptotic cells does not cause an inflammatory response. The apoptotic bodies are eventually engulfed by neighbouring cells and nearby phagocytes.

It is generally accepted that the cellular differentiation state is important factor modifying the sensitivity of the cell to the specific inducing factor. Cell death with the morphology of apoptosis occurs mostly in embryonic, developing or neoplastic tissues [34]. In the present study we found that a small but readily observable number of immature muscle cells display distinct structural changes compatible with apoptosis when the newborn rats muscle was subjected to a local injury.

It has also been found in metamorphosis [24, 25] and hormone induced atrophy [14]. In embryonic stage muscle apoptosis plays an important role in the regulation of tissue size and the number and shape of the cells [32, 33]. During normal myogenesis apoptosis occurs spontaneously and has been shown to be under genetic control. It is prominent during the early formation of muscle tissue and occurs in predictable form and time in myogenesis [15]. Recently the increasing evidence suggest that naturally occurring cell death in animals may be caused by the activation of a suicide program [28]. In higher animals the cells seem to need signals from other cells in order to survive. In their absence the cells kill themselves by activating an intrinsic suicide program. Muscle maturation requires specific trophic agents which promote survival and muscle cell differentiation such agents as fibroblast growth factor, growth hormone and insulin - like growth factors stimulate muscle tissue growth and promotes muscle cell survival. Muscle cells deprived of such signals die by typical programmed cell death [10].

In the present study we found that a small but readily observable number of immature muscle cells display distinct structural changes compatible with apoptosis when the newborn rats muscle was subjected to a local injury.

This may represents the effect of direct pathological effect of those injurious stimuli on immature rat skeletal muscle. It is also conceivable that toxic agents blocking enzymatic function of trophic factors promote muscle cell death. Immature muscle cells deprived a trophic factors necessary for further development start to synthesize molecules that would ultimately kill the muscle cells themselves. Our experiments show that rat skeletal muscle in its early period of postnatal development is relatively resistant to injury. Little is known about susceptibility of immature cells to necrosis. Chizzonite and Zak [8] have shown that the sensitivity of the cardiac myocytes to the calcium - induced cellular disruption changed from relative insensitivity before 6 days of age to a slight but constant sensitivity from 7 to 11 days of age, and to full sensitivity at 15 days of age. Similar resistance to necrosis of the neonatal rat muscle has been observed by us in this experiment. Rat skeletal muscle is immature at birth [17, 23] and in short period of postnatal development reaches full morphological maturity. Divergent pathological agents used by us in this experiment induced in neonatal muscle apoptosis, whereas later the same agents induced necrosis. Although mechanism of the myotoxic action of BPVC is not fully understood, it is widely used in the studies of experimental regeneration [3, 22]. Direct damage of the cell membrane and binding of the BPVC to the mitochondria is probably responsible for the cell necrosis [3]. Schulz and Lipton [30] noted that in muscle culture BPVC affected preferentially and irreversibly multinucleated muscle cells, whereas myoblasts and fibroblast were relatively resistant to BPVC. For the myotoxic action of notexin is responsible phospholipase A_2. This toxin induces massive muscle damage by affecting initially the plasma membrane [19]. Interestingly enough Harris [20] found that immature rat soleus muscle was insensitive to notexin. The mechanism of the noxious influence of the cold on muscle can be simply explained by a physical damage of plasma membrane and subsequent cascade of events leading to muscle cell necrosis.

The results presented here demonstrate that susceptibility of muscle fiber to apoptosis and necrosis depends on the state of cell maturation. Lower sensitivity of the immature muscle cells to pathological stimuli may be related to incomplete development of the surface membrane system, of the sarcoplasmic reticulum and myofibrils in the neonatal muscle. One might hypothesize that different protective

proteins such as trophic growth factors active in neonatal period may also prevent cellular damage induced by local injury.

Address correspondence to:

A.M. Kamińska, Department of Neurology, Medical Academy, ul. Banacha 1A, Warsaw 02-097, Poland, fax 4822 6688512.

References

[1] Barry MA, Eastman A: Endonuclease activation during apoptosis: The role of cytosolic Ca^{++} and pH. *Biochem Biophys Res Commun* 1992; 186: 782-789.

[2] Barry MA, Eastman A: Identification of deoxiribonuclease II as an endonuclease involved in apoptosis. *Arch Biochem Biophys* 1993; 300: 440-450.

[3] Benoit P, Belt WD: Some effects of local anaesthetic agent on skeletal muscle. *Exp Neurol* 1972; 34: 264-278.

[4] Bino GD, Lassota P, Darżynkiewicz Z: The S-phase cytotoxicity of Camptothecin. *Exp Cell Res* 1991; 193: 27-35.

[5] Bowen ID: Apoptosis or programmed cell death? *Cell Biol Int* 1993; 17: 365-380.

[6] Bowen ID, Morgan SM, Mullarkey K: Cell death in the salivery glands of metamorphosing Calliphora Vomitoria. *Cell Biol Int* 1993; 17: 13-33.

[7] Britton SL, Bowen ID: The effects of isotretinoin on the murine sarcoma S180. *Proc Roy Microsc Soc* 1987; 22: AH3.

[8] Chizzonite RA, Zak R: Calcium-induced cell death: susceptibility of cardiac myocytes is age-dependent science. 1981; 213: 1508-1510.

[9] Clarke PGH: Developmental cell death: morphological diversity and multiple mechanism. *Anat Embryol* 1990; 181: 195-213.

[10] Comella JX, Sanz-Rodrigez C, Aldea M, Esquerda JE: Skeletal muscle-derived trophic factors prevent motoneurons from entering an active cell death program in vitro. *J Neurosc* 1994; 14: 2674-2688.

[11] Cohen JJ, Duke RC, Chervenak R, Sellins KS, Olson LR: DNA fragmentation in targets of CT_b: an example of programmed cell death in the immune system. *Adv Exp Med Biol* 1985; 184: 493-508.

[12] Debbas M, White E: Wild type p 53 mediated apoptosis by T-A which is inhibited by E 1B. *Genes Dev* 1993; 7: 546-554.

[13] Evan GI, Wyllie AH, Gilbert CS: Induction of apoptosis in fibroblast by c-myc protein. *Cell* 1992; 63: 119-128.

[14] Ferguson DJP, Anderson TJ: Ultrastructural observations on cell death by apoptosis in the "resting" human breast. *Virchows Arch (Pathol Anat)* 1981; 393: 193-203.

[15] Fidziańska A, Goebel HH: Human ontogenesis III. Cell death in fetal muscle. *Acta Neuropath (Berlin)* 1991; 81: 572-

[16] Fidziańska A, Kamińska A: Apoptosis: a basic pathological reaction of injured neonatal muscle. *Pediatr Pathol* 1991; 11: 421-429.

[17] Finowicka J, Kamińska A: Effect of denervation on muscle maturation in rats. *Neuropat Pol* 1988; 26: 283-301.

[18] Freeman RS, Estus S, Johnson Jr EM: Analysis of cell cycle - related gene expression in postmitotic neurons: selective induction of cyclin D1 during cell death. *Neuron* 1994; 12: 343-345.

[19] Gutierrez JM, Ownby CL, Odell GV: Pathogenesis of myonecrosis induced by crude venom and a myotoxin of Bothrops asper. *Exp Mol Pathol* 1984; 40: 367-379.

[20] Harris JB: Notexin: Its action on skeletal muscle, in Sellin and Thesleff (eds): *Neuromuscular Junction.* Elsevier Science Publishers 1989, pp 467-479.

[21] Hotz MA, Traganos F, Darzynkiewicz Z: Changes in nuclear chromatin related to apoptosis or necrosis induced by the DNA topoisomerase II inhibitor fostrectin in MOLT-4 and HL-60 cells are revealed by altered DNA sensitivity to denaturation. *Exp Cell Res* 1992; 201: 184-191.

[22] Jirmanova I, Thesleff S: Ultrastructural study of experimental muscle degeneration and regeneration in the adult rat. *Z Zellforsch Mikrosk Anat* 1972; 131: 77-97.

[23] Kamińska A: Effect of denervation on muscle development in the rat. Doctoral Thesis (in Polish) Warsaw Medical Academy, 1979

[24] Kerr JFR, Harmon B, Searle J: An electron microscope study of cell deletion in the anuran tadpoke tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibres. *J Cell Sci* 1974; 14: 571-585

[25] Lockshin RA: Cell death in metamorphosis, in Bowen LD, Lockshin RA (eds): *Cell Death in Biology and Pathology.* London, Chapman and Hall 1981, 79-109.

[26] Lockshin RA, Zakeri Z: Programmed cell death and apoptosis, in Tomei DL, Cope FO (eds): *Apoptosis: The molecular basis of cell death.* Current Communications in Cell and Molecular Biology Vol. 3. Cold Spring Harbor Laboratory Press, New York, pp 47-60, 1991.

[27] Mc Conkey DJ, Hartzell P, Duddy SK, Hakansson H, Orrenius S: 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin Kills immature thymocytes by Ca^{2+}-mediated

endonuclease activation. *Science* 1988; 242: 256-259.

[28] Raff CR: Social controls on cell survival and cell death. *Nature* 1992; 356: 397-399.

[29] Rubin LL, Philpott KL, Brooks SF: The cell cycle and cell death. *Curr Biol* 1993; 3: 391-394.

[30] Schultz E, Lipton BH: The effect of marcaine on muscle and non-muscle cells in vitro. *Anat Rec* 1978; 191: 351-370.

[31] Sorenson CM, Barry MA, Eastman A: Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. *J Natl Concer Inst* 1990; 82: 749-755.

[32] Webb JN: Cell death in developing skeletal muscle: histochemistry and ultrastructure. *J Pathol* 1977; 123: 175-180.

[33] Webb JN: The development of human skeletal muscle with particular reference to muscle cell death. *J Pathol* 1972; 106: 221-228.

[34] Wyllie AH, Kerr JFR, Currie AR: Cell death: the significance of apoptosis, in Bourne GH, Danielli JF (eds): *Int Rev Cytol.* Academic Press, 1980, vol 68, pp 251-306.