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Cell death induced by gamma irradiation of developing skeletal muscle

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ABSTRACT

Newborn Sprague–Dawley rats were exposed to a single dose of 2 Gy gamma rays and killed from 6 h to 5 d later. Increased numbers of dying cells, characterised by their extreme chromatin condensation and often nuclear fragmentation were seen in skeletal muscle 6 h after irradiation. Dying cells decreased to nearly normal values 48 h later. In situ labelling of nuclear DNA fragmentation identified individual cells bearing fragmented DNA. The effects of gamma rays were suppressed following cycloheximide i.p. at a dose of 1 μg/g body weight given at the time of irradiation. Taken together, the present morphological and pharmacological results suggest that gamma ray induced cell death in skeletal muscle is apoptotic, and that the process is associated with protein synthesis. Finally, proliferating cell nuclear antigen-immunoreactive cells, which were abundant in control rats, decreased in number 48 h after irradiation. However, a marked increase significantly above normal age values was observed at the 5th day, thus suggesting that regeneration occurs following irradiation-induced cell death in developing muscle.

Key words: Rat; gamma irradiation; striated muscle; apoptosis; DNA fragmentation; proliferating cell nuclear antigen.

INTRODUCTION

Adult skeletal muscle is considered resistant to ionising radiation unless massive doses of radiation are applied, a fact which is attributed to the low numbers of radiosensitive proliferating cells in adulthood (Lewis, 1954; Khan, 1974; Wirtz et al. 1982; Gulati, 1987; Wakeford et al. 1991). Although developing tissues are highly sensitive to ionising radiation, little is known about radiation effects on developing muscle. In the present study, newborn rats were subjected to a single dose of gamma rays to investigate the vulnerability of developing skeletal muscle to ionising radiation. With this purpose, in addition to routine histological and electron-microscopical examination of dying cells, in situ labelling of nuclear DNA fragmentation, which recognises dying cells after the incorporation of biotin-16-2′-deoxyuridine-5′-triphosphate (biotin-16-dUTP) at sites of DNA breaks by means of a terminal deoxynucleotidyl transferase (TdT) (Gavrieli et al. 1992), was applied. A few animals were treated with cycloheximide at the time of irradiation to investigate the effects of this protein synthesis inhibitor on the process of gamma ray induced cell death. Finally, a monoclonal antibody against proliferating cell nuclear antigen (PCNA), which marks proliferating cells in the late G1 and S phases of the cell cycle (Mathews et al. 1984; Celis et al. 1987; Ogata et al. 1987; Kurki et al. 1988), was used to assess muscle regeneration following irradiation.

MATERIALS AND METHODS

Animals

Newborn Sprague–Dawley rats (n = 15) of both sexes were irradiated with a single dose of 2 Gy gamma rays delivered by a cobalt beam (Theratron 780) with a dose rate of 12 cGy/min from 2 opposite fields. The rats were killed 6 (n = 6), 24 (n = 2), 48 (n = 2) h, and 5 d (n = 2) after irradiation. Another group of animals

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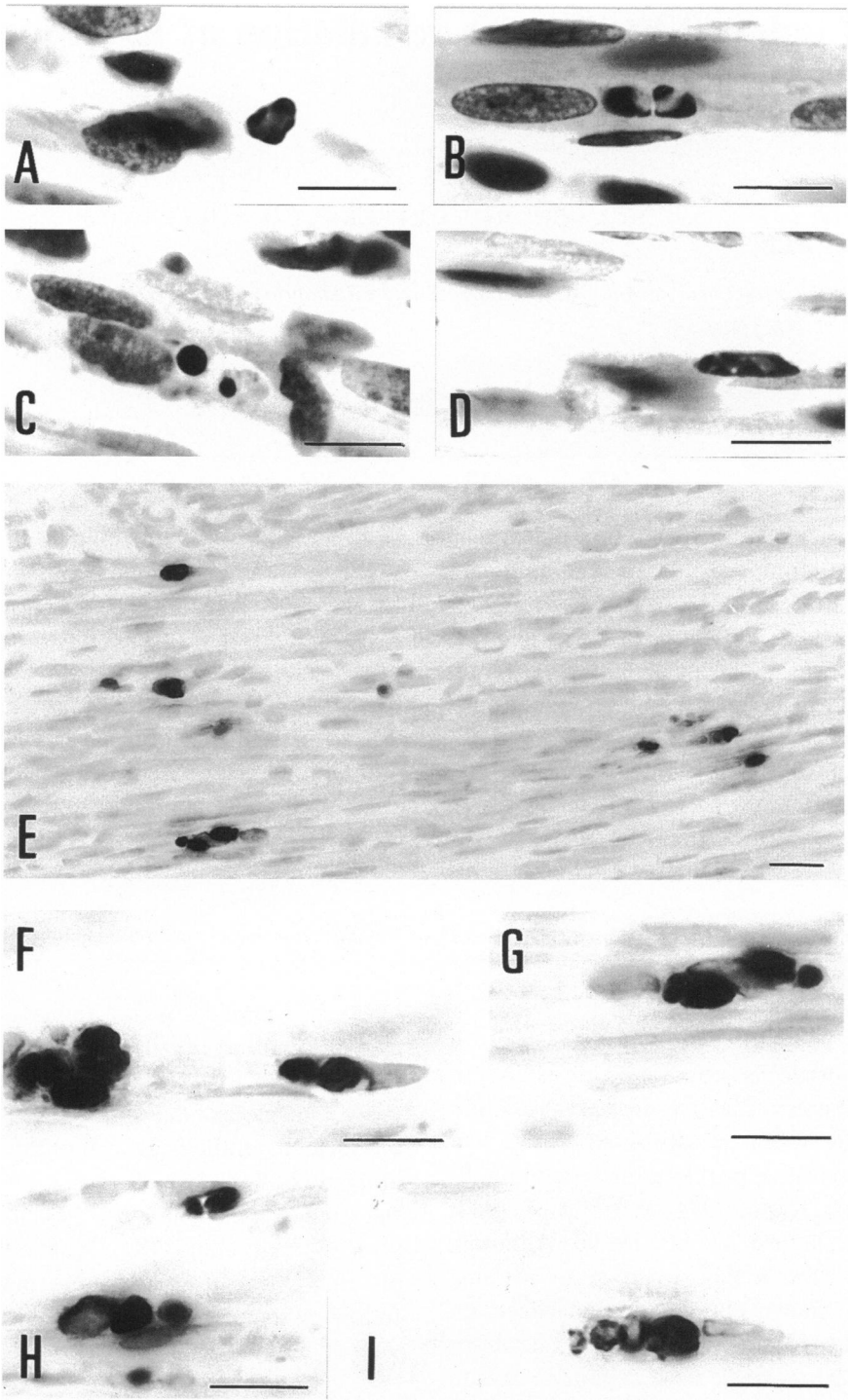

Fig. 1. Dying cells in normal muscle (*A*) and 6 h after irradiation (*B–D*) as revealed in paraffin sections stained with haematoxylin and eosin. The method of in situ labelling of nuclear DNA fragmentation recognises individual cells bearing fragmented DNA in irradiated (*E–H*) and normal age-matched controls (*I*) *A–D* and *F–H*: bar, 10 μm; E: bar, 25 μm.

(n = 3) received cycloheximide i.p. (Actidione; ICN Biochemicals) at a dose of 1 μg/g body weight dissolved in saline, immediately after irradiation. The rats were killed 6 h later. Age-matched rats (n = 3, for each age) were used as controls. Animal welfare was observed according to the regulations of the Real Decreto 223 (1988), which shares similar recommendations with the NYH guide for the care and use of laboratory animals.

Methods

The animals were killed with an overdose of diethyl ether and were perfused through the heart with saline

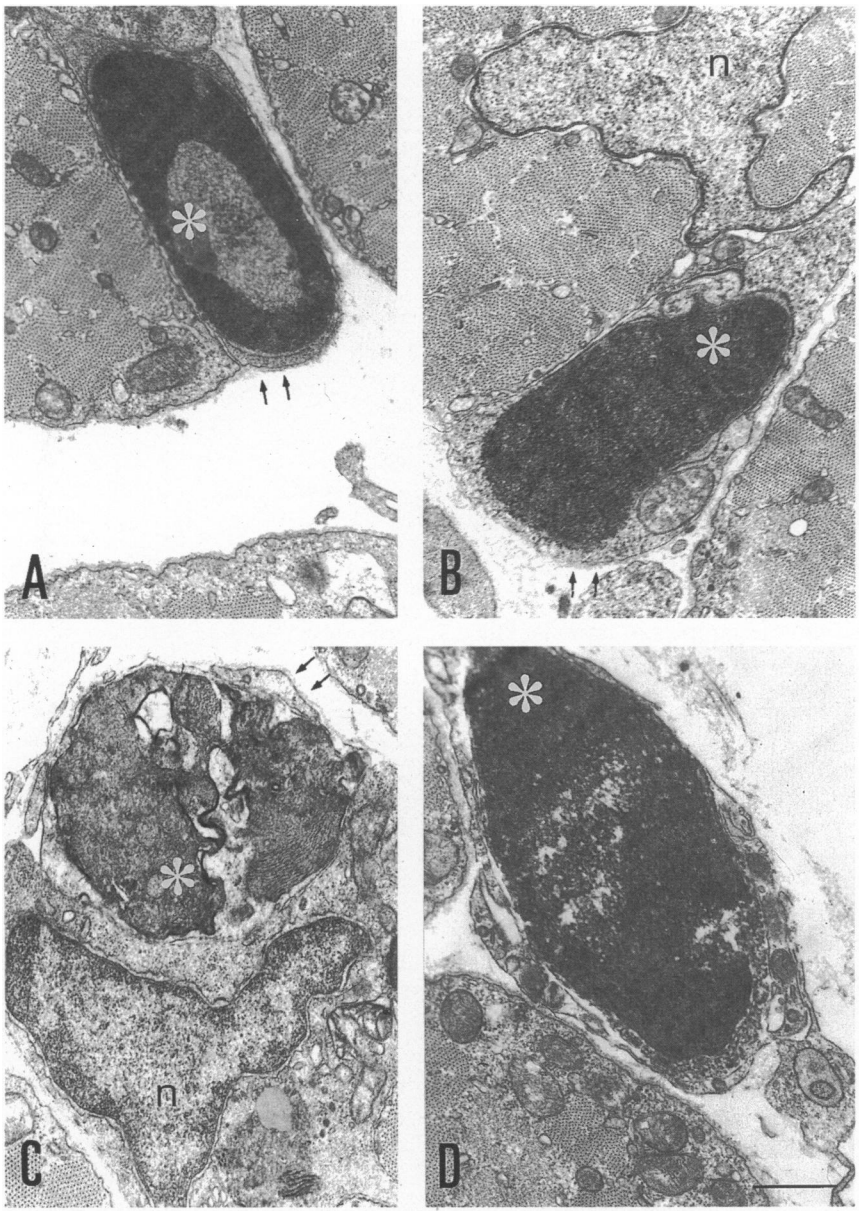

Fig. 2, *A–D*. Electron microscopy of dying cells 6 h after gamma ray irradiation. Note the extreme condensation of the chromatin (asterisk) when compared with normal nuclei (n). The basement membrane around dying cells is marked by arrows. Bar, 1 μm.

followed by 4% paraformaldehyde in phosphate buffer. Immediately afterwards, the 4 limbs of each animal were removed and fixed with 4% paraformaldehyde at 4 °C for 24 h. The samples were embedded in paraffin wax. Longitudinal sections were obtained with a sliding microtome and stained with haematoxylin and eosin, or else processed for proliferating cell nuclear antigen (PCNA) immunocytochemistry or for in situ nuclear DNA fragmentation study. PCNA immunocytochemistry was carried out following the avidin–biotin–peroxidase procedure (ABC, Vectastain, Vector Labs). Briefly, after blocking endogenous peroxidase with 0.3% hydrogen peroxide and 10% methanol for 15 min, the sections were treated with 3% normal horse serum for 2 h, and then incubated overnight at 4 °C with a monoclonal antibody against PCNA (Ab-1; Oncogene Science) used at a dilution of 1:500, followed by biotinylated horse antimouse IgG (Vector Labs) at a dilution of 1:150 for 1 h, and finally with the ABC complex at a dilution of 1:100 for 1 h. The peroxidase reaction was visualised with 0.05% diaminobenzidine and 0.01% hydrogen peroxide.

In situ labelling of nuclear DNA fragmentation was carried out following the protocol used in the original description (Gavrieli et al. 1992) with the following modifications. Dewaxed sections were incubated with 20 μg/ml proteinase K for 15 min at room temperature, washed in distilled water, and treated with 2% H_2O_2 for 5 min. Immediately afterwards, the

sections were washed with distilled water and immersed in TdT (terminal deoxynucleotidyl transferase) buffer (25 mM trizma base at pH 6.6, 200 mM cacodylic acid, and 200 mM potassium chloride) for 15 min at room temperature. The sections were then incubated with 12 µl of 25×10^3 units/ml TdT, 36 µl of 1 mM biotin-16-dUTP in 1000 µl of TdT buffer at 37 °C for 60 min, and finally with a buffer solution composed of 0.3 M sodium chloride and 0.03 M sodium citrate for 15 min. The sections were rinsed in distilled water, covered with a 2% aqueous solution of bovine serum albumin for 10 min, rinsed in distilled water, and immersed in phosphate buffered saline (PBS) for 5 min. Finally, the sections were incubated with the ABC complex at 37 °C for 30 min at a dilution of 1:25 in distilled water. Peroxidase reaction was visualised with diaminobenzidine. Biotin-16-dUTP and TdT were obtained from Boehringer Mannheim.

In addition, a few muscle samples of animals killed 6 h after irradiation were used for ultrastructural examination. After 2% glutaraldehyde fixation for 24 h, small blocks were postfixed with 1% osmium tetroxide for 1.5 h, dehydrated in ethanol and propylene oxide, and embedded in Araldite. Selected ultrathin sections were stained with uranyl acetate and lead citrate.

Quantitative analyses were performed on longitudinal paraffin sections directly through the ocular of the microscope at a magnification of ×400. The following counts were made: dying cells in haematoxylin and eosin-stained sections, dying cells identified by in situ labelling of nuclear DNA fragmentation, and PCNA-immunoreactive cells. Counts were made in irradiated, irradiated plus cycloheximide, and control animals at different ages. Results were expressed as the mean values ± S.D. per 1 mm² obtained from 5 sections for each animal. The results were processed statistically (2-way and 1-way ANOVA and post hoc test) using the Statigraphic statistical package.

RESULTS

Gamma ray induced cell death in developing skeletal muscle

Dying cells, recognised by their extremely dark and often fragmented nuclei, appeared to be distributed at random or in small clusters (Fig. 1 *A–D*). On ultrastructural examination, most dying cells were myoblasts and satellite cells (Fig. 2). Biotin-16-dUTP labelling was observed in dying cells which appeared clearly distinguished from healthy cells by their extremely condensed chromatin or fragmented nuclei (Fig. 1 *E–I*). The number of dying cells revealed by biotin-16-dUTP was greater than observed in haematoxylin and eosin sections (ANOVA test; $P = 0.0001$). Although a small number of dying cells was seen in control animals, the number of dying cells was markedly increased in irradiated animals, with peak values obtained 6 h after irradiation. The number of dying cells decreased 48 h later. The number of dying cells in irradiated animals was similar to that found in age-matched controls by d 5 after irradiation (see Table). The number was drastically reduced following cycloheximide (1 µg/g body weight i.p. immediately after irradiation) (LSD test; $P = 0.01$). The number of dying cells in these animals was similar to that found in control rats (Table).

Table. *Number of dying cells as revealed by haematoxylin and eosin staining (H+E) and in situ labelling of nuclear DNA fragmentation (DNA), and number of PCNA-immunoreactive cells (PCNA) in skeletal muscle of newborn irradiated rats killed at different intervals after irradiation (6 h, 48 h, 5 d), irradiated animals treated with cycloheximide and killed 6 h after irradiation, and age-matched controls*

	Control	2 Gy	2 Gy + Cycloheximide
H+E			
Newborn	6.8±7.8	—	—
6 h	—	87.0±42.6*	7.8±8.6**
48 h	3.1±4.3	7.6±5.2	—
5 d	1.1±3.0	1.2±3.1	—
DNA			
Newborn	15.6±7.2	—	—
6 h	—	181.0±12.9*	8.7±7.5**
48 h	7.4±7.5	13.0±6.1	—
5 d	2.6±4.2	3.4±4.4	—
PCNA			
Newborn	146.2±27.4	—	—
6 h	—	149.7±22.5	—
48 h	181.1±29.7	30.4±9.3*	—
5 d	132.3±19.1	371.2±47.9*	—

Results are expressed as mean values ± S.D. in an area of 1 mm².
* Statistical difference between controls and irradiated animals, LSD test, $P = 0.01$.
** Statistical difference between animals irradiated alone, and animals irradiated and treated with cycloheximide, LSD test, $P = 0.01$.
The number of dying cells as revealed by in situ labelling of nuclear DNA fragmentation is larger than observed with haematoxylin and eosin staining. ANOVA test, $P = 0.0001$.

PCNA-immunoreactive cells were present in control animals and in rats irradiated and killed 6 h later (Fig. 3). The number of PCNA-immunoreactive cells was markedly decreased in irradiated animals killed 48 h later (LSD test; $P = 0.01$). However, an increase in the number of PCNA-immunoreactive cells when compared with age-matched controls was observed in

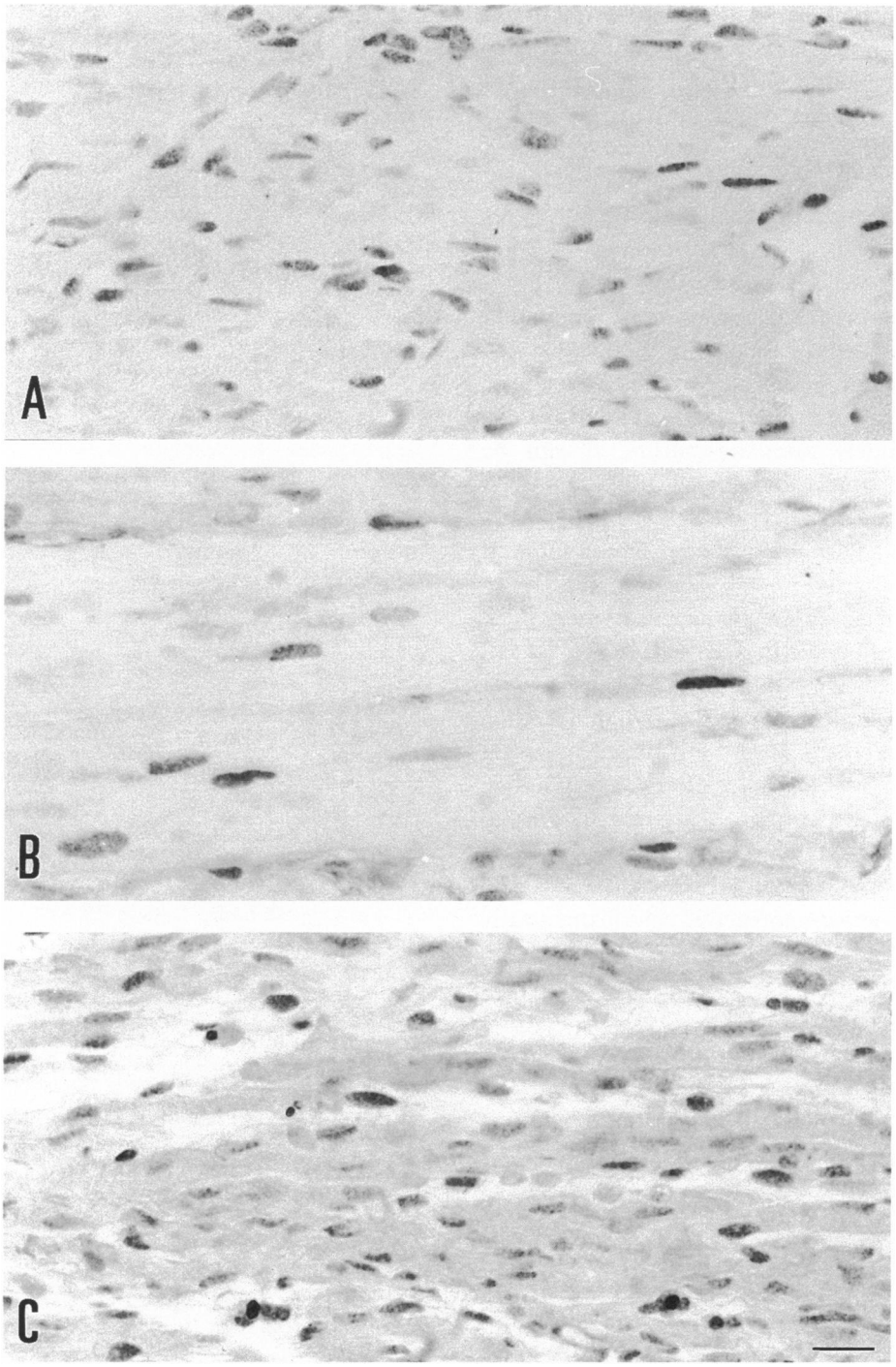

Fig. 3. Proliferating cell nuclear antigen-immunoreactive cells in skeletal muscle of newborn rats (*A*), rats 6 h (*B*) and 5 d (*C*) after irradiation. Bar, 25 μm.

irradiated animals 5 d after irradiation (LSD test; $P = 0.01$) (Table).

DISCUSSION

Dying cells are seen in skeletal muscle of neonatal rats, a feature which is in accordance with similar observations in developing human skeletal muscle (Webb, 1972; 1977; Fidzianska & Goebel, 1991). In fact, the maturation of the skeletal muscle in the neonatal rats is similar to that observed in human embryos between the 15th and 20th wk of gestation. A marked increase in the number of dying cells is observed in newborn rats after irradiation, indicating that developing skeletal muscle is highly sensitive to ionising radiation. The effects of gamma rays are not massive; on the contrary, they affect single cells, or cells in small clusters. Dying cells are characterised by

their extreme chromatin condensation and, often, nuclear fragmentation. In addition, in situ labelling of nuclear DNA fragmentation demonstrated biotin-16-dUTP labelling in dying cells. The term apoptosis designates a type of cell death characterised by nuclear fragmentation followed by cellular breakdown without an inflammatory reaction (Cotter et al. 1990; Wyllie et al. 1990; Kerr & Harmon, 1991; Gerschenson & Rotello, 1992).

The present study has demonstrated that gamma ray induced cell death in developing muscle has the morphological features of apoptosis. Since the effects of gamma rays are suppressed when cycloheximide is injected immediately after irradiation, it can be suggested that gamma ray induced cell death is an active process associated with protein synthesis. A similar reduction in the number of radiation-induced dead cells is observed in the developing brain following cycloheximide injection immediately after irradiation (Ferrer, 1992; Ferrer et al. 1993).

The monoclonal antibody against-PCNA recognises a nuclear protein which is found in the late G1 and S phases of the cell cycle. PCNA is synthesised in variable amounts by proliferating cells, but is absent or found in very low amounts in normal nondividing tissues (Mathews et al. 1984; Celis et al. 1987; Ogata et al. 1987; Kurki et al. 1988). The number of PCNA-immunoreactive cells is drastically reduced in irradiated animals 48 h after irradiation, indicating that DNA replication is impaired following gamma ray exposure. However, a marked increase above normal values in the number of PCNA-immunoreactive cells occurs by d 5 after irradiation, thus suggesting a regenerative capacity of developing muscle following exposure to ionising radiation.

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