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Double-stranded Internucleosomal Cleavage of Apoptotic DNA Is Dependent on the Degree of Differentiation in Muscle Cells*

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Apoptotic cell death has been correlated to DNA fragmentation into discrete segments corresponding to the length of nucleosomal protected fragments of 180–200 base pairs or multiples of it. This DNA degradation has been ascribed to endonuclease activity that cleaves internucleosomally, thus giving rise to a ladder distribution upon electrophoretic migration. This strict correlation was, however, shown to have notable exceptions, since in some cases only single strand cleavage in the internucleosomal DNA regions has been observed (Tomei, D. L., Shapiro, P. J., and Cope, O. F. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 853–857). In the present work we show that mouse muscle cells, able to differentiate *in vitro*, if subjected to apoptosis present no DNA degradation into ladder form unless differentiation is previously induced. Furthermore, C3H/10T1/2 fibroblast cells, known to undergo apoptosis without DNA ladder formation, if converted to a myogenic program by MyoD expression, display internucleosomal DNA degradation upon induction of differentiation.

A set of biochemical and morphological features characterizes the process of cell death by apoptosis (1). This phenomenon, associated with several developmental processes and tissue homeostasis, is often called programmed cell death to indicate a physiological, naturally occurring cell death (2, 3). In addition to physiological stimuli, apoptosis can also be induced both *in vivo* and *in vitro* by a variety of cytotoxic drugs and physical stimuli (4–6) and is characterized by some typical parameters such as cell shrinkage, chromatin condensation, nuclear apoptotic bodies, and DNA degradation that clearly differentiate this cell death from necrosis (for reviews on apoptosis and programmed cell death see Refs. 1 and 7–9). For many years it was thought that the strictest marker of apoptosis was the DNA degradation in fragments of oligonucleosomal size (multiples of approximately 180 base pairs). This fragmentation is easily evidenced by the electrophoretic migration of DNA into a ladder configuration. Although internucleosomal DNA degradation has long been considered the cause of chromatin morphological alterations, in the last years many investigators have observed apoptotic phenotypes in the absence of ladder formation. In these cases, however, other types of DNA modification have been observed, such as single strand breaks (10) or cleavage into large DNA fragments of 50–300 kilobase pairs (11) (see also Table 1 in Ref. 9). Evidence now suggests that DNA degradation could be a late, dispensable step in the apoptotic process, perhaps important *in vivo* for a safe elimination of apoptotic cells by the organism (12–14).

Several nucleases have been invoked as responsible for apoptotic DNA degradation (15–17), but their involvement in this process is supported only by indirect evidence. It is not clear yet whether it is always the same or there are different nucleases that participate in DNA fragmentation in different cell systems and in different apoptosis inducing conditions or whether this enzymatic activity results from the activation of a preexisting nuclease or from its *de novo* synthesis. Our present results deal with the observation that apoptosis in myoblast cells displays different degradation patterns correlated with the degree of cell differentiation. The possibility to modulate the pattern of apoptosis renders this cell system useful for the investigation of the pathways leading to DNA fragmentation.

EXPERIMENTAL PROCEDURES

Cell Cultures—Mouse myoblast C2 cell clone 7 was obtained from Dr. M. Buckingham, Institute Pasteur, Paris, France; the subline expressing Polyomavirus large T (LT.N2) was derived from parental C2.7 myoblasts (18); embryonic fibroblasts C3H-10T1/2 expressing the myogenic determinant MyoD were kindly provided by Dr. M. Caruso (19). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS)[1] (and under constant selection of 400 μg/ml geneticin (Sigma) in the case of N2 cell line and C3H-10T½-MyoD) in a humidified 5% carbon dioxide atmosphere. Cells were passaged by standard trypsinization and seeded directly onto tissue culture plastic. To induce cell differentiation 3×10^3 cells/cm^2 were seeded and grown to confluence in 20% FCS for 4 days and then were shifted to a lower serum concentration (0.5% FCS) or to a medium containing 10% horse serum (HS). In the case of 10TMyo cells medium was supplemented with 5 μg/ml insulin.

Drug Treatment—In the cases of induction of cell death with apoptotic agents, etoposide (0.1 μM) or puromycin (1 μg/ml) was added to proliferating cells or to differentiated cells kept 48 h in 10% HS medium. The induction of apoptosis was analyzed after 16 and 36 h of treatment.

Morphological Examination of Cells—For determination of cell viability and chromatin condensation, ethidium bromide (4 μg/ml, Sigma) was added to the culture medium (20); the dye uptake by dead cells was instantaneous. Microscopic observation of cells was then performed, without fixation, under conditions of normal illumination or fluorescent light using a phase contrast inverted microscope.

Indirect Immunofluorescence Staining—Cells grown on glass coverslips were fixed by immersion in methanol/acetone (3:7, v/v) for 15 min

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[1] The abbreviations used are: FCS, fetal calf serum; HS, horse serum; MHCe, embryonic myosin heavy chain; PBS, phosphate-buffered saline; DAPI, 4′,6′-diamine-2′-phenylindole dihydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MHC, myosin heavy chain.

at −20 °C and then air dried. Coverslips were then incubated for 1 h at room temperature with a mouse monoclonal anti-embryonic myosin heavy chain antibody (MHCe) MF20 (21) as undiluted hybridoma supernatant. After three washes with PBS, the coverslips were incubated with a goat anti-mouse IgG rhodamine-conjugated IgG fraction (Cappel Immunochemicals) diluted 1:100 in PBS with 3% bovine serum albumin. Cells were then washed repeatedly with PBS. A final stain of 10 min with 1 μg/ml DNA binding fluorochrome 4′,6′-diamine-2′-phenylindole dihydrochloride (DAPI, Boehringer Mannheim) was carried out. The coverslips were washed and mounted with 70% glycerol in PBS. The samples were analyzed under phase contrast and appropriate fluorescent light.

DNA Analysis—To examine nucleosome laddering, an equal number of cells was seeded as described before in 60-mm diameter culture dishes. At different times after starvation or induction of cell death with apoptotic agents, both attached and floating cells were collected, centrifuged, and washed with PBS (containing 5 mM $MgCl_2$) twice. DNA was extracted as described in Ref. 22. In brief, the pelleted cells were resuspended and disrupted in cold Tris/EDTA buffer (5 mM Tris-HCl, pH 8, 10 mM EDTA) containing 0.5% Triton. The lysates were centrifuged for 20 min to separate fragmented DNA (soluble) from intact chromatin. At that point the samples were incubated for 1 h in the presence of 20 μg/ml RNase. Proteinase K was then added to the mixture adjusted to 100 μg/ml in 1% SDS and incubated for 3 h at 50 °C. The DNA was isolated using phenol/chloroform extraction and ethanol precipitation, using glycogen as coprecipitating agent. Pellets were air dried and resuspended in distilled water. Neutral agarose gels (1.5%) were prepared using multipurpose agarose according to the manufacturer's recommendations. DNA samples were prepared in a neutral loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol ff, and 30% glycerol in water), and the DNA samples were then added to the gel. Electrophoretic separation was performed at 100 V for 30–60 min. The DNA was visualized by UV illumination after ethidium bromide staining.

Northern Blot Analysis—Cells were assayed for expression of myosin by Northern blot analysis. Total RNA was isolated from cultured cell lines by acidic guanidinium thiocyanate/phenol/chloroform extraction as described (23) at different times after serum starvation. Twenty μg of total RNA were loaded per lane and electrophoresed in formaldehyde/agarose gel in MOPS buffer. The gels were blotted on nylon filters (Hybond, Amersham Corp.) in 10 × SSC (saline sodium citrate) for 16–20 h, cross-linked, and prehybridized in a buffer containing 50% formamide, 6 × SSC, 1% SDS, 10% dextran sulfate, and 0.5% nonfat dry milk for 2 h.

[α-^{32}P]dATP random primed cDNA probe generated from a *Pst*I fragment of the plasmid MHCe 2.2 (24), specific for embryonic mouse myosin heavy chain transcript, was used for Northern blot analysis. To provide evidence that the RNA samples were not degraded and to normalize their amounts, the filter was rehybridized with a probe for the constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (25). The probe was hybridized to the immobilized RNA for 16–20 h at 42 °C in the prehybridization buffer containing 500 μg/ml denatured salmon sperm DNA. The membrane was washed (under high stringency conditions according to the manufacturer's instructions) four times. The first wash was with 2 × SSPE (sodium saline phosphate EDTA), 0.1% SDS for 30 min at 42 °C, then at 65 °C for 30 min, and finally with 1 × SSPE, 0.1% SDS for 30 min at 65 °C. The last wash was with 0.2 × SSPE, 0.1% SDS for 15 min at room temperature.

RESULTS

In the course of our studies on C2 myoblast differentiation (18) we observed that the induction of differentiation by mitogen deprivation (0.2–0.5% FCS) was accompanied by some cell death showing many of the characteristics of apoptosis.

In order to verify whether the observed cell death could be ascribed to an apoptotic phenomenon, confluent grown cells were deprived of mitogens and DNA fragmentation was analyzed for 4 consecutive days, until full myogenic differentiation was obtained. No typical ladder distribution of the DNA was observed until 48 h after lowering the serum concentration to 0.5% (Fig. 1*a*), although all other apoptotic characteristics may be observed from 12 h on (Fig. 1*b*). Thereafter, the internucleosomal degradation of DNA augmented until it reached the normal apoptotic DNA fragmentation. This increase went along with differentiation, as shown by the expression of the muscle-specific marker myosin heavy chain (MHC) (Fig. 1*c*).

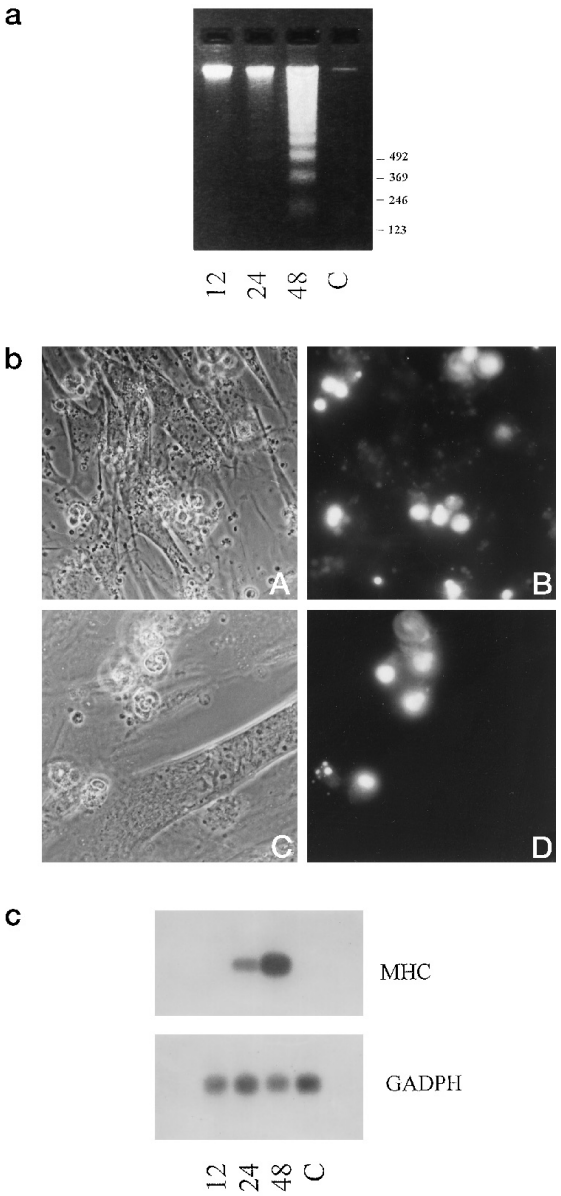

FIG. 1. **Correlation between myoblast differentiation and oligonucleosomal DNA fragmentation.** Confluent C2.7 cells were shifted to 0.5% FCS and, at different times, analyzed for DNA fragmentation, apoptotic morphology, and expression of a differentiation marker. *a*, kinetics of DNA fragmentation after mitogen deprivation. DNA was extracted as described under "Experimental Procedures" and analyzed by gel electrophoresis. *Numbers* below the *lanes* indicate the hours after mitogen deprivation. *C* represents the DNA from a proliferating culture (kept in 20% FCS) used as a negative control. Molecular size markers, in base pairs, are indicated on the right. *b*, apoptotic morphology after mitogen deprivation. Phase contrast (*A* and *C*) and fluorescence (*B* and *D*) micrograph of C2.7 cells stained with ethidium bromide 12 h (*A* and *B*) and 48 h (*C* and *D*) after the shift to 0.5% FCS. *c*, kinetics of MHC RNA expression after mitogen deprivation. Total RNA was extracted from cultures parallel to those described in *a* and analyzed by Northern blot hybridization for MHC expression. The same filter was hybridized with a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific probe to ensure that the same RNA amounts were loaded on the gel.

Induction of C2 Cell Death—To better analyze the possible correlation between internucleosomal DNA degradation and the degree of differentiation of muscle cells, we treated C2 cells with apoptotic agents at different times after the induction of differentiation. Myoblast differentiation is dependent on the arrest of cell cycle (26), and this is generally obtained by re-

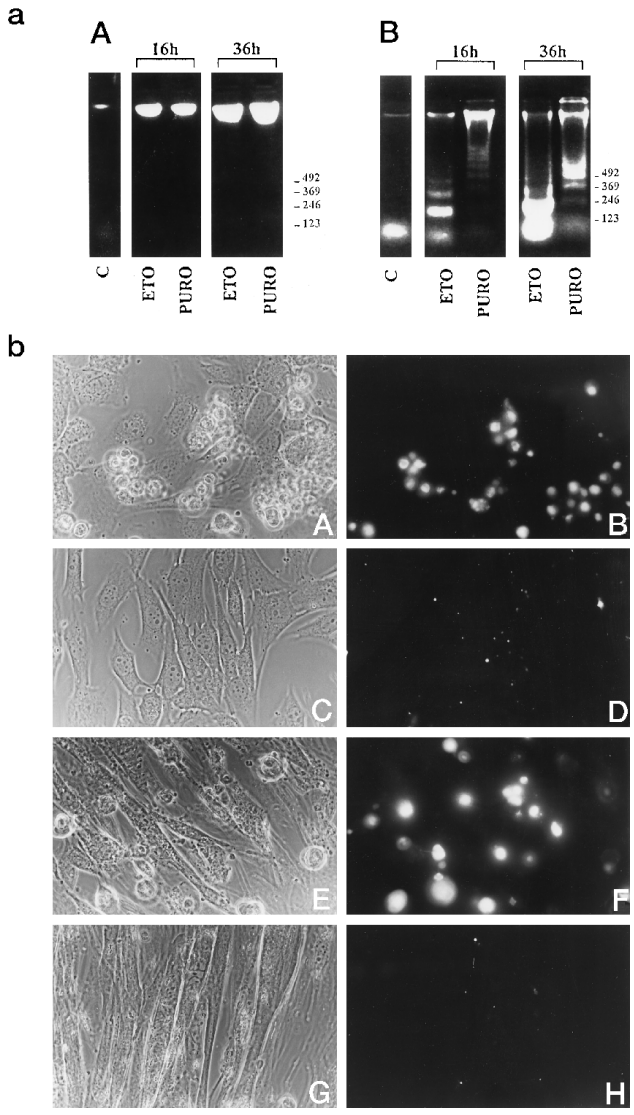

FIG. 2. **Effect of apoptotic agents in relation to the differentiative stage.** Undifferentiated (growing in 20% FCS) or differentiated (kept 48 h in 10% HS) myoblasts were treated with apoptotic agents as described under "Experimental Procedures" and then analyzed for DNA fragmentation and for apoptotic morphology. *a*, agarose gel analysis of DNA extracted from undifferentiated (*A*) and differentiated (*B*) myoblasts after treatment with etoposide (*ETO*) or puromycin (*PURO*); *16 h* and *36 h* indicate the hours of treatment; both untreated controls (*C*) were collected at 36 h. Molecular size markers, in base pairs, are indicated on the *right*. The low molecular weight band, under 123 base pairs, represents some residual RNA present in DNA samples. *b*, phase contrast (*A*, *C*, *E*, and *G*) and fluorescence (*B*, *D*, *F*, and *H*) micrographs of cells stained with ethidium bromide. *A*, *B*, *E*, and *F*, etoposide-treated cells; *C*, *D*, *G*, and *H*, untreated controls. *A–D*, undifferentiated cells; *E–H*, differentiated cells. The cells were analyzed after 16 h of treatment. The same morphology was observed after puromycin treatment (data not shown).

moving growth factors from the medium. Contact inhibition, however, also contributes to the growth arrest so that confluent cultures can still differentiate in the presence of small amounts of mitogens. In this case differentiation was induced with 10% horse serum, a condition that does not cause cell death and still allows differentiation. As apoptotic agents we used the topoisomerase II inhibitor etoposide (5) or the protein synthesis inhibitor puromycin (6). Results reported in Fig. 2 show that treatment with both agents resulted in oligonucleosomal DNA fragmentation only in cells previously kept in differentiation medium, despite the fact that the apoptotic phenotype was induced in both undifferentiated and differentiated cultures. A prolonged exposure (36 h) of proliferating myoblasts to apoptotic agents showed that oligonucleosomal fragmentation was completely absent and not simply delayed with respect to differentiated myoblast in which DNA ladder was already present after 16 h of treatment. The extensive cell death and the irreversible damage of cells undergoing apoptosis in the absence of oligonucleosomal DNA fragmentation was confirmed by a strong reduction in their clonogenic potential; the plating efficiency (clonogenic survival) of etoposide-treated, undifferentiated cells was more than 10-fold lower with respect to that of untreated controls, after only 16 h of treatment (data not shown). These results show the correlation of DNA ladder formation with the degree of cell differentiation. In all cases in which apoptosis was induced in the absence of DNA ladder formation the DNA of dead cells appeared in a high molecular weight soluble form.

Absence of Double-strand Cleavage in C2 Cells Expressing Polyomavirus Large T Oncogene—In a previous work we have shown that C2 cells constitutively expressing the Polyomavirus large T oncogene were unable to terminally differentiate, despite their ability to perform some early steps of differentiation (18). Therefore, to further correlate the internucleosomal DNA fragmentation with muscle differentiation, LT.N2 cells were subjected to treatment with etoposide or puromycin in proliferating or differentiating conditions. The obtained results demonstrate that the inhibition of differentiation correlates with the absence of the DNA internucleosomal degradation (Fig. 3) in spite of the presence of apoptotic phenotype (data not shown). When differentiation is induced in low serum medium (0.2–0.5% FCS), LT.N2 cells undergo a consistent phenomenon of cell death, much more pronounced even than that observed in the parental C2 cell line; also in this case there is no trace of DNA ladder (data not shown). Again, the DNA of apoptotic LT.N2 cells appeared in a soluble form and at high molecular weight.

Ladder Formation in C3H/10T1/2 Fibroblast Cells Converted to Muscle Cells upon Transfection with MyoD—C3H/10T1/2 cells were shown to undergo apoptosis in the absence of internucleosomal double-stranded DNA degradation (10). These fibroblast cells can be converted to myoblast cells by the expression of the exogenous myogenic determinant MyoD (27). It therefore appears a suitable system for testing the correlation between the kind of DNA degradation and muscle differentiation. C3H/10T1/2 cells stably transfected with a MyoD expression vector (10TMyo) and their parental untransfected cells were analyzed for their ability to degrade internucleosomally their apoptotic DNA. Confluent grown cells were transferred to low serum medium, and at different intervals DNA degradation was determined. Results reported in Fig. 4*a* show that myogenic conversion confers to C3H/10T1/2 apoptotic cells the ability to degrade DNA internucleosomally upon induction of differentiation. The degree of myogenic differentiation was determined by muscle-specific gene expression. Furthermore, by immunofluorescence microscopy (Fig. 4*b*) we observed that the nuclei included in myotubes remain always intact and that the apoptotic nuclei are restricted to the differentiated mononucleated cells.

DISCUSSION

In this report we have described how the induction of differentiation of cultured myoblast cells alters the pattern of apoptotic DNA degradation. Undifferentiated myoblasts, when treated with apoptosis-inducing agents, undergo cell death showing the typical apoptotic morphology, characterized by chromatin condensation and nuclear fragmentation, but lack the typical internucleosomal DNA fragmentation. This finding is not surprising, since apoptosis in the absence of ladder dis-

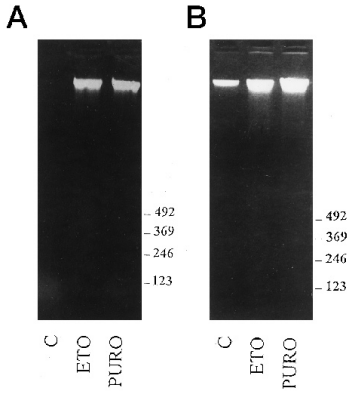

FIG. 3. **Effect of apoptotic agents on differentiation-defective myoblasts.** Agarose gel analysis of DNA extracted from LT.N2 cells after 16 h of treatment with etoposide (*ETO*) or puromycin (*PURO*) and from untreated controls (*C*). *A*, cells growing in 20% FCS. *B*, cells kept 48 h in differentiation medium (10% HS). Molecular size markers, in base pairs, are indicated on the *right*.

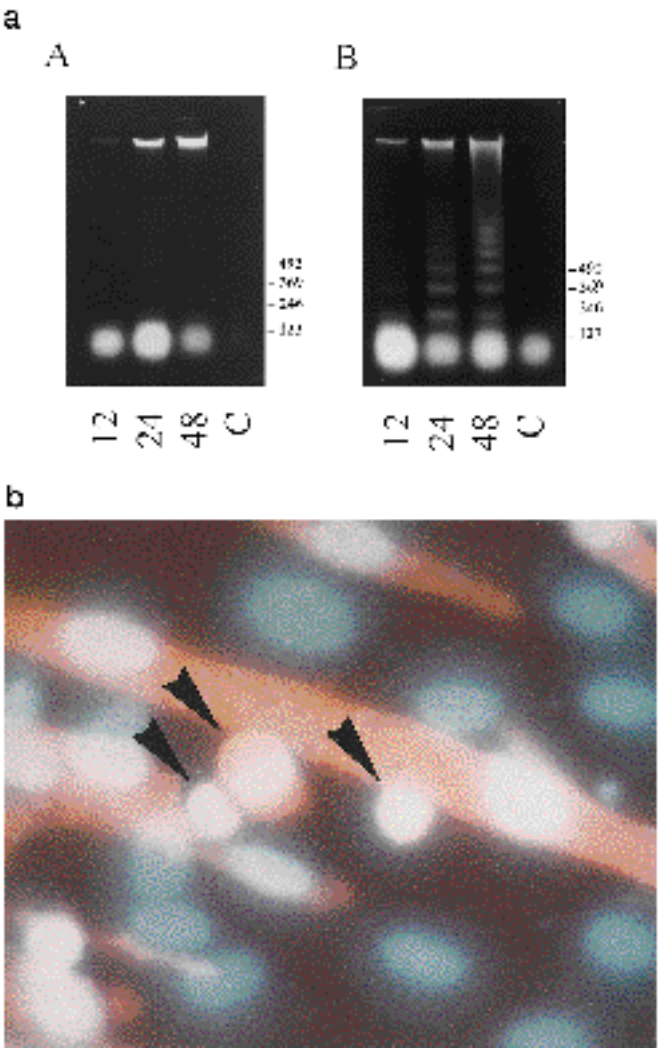

FIG. 4. **Correlation between myogenic conversion and oligonucleosomal DNA fragmentation.** *a*, agarose gel analysis of DNA extracted from C3H10T1/2 (*A*) and from its myogenic derivative 10TMyo (*B*) at different times after the shift to low serum medium (0.5% FCS + insulin). *Numbers* below the *lanes* indicate the hours after the shift. *C* represents the DNA from a proliferating culture (20% FCS) used as a negative control. Molecular size markers, in base pairs, are indicated on the *right*. The low molecular weight band, under 123 base pairs, represents some residual RNA present in DNA samples. *b*, fluorescence micrograph of 10TMyo cells double-stained with anti-MHC antibody and DAPI. Cells were fixed and stained 48 h after the shift to low serum medium. *Arrows* indicate apoptotic nuclei in differentiated mononucleated cells.

tribution of degraded DNA has been already observed in other cell systems, mainly in epithelial and fibroblast cell lines (9). Our finding, therefore, supports the hypothesis that the changes in nuclear morphology occurring during apoptosis are not dependent on a previously activated internucleosomal DNase activity. The high molecular weight, soluble DNA extracted from undifferentiated apoptotic myoblasts is likely to correspond to the large DNA fragments of 50–300 kilobase pairs, described as an early event in many forms of apoptosis (11, 14) and probably correlated with the size of chromatin loop domains.

After the induction of differentiation, myoblast cells acquire the ability to degrade their DNA into oligonucleosomal fragments. The correlation between myogenic differentiation and such an endonucleolytic activity is strongly supported by the effect of myogenic conversion of C3H10T1/2 fibroblast cells by MyoD expression. Apoptotic DNA from C3H10T1/2 is not degraded in oligonucleosomal form (10) whereas its myogenic derivative (10TMyo), if induced to differentiate, displays a ladder distribution of its DNA. Moreover, Polyomavirus large T oncogene-transformed myoblasts, inhibited in the myogenic program, lose the ability to activate the internucleosomal DNA fragmentation. Interestingly, differentiated apoptotic cells, from both C2 and 10TMyo cell lines, have been found to be mononucleated. This observation could probably reflect that apoptotic DNA fragmentation occurs in a differentiative stage preceding the fusion into myotubes where nuclei are protected from apoptosis. This finding is consistent with the view that apoptosis represents a mechanism to eliminate cells that do not perform correctly their differentiative program.

The ability of differentiated cells to degrade their DNA into small fragments could reflect a modification in the level or in the activity of a nuclease or, alternatively, a modification in the chromatin structure that allows the access of some nuclease to the DNA. Nuclear extracts from both differentiated and undifferentiated myoblasts contain a high nuclease activity migrating with an 18-kDa molecular mass, as we determined by the gel nuclease assay used by Gaido and Cidlowski (28) to identify the nuclease NUC 18 from apoptotic thymocytes (data not shown). This result excludes only the possibility of myoblast differentiation being associated with an increased level of this nuclease, leaving open all possible activity modifications including a different availability of the substrate. A more detailed biochemical analysis is necessary to clarify this issue.

Apoptosis in muscle cells has not been previously described, and the role of programmed cell death in muscle development has not been defined (the behavior of C2 myoblasts could be a particular characteristic of an immortal cell line). However, in the past decades, consistent phenomena of cell death have been observed during the development of many tissues, included myotome and embryonic muscle (29). The acquisition of the ability, by muscle cells, to perform the ultimate step of apoptosis, which may be necessary in particular physiological contexts, could reflect a possible role for this process during *in vivo* differentiation.

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