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The Activity of Differentiation Factors Induces Apoptosis in Polyomavirus Large T-Expressing Myoblasts

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It is commonly accepted that pathways that regulate proliferation/differentiation processes, if altered in their normal interplay, can lead to the induction of programmed cell death. In a previous work we reported that Polyoma virus Large Tumor antigen (PyLT) interferes with in vitro terminal differentiation of skeletal myoblasts by binding and inactivating the retinoblastoma antioncogene product. This inhibition occurs after the activation of some early steps of the myogenic program. In the present work we report that myoblasts expressing wild-type PyLT, when subjected to differentiation stimuli, undergo cell death and that this cell death can be defined as apoptosis. Apoptosis in PyLT-expressing myoblasts starts after growth factors removal, is promoted by cell confluence, and is temporally correlated with the expression of early markers of myogenic differentiation. The block of the initial events of myogenesis by transforming growth factor β or basic fibroblast growth factor prevents PyLT-induced apoptosis, while the acceleration of this process by the overexpression of the muscle-regulatory factor MyoD further increases cell death in this system. MyoD can induce PyLT-expressing myoblasts to accumulate RB, p21, and muscle- specific genes but is unable to induce $G0_0$ arrest. Several markers of different phases of the cell cycle, such as cyclin A, cdk-2, and cdc-2, fail to be down-regulated, indicating the occurrence of cell cycle progression. It has been frequently suggested that apoptosis can result from an unbalanced cell cycle progression in the presence of a contrasting signal, such as growth factor deprivation. Our data involve differentiation pathways, as a further contrasting signal, in the generation of this conflict during myoblast cell apoptosis.

INTRODUCTION

A large body of evidence has been accumulated showing that the disruption of growth control can lead, in addition to uncontrolled proliferation and defective differentiation, to apoptotic cell death. Several indications now suggest that signals controlling apoptosis or cell survival are strictly related to the pathways regulating proliferation/differentiation processes. Myogenic cell lines represent a valuable system for the study of the interdependence between terminal differentiation and cell cycle control. Skeletal muscle differentiation is regulated by the MyoD family of muscle regulatory factors (MRFs)[1] that includes MyoD, myogenin, myf 5, and MRf4, identified on the basis of their ability to activate muscle-specific genes in several non-

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[1] Abbreviations used: bFGF, basic fibroblast growth Factor; BrdU, 5-bromodeoxyuridine; CAT, chloramphenicol acetyl transferase; cdk, cyclin-dependent kinase; IGF-I, insulin-like growth factor I; MHC, myosin heavy chain; MRF, muscle-regulatory factor; PyLT: polyomavirus large tumor antigen; RB, retinoblastoma antioncogene product.

muscle cell types and to determine the entire differentiative program (for reviews see Weintraub, 1993; Olson and Klein, 1994). Several different mechanisms by which myogenesis is modulated by signals involved in growth control have been identified (reviewed by Maione and Amati 1997). MRFs' activity is inhibited, for example, by direct interaction with some proteins, such as Id and jun, whose levels depend on the activation of growth factors and oncogenic pathways. Posttranslational modifications also seem to play an important role in the inhibition of MRFs' activity. Both PKC and PKA have been involved in the phosphorylation of MRFs after the activation of signal transduction pathways and, more recently, also some members of the cyclin-dependent-kinase (cdk) family have been suggested as possible regulators of MyoD. A more direct mechanism that contributes to the dependence of muscle differentiation on growth arrest consists in the positive regulation of MRFs by factors already recognized as important negative regulators of the cell cycle. Much information in this regard has been obtained by using DNA tumor virus oncoproteins, such as Adenovirus E1A, SV40, and Polyomavirus Large T antigen, that bind and inactivate the retinoblastoma family of growth suppressors (RB, p107, and p130) and the unrelated protein p300 (for a review see Nevins, 1994). These interactions, in addition to being critical for their transforming activity, are also involved in the mechanism by which the viral products inhibit myogenic differentiation (Mymryk *et al.*, 1992; Caruso *et al.*, 1993; Maione *et al.*, 1994; Tedesco *et al.*, 1995). A direct interaction between myogenic factors and RB-family proteins, which would be prevented by viral oncoproteins, has been suggested as a mechanism supporting MRFs' activity (Gu *et al.*, 1993; Schneider *et al.*, 1994) and, more recently, it has been shown that p300 can function as a transcriptional coactivator of MyoD (Eckner *et al.*, 1996).

The appearance of differentiation markers is associated with an irreversible withdrawal of myoblast cells from the cell cycle that prevents them from reinitiating proliferation even upon growth factor stimulation (Endo and Nadal-Ginard, 1986). Myogenic factors are also involved in the maintenance of the G_0 phase of the cell cycle in differentiated muscle cells through their property to exert a growth-suppressive action (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990). The antioncogene product RB seems to play a fundamental role in mediating this effect. Direct binding by myogenic factors has been suggested to lock RB in its hypophosphorylated, growth-suppressive form (Gu *et al.*, 1993). MyoD has been also implicated in the RB gene transcriptional activation occurring concomitantly with the onset of differentiation (Martelli *et al.*, 1994). More recently, it has been shown that the cdk inhibitor p21 is transcriptionally activated during myoblast differentiation, and it has been proposed that this up-regulation can be mediated by MyoD (Halevy *et al.*, 1995). One of the functions of cyclin/cdk-complex inhibition is expected to be the prevention of RB phosphorylation by cdks (Sherr and Roberts, 1995), so that several mechanisms ensure the accumulation of high levels of functional RB in differentiated cells.

There are a number of indications that a defective cell cycle arrest can lead to apoptotic cell death. For example, the expression of DNA tumor virus oncoproteins such as adenovirus E1A (Debbas and White, 1993; Lowe and Ruley, 1993) and human papillomavirus E7 (Pan and Griep, 1994), or the constitutive activation of the cellular proto-oncogene c-*myc* (Evan *et al.*, 1992), have been shown to induce apoptosis in several systems. The ability of viral and cellular oncogenes to induce cell death is related to their ability to alter the levels and/or the activity of cell cycle regulators. Accordingly, extensive proliferation and cell death have been observed in several developing tissues of RB-lacking embryos (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992, 1994; Morgenbesser *et al.*, 1994; Zacksenhaus *et al.*, 1996). Moreover, an increased susceptilbility to apoptosis has been described also for in vitro cell systems (Almasan *et al.*, 1995; Haas-Kogan *et al.*, 1995). In line with the above observations, E2F overexpression, which overrides RB function, both increases S phase transition and induces apoptosis (Shan and Lee, 1994; Kowalik *et al.*, 1995). Furthermore, increased levels of cyclin D1, the catalytic subunit of an RB-inactivating kinase, cause cell death in different cell types and have been suggested as a physiological mechanism regulating apoptosis in postmitotic neurons (Kranenburg *et al.*, 1996; Sofer-Levi and Resnitzky, 1996). A general picture emerges in which cell death would result from an abnormal progression through the cell cycle due to the simultaneous activation of proliferative and growth arrest pathways. It is not yet clear how cells detect the presence of these contradictory signals and the detailed mechanisms leading to the execution of the apoptotic response. An important role in this process has been attributed to the p53 tumor suppressor protein (reviewed by Ko and Prives, 1996; Levine, 1997), although evidence suggests the existence of both p53-dependent and p53-independent pathways in oncogene-induced apoptosis (Pan and Griep, 1995; Sakamuro *et al.*, 1995; Teodoro *et al.*, 1995).

Programmed cell death in vivo occurs at defined stages of development and differentiation (Ellis *et al.*, 1991; Jacobson *et al.*, 1997). Many observations obtained in vitro also suggest that both normal and transformed cells change their susceptibility to apoptosis and their survival factor requirement along with differentiation (Solary *et al.*, 1993; Bhatia *et al.*, 1995; Ishizaki *et al.*, 1995). Programmed cell death in skeletal muscle cells has also been described. Most information regards metamorphosis processes (Schwartz, 1992) and, more recently, it has

been suggested that apoptosis can play a role in some muscle pathologies in mammals (Lockshin *et al.*, 1995). The regulatory pathways controlling muscle death and survival are even less clear with respect to other cell systems, even though it has been recently demonstrated that RB plays an important role in in vivo myoblast cell survival during development (Zacksenhaus *et al.*, 1996).

We have previously described that C2 mouse myoblast cells expressing polyomavirus large T antigen (PyLT) are unable to terminally differentiate, despite their ability to perform some early steps of differentiation (Maione *et al.* 1992a,b). This inhibition is correlated with the ability of the viral oncogene to bind and inactivate RB (Maione *et al.*, 1994). Here we report that PyLT-dependent inhibition of terminal differentiation is accompanied by the induction of apoptosis and that this apoptosis is strictly related to the induction of the early steps of differentiation. Treatment with growth factors that prevent muscle gene expression promotes the survival of PyLT-expressing myoblasts whereas the overexpression of the muscle-regulatory factor MyoD further increases cell death. Our data suggest that apoptosis in this system may result from the activity of myogenic factors that, in the presence of the viral oncogene, induce abnormal differentiation pathways.

MATERIALS AND METHODS

Cell Cultures

Mouse myoblast C2 cells, clone 7 (Yaffe and Saxel, 1977), and the derivative subclones expressing PyLT, LT.N2, and LT.R13 (Maione *et al.*, 1994) were maintained in DMEM supplemented with 10–20% FCS (and under constant selection of 400 μg/ml geneticin [Sigma Chemical, St. Louis, MO) in the case of LT.N2 and LT.R13) in humidified 5% carbon dioxide atmosphere. Cells were passaged by standard trypsinization and seeded directly onto tissue culture plastic plates. To induce differentiation, cells were grown to confluence and then shifted to a low serum concentration. Optimal differentiation of C2 cells can be induced in the presence of FCS concentrations ranging from 0.5% to 2%, or even in the presence of 10% horse serum. However, in all these experiments we used DMEM-0.5% FCS as the differentiation medium, which allows us to better appreciate apoptotic cell death of LT.N2 cells.

For 5-bromodeoxyuridine (BrdU) incorporation assays, cells kept in 0.5% FCS were incubated with 10 μM BrdU (Sigma) added to the medium either 1 h or 5 h before fixation. BrdU-positive cells were detected by indirect immunofluorescence as described below.

Morphological Examination of Cells

For determination of cell viability and chromatin condensation, ethidium bromide (4 μg/ml, Sigma) was added to LT.N2 culture medium 24 h after the shift to low serum conditions (0.5% FCS); the dye uptake by dead cells was instantaneous. In some experiments, cell viability was also assessed by trypan blue exclusion. Microscopic observation of cells was performed under conditions of normal illumination or fluorescent light using a phase contrast inverted microscope.

Flow Cytomery

Cell cycle distribution and apoptosis were analyzed by using the acridine orange (AO, Polysciences, Warrington, PA) flow cytometric tecnique, as previously described (Tafuri and Andreeff, 1990). Briefly, cell suspensions (0.2 ml) containing 0.1 to 0.4 $\times$ 10^6 cells in PBS were mixed with 0.2 ml of 0.05 N hydrocloric acid (HCl), 0.15 M sodium chloride (NaCl), and 0.1% Triton X-100 (Sigma). After 30 s, 0.6 ml of chromatographically purified AO (6 μg/ml) in 1 mM disodium-EDTA (EDTA-Na), 0.15 N NaCl, and 0.1 M phosphate-citrate buffer (pH 6) was added. Pretreatment with Triton X-100 makes the cells permeable to the dye, and, at a low pH, nucleic acids remain insoluble. Subsequent staining with AO in the presence of a chelating agent (EDTA) which makes cellular RNA single-stranded results in metachromatic red staining of RNA, whereas the native DNA intercalates the dye and stains orthochromatically green. Myoblasts undergoing apoptosis were detected as a subG_1 peak on DNA frequency histograms, since their stainability diminished with DNA-specific fluorochromes (Darzynkiewicz *et al.*, 1992). AO staining allows discrimination between necrotic and apoptotic cells because of decreased stainability of apoptotic elements in DNA green fluorescence coupled with higher red fluorescence (which is common to chromatin condensation and higher content of single-stranded DNA) (Lemoli *et al.*, 1997). Cell debris was excluded from the analysis on the basis of its forward light scatter properties.

Modified FACScan equipment (Becton Dickinson, Franklin Lakes, NJ) was used to measure fluorescence upon excitation at 488 nm. Five thousand cells were measured for each analysis at separate wavelength bands for green (F530-DNA) and red (F>620-RNA). Samples were analyzed using a Hewlett Packard (Palo Alto, CA) microcomputer and Becton Dickinson software including Cellfit and Lysis II.

Indirect Immunofluorescence Staining

Cells grown on glass coverslips were fixed by immersion in methanol/acetone (3:7, vol/vol) for 15 min at −20°C and then air dried. Coverslips were then incubated for 1 h at room temperature or 30 min at 37°C in humidified atmosphere with the primary antibody. After three washes with PBS, the coverslips were incubated with the secondary fluorochrome-conjugated antibody diluted in PBS plus 3% BSA, washed repeatedly with PBS, and mounted with 70% glycerol in PBS. The samples were analyzed under phase contrast and appropriate fluorescent light.

The following primary antibodies were used: to detect desmin, the mouse monoclonal antibody DE-B-5 (Boehringer Mannhein, Indianapolis, IN) undiluted; to detect embryonic myosin heavy chain (MHC) the mouse monoclonal antibody MF20 (Bader *et al.*, 1982) as undiluted hybridoma supernatant; to detect bromodeoxyuridine incorporated in cellular DNA, the mouse monoclonal antibody BU-1 (Amersham, Arlington Heights, IL) undiluted (in this case a 30 min incubation with 1.5 N HCl at room temperature was performed before the incubation with the primary antibody).

As secondary antibodies we used a fluorescein-conjugated goat affinity purified antibody to mouse IgG diluted 1:10 and a rhodamine-conjugated goat IgG fraction to mouse IgG diluted 1:100, both from Cappel Immunochemical (Cochranville, PA).

Double immunofluorescence staining of BrdU and MHC was performed by sequential incubation of coverslips with anti-BrdU, rhodamine-conjugated secondary antibody, anti-MHC, and finally, fluorescein-conjugated secondary antibody. A final stain of 10 min with 1 μg/ml of the DNA binding fluorochrome DAPI (Boehringer Mannheim) was carried out to visualize total nuclei.

Production of Recombinant Retroviruses and Retroviral Infections

Retroviral vectors coding for wild-type or mutant MyoD were constructed by inserting the *Eco*RI fragments from either pEMSV-MyoD or pEMSV-B2proB3, containing the respective coding sequences (Davis *et al.*, 1990) into the *Eco*RI site of the vector pBabe-Puro (Morgenstern and Land, 1990), made available by Dr. B. Amati (Swiss Institute for Cancer Research, Epalinges, Switzerland). BOSC

23 ecotropic packaging cells (Pear *et al.*, 1993), kindly made available by Dr. P.G. Pelicci (European Institute of Oncology, Milan, Italy), were maintained in DMEM supplemented with 10% FCS in humidified 5% carbon dioxide atmosphere. To obtain recombinant retroviruses BOSC 23 cells were transfected as described (Pear *et al.*, 1993). Briefly, 6×10^6 cells were seeded onto 100-mm tissue culture dishes in DMEM supplemented with 10% FCS and grown for 24 h. Just before transfection, 25 μM chloroquine was added to the culture medium and 20 μg of plasmid/100-mm dish were transfected with the calcium phosphate precipitation method. After 10 h the medium was changed and cells were incubated for an additional 16 h in DMEM-10% FCS. Medium was again replaced with a smaller volume. The retroviral supernatant was harvested 24 h later and, after removal of cell debris, frozen at −80°C for later use.

For transient retroviral MyoD expression, 3×10^5 C2.7 and LT.N2 cells were plated onto 60-mm dishes 24 h before infection. Cells were then incubated with 1 ml of BOSC 23 retroviral supernatant supplemented with 4 μg/ml polybrene for 10 h and then refed with fresh medium. Forty eight hours later, cells were either collected for analysis or further incubated in DMEM supplemented with 0.5% FCS and analyzed at different times after the shift to low serum medium as described in RESULTS.

Western Blot Analysis

C2.7 and LT.N2 kept either in proliferative (GM) or differentative (DM) conditions were washed twice with ice-cold phosphate saline buffer (PBS) and then lysed for 30 min on ice in EBC buffer containing 50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 100 mM NaF supplemented with various protease and phosphatase inhibitors (leupeptin, aprotinin, PMSF, sodium-orthovanadate). After clearing by centrifugation at 12,000 rpm for 20 min at 4°C, protein content of samples was quantified by the Bio-Rad (richmond, CA) method. Similar results were obtained by extraction with boiling sample buffer (2% SDS, 10% glycerol, 60 mM Tris, pH 6.8, 5% β-mercaptoethanol, 0.01% bromophenol blue) directly added to the plates. Lysates were scraped into microcentrifuge tubes and then heated at 90°C for 10 min. Aliquots corresponding to equivalent protein amounts for each sample were separated by SDS-PAGE and transferred to nitrocellulose filters. After blocking with 5% nonfat dry milk in Tris-buffered saline, membranes were incubated with the following primary antibodies: 1) for PyLT, a 1:500 dilution of the 440 rabbit polyclonal serum (kindly provided by Dr. B. Schaffhausen, Tufts University School of Medicine, Boston, MA); 2) for MyoD, a 1:500 dilution of the rabbit polyclonal anti-MyoD antibody M-318 (sc-760; Santa Cruz Biotechnology, Santa Cruz, CA); 3) for myogenin, the monoclonal anti-myogenin antibody IF5D as undiluted hybridoma-supernatant (Wright *et al.*, 1991) made available by Dr. G. Cossu (University of Rome La Sapienza); 4) for MHCe, the monoclonal antibody MF-20 as undiluted hybridoma supernatant (Bader *et al.*, 1992); 5) for RB, a 1:200 dilution of the affinity-purified monoclonal antibody to RB, PMG3–245 (Pharmingen, San Diego, CA); 6) for p21, a 1:100 dilution of the rabbit polyclonal antibody C-19 (sc-397-G Santa Cruz); 7) for cyclin D1 a 1:500 dilution of the mouse monoclonal antibody 72–13G (sc-450 Santa Cruz); 8) for cyclin E, a 1:500 dilution of the rabbit polyclonal antibody M-20 (sc-481 Santa Cruz); 9) for cyclin A, a 1:1000 dilution of the rabbit polyclonal antibody C-19 (sc-596 Santa Cruz); 10) for cdk-2 and cdc-2, a 1:500 dilution of the respective rabbit polyclonal antisera, kindly provided by Dr. Michele Pagano (Mitotix, Cambridge, MA). The detection system used was enhanced-chemiluminescence (ECL, Amersham)

RESULTS

Py LT-Expressing Myoblasts Undergo Apoptosis

Myogenic differentiation of C2 skeletal myoblast cells is dependent on the arrest of the cell cycle and is generally achieved by reducing the mitogen concentration in the medium of confluent cultures. As we previously reported, when C2 myoblasts expressing wild-type PyLT are shifted to differentiation medium, they do not undergo the typical myogenic differentiation as do, in contrast, control cells and C2 myoblasts expressing a mutant PyLT unable to bind RB (Maione *et al.*, 1992a,b, 1994). Here we show that, in these conditions, a significant fraction of wild-type PyLT-expressing myoblasts undergoes a phenomenon of cell death. Figure 1A shows the morphological appearance of LT.N2 cells (a representative clone expressing wild-type PyLT), LT.R13 cells (a representative clone expressing an RB-binding mutant PyLT), and C2 parental cells, examined in growth conditions and at two different times after the shift to differentiation medium. Floating cells appeared in LT.N2 plates starting a few hours after the removal of growth factors and reaching the maximum level between 24 and 48 h. The same phenomenon was observed in other clones derived from independent transfections and was proportional to the level of wild-type PyLT expressed (our unpublished observations). LT.R13 and C2 parental myoblasts also undergo some cell death when induced to differentiate, but this phenomenon can be minimized by allowing cells to reach full confluence before the shift to low serum medium. These results indicate, as expected, that the binding and the consequent inactivation of RB are also necessary for PyLT to interfere with myoblast cell survival.

Different analytical approaches suggest that cell death of Py LT-expressing myoblasts occurs by apoptosis. One of the typical features of apoptotic cells is the appearance of a hypodiploid DNA content, measurable by DNA staining. Figure 1B shows the flow cytometric analysis of AO-stained LT.N2, compared with the parental C2 cells. As expected from the comparable rate of proliferation of the two cell lines, they show a similar cell cycle profile in growth conditions. In contrast, after the shift to 0.5% FCS-containing medium, a large percentage of LT.N2 cells accumulates in the hypodiploid region of the cell cycle, whereas the parental C2 cells undergo the expected cell cycle arrest, normally associated with terminal differentiation. The chromatin state of apoptotic cells was assessed by ethidium bromide staining as reported in Figure 1C, which shows dead cells with nuclear condensation and fragmentation, typical characteristics of apoptotic death. The electrophoretic analysis of DNA extracted from LT.N2 cells in apoptosis-inducing conditions showed, instead of the typical internucleosomal DNA fragmentation, the presence of digested DNA of high molecular weight. The absence of DNA ladder formation is due to the inability of apoptotic undifferentiated myoblasts to perform this kind of DNA degradation, as we previously reported (Fimia *et al.*, 1996).

Apoptosis in PyLT-expressing Myoblasts Is Inhibited by Cytokines That Prevent Myogenic Differentiation

It has long been recognized that growth factors, in addition to regulating cell proliferation and differentiation, also play an important role in cell survival. It is now clear that this control can act through the suppression of apoptosis (Baserga, 1994). Serum deprivation causes apoptosis in various cell types, and for each system there are specific cytokines able to promote survival. This specificity could reflect not only differential activities of distinct signal transduction pathways in different cell systems but also the existence of different apoptotic mechanisms. PyLT-expressing myoblasts undergo apoptosis after growth factor withdrawal. In this condition LT.N2 cells, although unable to terminally differentiate, acivate some early steps of myogenesis (Maione *et al.*, 1994), such as the down-regulation of the inhibitory factors Id and c-*myc*, the up-regulation of desmin, a muscle-specific intermediate filament protein, and that of the inhibitors of cell proliferation RB and p21 (see also Figures 3 and 6A). To elucidate the events leading to apoptosis in PyLT-expressing myoblasts, we investigated the action of a set of growth factors known to affect, in different ways, proliferation and/or differentiation of myoblast cells. LT.N2 cells were grown in 10% FCS until they reached confluence and then transferred to low serum-containing medium, either alone or supplemented with one of the following cytokines: platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ), insulin-like growth factor-I (IGF-I), insulin, and basic fibroblast growth factor (bFGF). Cell death was analyzed by microscopic observation during the following 24 h. As shown in Figure 2, either bFGF or TGFβ efficiently protected LT.N2 cells from apoptosis until at least 24 h after the shift to low serum, at which time the maximum level of cell death was detected in control cells. In contrast, PDGF, IGF-I, and insulin do not prevent LT.N2 cell death. Previous studies have pointed out the importance of several growth factors in regulating proliferation and differentiation of C2 myoblast cells (Florini and Magri, 1989 and our unpublished observations). In particular, bFGF stimulates C2 cell proliferation and strongly inhibits their differentiation; TGFβ is not mitogenic for these cells, yet it is a potent inhibitor of myogenesis as well; insulin and IGF-I stimulate both proliferation and differentiation; PDGF does not significantly affect C2 cells except for a slightly increased proliferation. LT.N2 cells respond to growth factors similarly to the parental C2 cells. An increased DNA synthesis was observed in LT.N2 cells after treatment with bFGF, insulin, and IGF-I, but not with TGFβ (Table 1). In addition, the initiation of the myogenic program is inhibited, as in C2 parental cells, by FGF or TGFβ and not by IGF-I or insulin. Figure 3 shows, for example, that the up-regulation of desmin, a differentiation property retained by LT.N2 cells, was completely inhibited only in the presence of the first two growth factors. Similar inhibitory effects, regarding both differentiation and cell death, were observed by treating LT.N2 cells with the PKC activator phorbol dibutyrate or by expressing an activated form of the Ras proto-oncogene (our unpublished results), both known as potent inhibitors of muscle differentiation (Lassar *et al.*, 1989; Li *et al.*, 1992). These results suggested an interesting correlation between the block of the early steps of myogenesis and the protection from cell death in PyLT-expressing myoblasts.

MyoD Overexpression Triggers Apoptosis in PyLT-expressing Myoblasts

The growth factors bFGF and TGFβ completely block myogenic differentiation by repressing both the expression and the functional activity of myogenic factors such as MyoD (Vaidya *et al.*, 1989). LT.N2 cells retain some activity of MRFs, as deduced by the expression of early differentiation markers, such as desmin, RB, and p21 known to be transcriptionally activated by MyoD (Li and Capetanaki, 1993; Martelli *et al.*, 1994; Halevy *et al.*, 1995). The observation that the onset of myogenesis parallels the appearance of apoptosis, and that the inhibition of the early steps of differentiation correlates with cell survival, prompted us to investigate the possible role of MyoD activation in the apoptosis induced by serum deprivation in PyLT-expressing myoblasts. For this purpose we analyzed the effects of MyoD overexpression in LT.N2 cells. MyoD cDNA sequences were subcloned in pBabePuro retroviral vector, and high-titer retroviruses were obtained, enabling quantitative infections of large cell populations in transient assays. C2.7 and LT.N2 cells were infected with pBabeMyoD retrovirus or, as a control, with the empty retrovirus. Starting 48 h postinfection, they were then analyzed for differentiation and survival. The effective overexpression of MyoD protein was determined by Western blot with an anti-MyoD antibody (Figure 4A). Microscopic examination of infected populations (Figure 4B) shows that MyoD overexpression, as expected, reduces the rate of proliferation and accelerates differentiation of C2 parental cells: MyoD-infected C2 cells did not reach confluence 48 h postinfection as did, in contrast, mock-infected C2 cells; moreover, even before the shift to differentiation medium, several long-shaped cells, reflecting the typical differentiated morphology, appeared. The same effects were observed in LT.R13 cells (our unpublished observations). Completely different was the scenario in LT.N2 plates. In fact, MyoD-infected LT.N2 cells began to round and detach since 36–48 h postinfection when cells were still in the

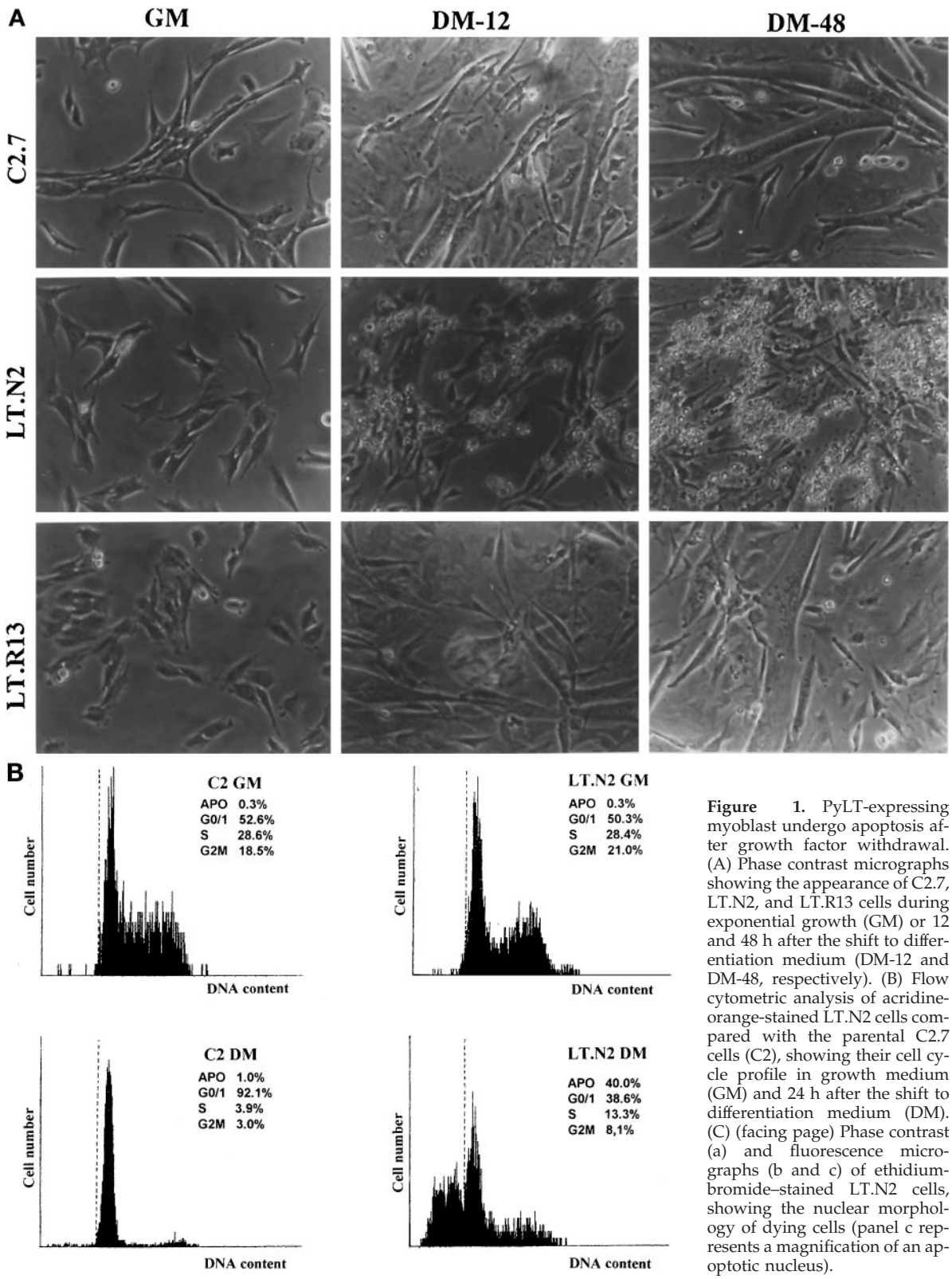


Figure 1. PyLT-expressing myoblast undergo apoptosis after growth factor withdrawal. (A) Phase contrast micrographs showing the appearance of C2.7, LT.N2, and LT.R13 cells during exponential growth (GM) or 12 and 48 h after the shift to differentiation medium (DM-12 and DM-48, respectively). (B) Flow cytometric analysis of acridine-orange-stained LT.N2 cells compared with the parental C2.7 cells (C2), showing their cell cycle profile in growth medium (GM) and 24 h after the shift to differentiation medium (DM). (C) (facing page) Phase contrast (a) and fluorescence micrographs (b and c) of ethidium-bromide–stained LT.N2 cells, showing the nuclear morphology of dying cells (panel c represents a magnification of an apoptotic nucleus).

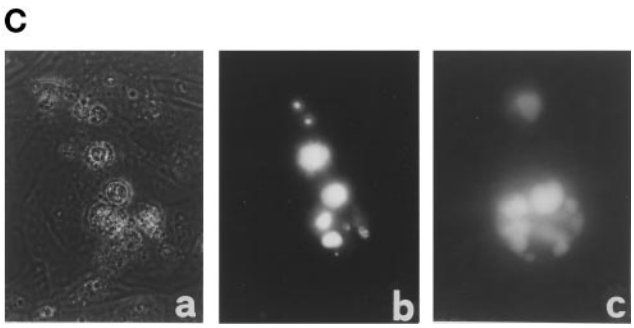


Figure 1 (cont).

presence of a high serum concentration. After the shift to differentiation medium the phenomenon dramatically increased and, in the space of a few hours, a much higher percentage of nonviable cells was detected in MyoD infected with respect to mock-infected cultures (Table 2). Cytofluorometric analysis shows that MyoD-overexpressing LT.N2 cells accumulate in the hypodiploid region of the cell cycle, thus confirming that cell death occurs by apoptosis (Figure 5B). These data demonstrate that forced expression of MyoD accelerates the activation of the apoptotic process in PyLT-expressing myoblasts and also suggest that the activity of endogenous myogenic factors can be involved in the cell death of uninfected LT.N2 cells. We also analyzed, using recombinant retrovirus infection, the effect of a MyoD mutant, B2proB3, in which a single amino acid substitution abolishes the myogenic functions without preventing the protein's effect on cell growth (Crescenzi *et al.*, 1990; Davis *et al.*, 1990; Sorrentino *et al.*, 1990). Figure 5 shows that, after high-titer retrovirus infection, this mutant does not significantly increase the percentage of apoptotic cells with respect to mock-infected control or after the shift to low serum medium. This suggests that the differentiation activity of MyoD can promote apopotosis in PyLT-expressing myoblasts.

MyoD Overexpression Induces Abortive Differentiation in PyLT-expressing Myoblasts

Terminal differentiation induced by myogenic factors is normally characterized by the activation of muscle-specific genes and, concomitantly, by increased levels and functional activities of inhibitors of cell proliferation such as RB and the cdk inhibitor p21. Moreover, the levels of some cyclins and cdks are also down-regulated (Rao *et al.*, 1994; Guo *et al.*, 1995) so that several mechanisms assure that cell cycle progression is efficiently and permanently blocked in differentiating cells. To analyze the functional activity of MyoD in LT.N2 cells, in which the attempt to force differentiation causes apoptosis, we determined the expression pattern of several differentiation markers and cell cycle-regulatory genes after MyoD retrovirus infection. As shown in Figure 6A, MyoD overexpression is able to induce LT.N2, as well as C2 cells, to accumulate early differentiation markers, such as myogenin, RB, and p21, already in proliferative conditions. Later differentiation markers, such as MHC, are also induced, but only after the shift to differentiation medium. Immunofluorescence analysis demonstrates that almost the totality of cells express MHC in these conditions although their morphology is abnormal with respect to the large multinucleated myotubes formed by C2 cells (see also Figure 8). As shown in Figure 6B, the levels of the muscle-specific protein were comparable in surviving and in dying cells, as determined by Western blot of attached and detached cells separately analyzed. This finding is consistent with the activation of differentiation pathways in dying cells.

To gain insights into the status of the cell cycle-regulatory apparatus of LT.N2 cells during differentiation-induced apoptosis, we determined the levels of some cyclins and cdks, known to be regulated concomitantly with the onset of differentiation when cells arrest in the G_0/G_1 phase of the cell cycle. Figure 7 shows, as expected, that cyclin D1 and cyclin A are down regulated in C2 cells after the shift to differentiation medium or, even more, after MyoD retrovirus infection. LT.N2 cells overexpress all these cyclins,

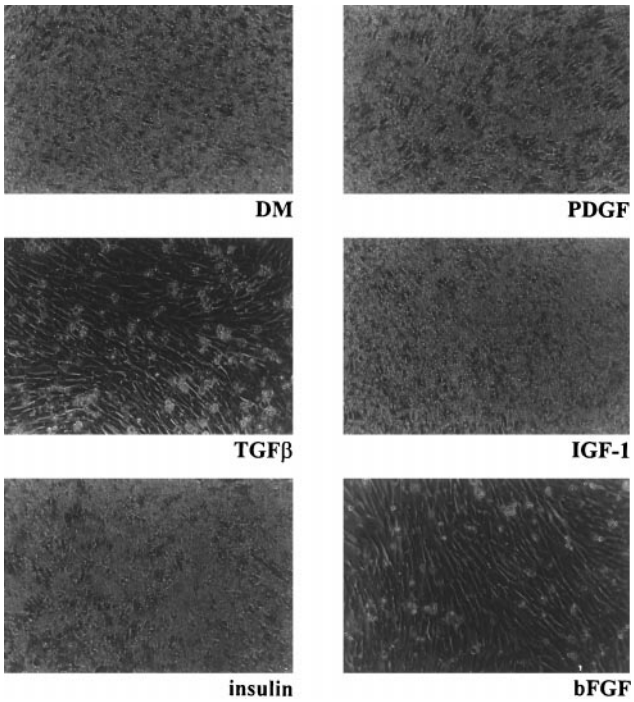


Figure 2. Apoptosis in PyLT-expressing myoblasts cells is inhibited by bFGF and TGFβ. Phase contrast micrographs of LT.N2 cells 24 h after the shift to differentiation medium, either alone or supplemented with each of the indicated growth factors: PDGF (50 ng/ml), TGFβ (10 ng/ml), IGFI (50 ng/ml), insulin (10 μg/ml), or bFGF (50 ng/ml).

Table 1. Effects of growth factors on DNA synthesis in LT.N2 cells

Treatment	BrdU-positive (%)
None (0.5% FCS)	23 ± 3
bFGF (50 ng/ml)	47 ± 4
TGFβ (10 ng/ml)	29 ± 5
IGF-I (50 ng/ml)	48 ± 2
Insulin (10 μg/ml)	53 ± 4

LT.N2 cells, grown to confluence, were shifted to DM, either alone or supplemented with each of the indicated growth factors. Twenty-four hours later they were fixed, after 1 h of BrdU incorporation. BrdU was detected by immunofluorescence staining and the percentage of positive nuclei was calculated with respect to total nuclei, visualized by DAPI staining. At least 400 nuclei were counted for each sample, and the results are the mean of three independent experiments.

with respect to parental cells, in growth medium. In addition, they fail to down-regulate cyclins E and A, even in the presence of the strongest differentiation stimulus, that is MyoD overexpression followed by the shift to differentiation medium. The major catalytic subunit regulated by cyclins A and E, cdk2, is also expressed by LT.N2 cells and at higher level with respect to C2 cells. Interestingly, cdc2 kinase, involved in the entry into mitosis and strongly down-regulated during C2 differentiation (Wang and Nadal-Ginard, 1995), persists unaltered in LT.N2 cells. Single-cell analysis, by immunofluorescence staining, showed that the majority of cells simultaneously express high levels of cyclins, cdks, and their inhibitors (our unpublished observations). The activity of cyclin–cdk complexes in this system has not yet been determined. However, the concomitant overexpression of markers of different phases of the cell cycle is no doubt indicative of an abnormal cell cycle progression. To analyze the ability of differentiated LT.N2 cells to synthesize DNA, cells were infected with MyoD retrovirus and, 48 h later, shifted to differentiation medium. After an additional 24 h, they were analyzed by double immunofluorescence staining for MHC expression and in-

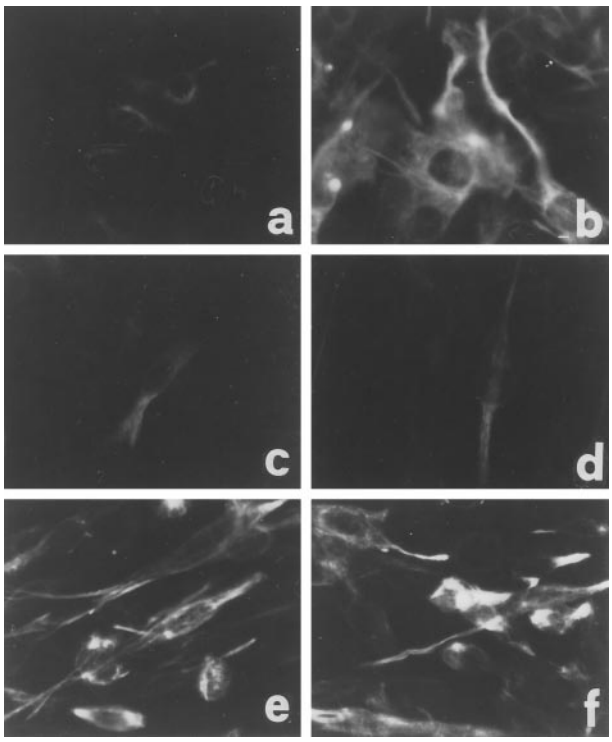


Figure 3. The onset of differentiation in LT.N2 cells is prevented only by bFGF and TGFβ. Immunofluorescence staining with anti-desmin antibodies performed on LT.N2 cells kept in growth medium (a) and 24 h after the shift to differentiation medium either alone (b) or supplemented with 50 ng/ml bFGF (c), 10 ng/ml TGFβ (d), 50 ng/ml IGF-I (e), or 10 μg/ml insulin (f).

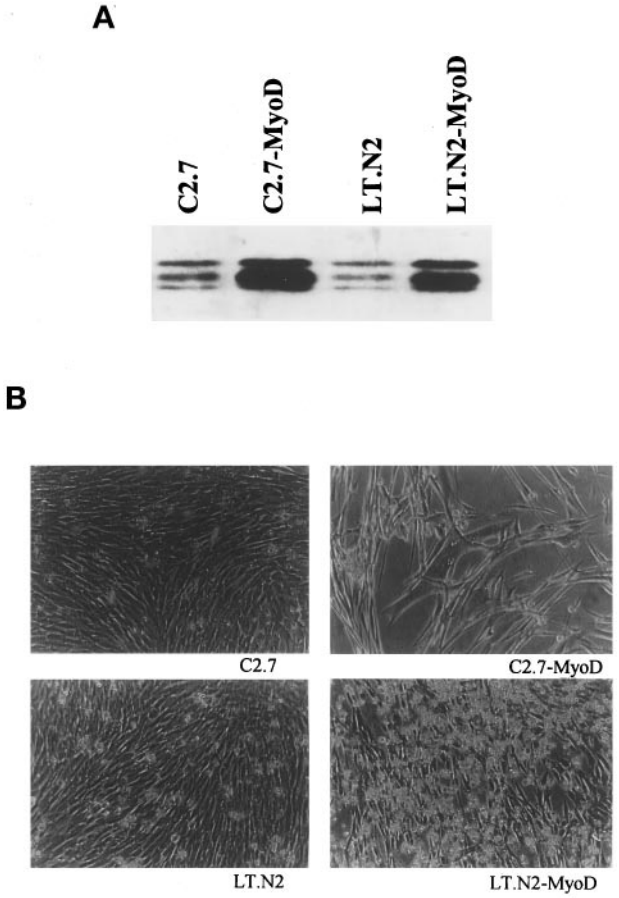


Figure 4. MyoD overexpression triggers cell death in PyLT-expressing myoblasts. (A) Western blot analysis showing MyoD levels in C2.7 and LT.N2 cells 48 h after infection with either pBabeMyoD retrovirus (C2.7-MyoD and LT.N2-MyoD) or with an empty vector as controls (C2.7 and LT.N2). (B) Phase contrast micrographs of C2.7 (a and b) and LT.N2, kept 48 h in 10% FCS after pBabeMyoD-infection or mock infection.

Table 2. MyoD overexpression increases the kinetics of cell death in LT.N2 cells

	0[a]	3	6	9
Mock	0.5%	3%	5.2%	12%
MyoD	5%	19%	26%	36%

LT.N2 cells were either infected with MyoD recombinant retrovirus or mock-infected. Forty-eight hours later they were shifted to DM and, at different times, the percentage of nonviable cells was determined by Trypan blue staining.

[a]Numbers indicate the hours after shift to low-serum medium.

corporation of BrdU (added to culture medium for the last 5 h). Table 3 and Figure 8 show not only a much higher percentage of BrdU-positive nuclei in LT.N2 with respect to C2 cells, but also the occurrence of DNA synthesis in MHC-positive LT.N2 cells (whereas in the control it is only detected in MHC-negative cells). All these data indicate that increased differentiation stimuli in PyLT-expressing cells, obtained by myogenic factor overexpression, are able to overcome, at least in part, the block of muscle-specific gene expression, but do not prevent a disorderly cell cycle progression. This also provides evidence that apoptosis occurs in cells undergoing differentiation in the absence of a correct cell cycle arrest.

DISCUSSION

In previous reports we described that PyLT oncogene inhibits terminal differentiation of C2 myoblast cells (Maione *et al.*, 1992a–1994). Here we show that the abnormal transition from a growing myoblast to a terminally differentiated muscle cell is converted into an apoptotic response. PyLT-expressing LT.N2 myoblast cells, when deprived of mitogens, a condition that normally triggers differentiation, undergo a phenomenon of cell death that increases with the lowering of serum concentration and prolonged cell confluence. LT.N2 cell death can be defined as apoptosis since it is characterized by nuclear condensation and by the acquisition of a hypodiploid DNA content.

The main mechanism by which PyLT is thought to affect cell growth control is the binding and the consequent inactivation of the RB antioncogene product and its related proteins (Holman *et al.*, 1994). PyLT mutants unable to bind RB lose the ability to immortalize primary cells (Larose *et al.*, 1991; Freund *et al.*, 1994) to transactivate DNA-synthesis genes (Mudrak *et al.*, 1994), to inhibit myogenic differentiation (Maione *et al.*, 1994), and to induce apoptotic cell death (this work). Our observation that RB binding by PyLT is deleterious for myoblast cell survival is in agreement with recent findings showing that functional RB and cdk inhibitors enhance the resistance of C2 myoblasts to apoptosis (Wang and Walsh, 1996; Wang *et al.*, 1997).

A deficiency in the RB growth-suppressive function or, similarly in some respects, a constitutive and enhanced E2F activity, can lead either to increased proliferation and neoplastic growth or to programmed cell death, depending on the cell context. It has been proposed that the presence of functional p53 is determinant in this choice (White, 1994). Preliminary results in our laboratory suggest that apoptosis in PyLT-expressing myoblasts does not require p53, since it is not affected by the expression of a p53 dominant-negative mutant. Work is in progress to better clarify this point.

Our results support the hypothesis that the induction of apoptosis in PyLT-expressing myoblasts involves the activity of myogenic factors. Cell death starts after mitogen removal, is promoted by cell confluence, and is temporally correlated with the expression of early markers of myogenic differentiation, some of which have been shown to be transactivated by MyoD (Li and Capetanaki, 1993; Martelli *et al.*, 1994; Halevy *et al.*, 1995). Furthermore, this apoptosis

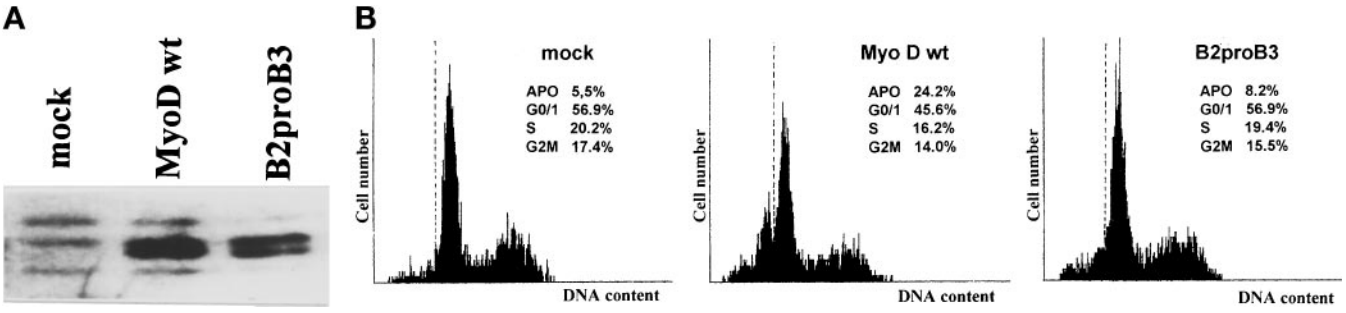


Figure 5. A nonmyogenic MyoD mutant does not affect LT.N2 cell survival. (A) Western blot analysis showing the levels of wild-type and mutant MyoD, 48 h after infection of LT.N2 cells with the empty retrovirus (mock), pBabeMyoD (wt MyoD), and pBabeB2proB3 (B2proB3). (B) Cells were infected as above and, 48 h later, shifted to differentiation medium. After an additional 6 h they were then acridine-orange stained and analyzed by flow cytometry.

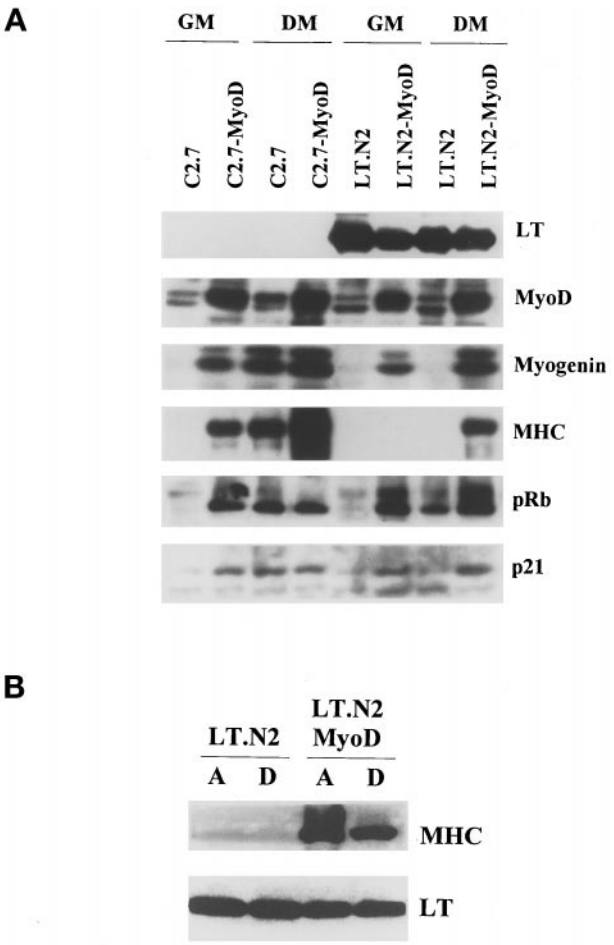


Figure 6. MyoD induces the expression of differentiation markers in LT.N2 cells. (A) C2.7 and LT.N2 cells were either infected with pBabeMyoD (C2.7-MyoD and LT.N2-MyoD) or mock-infected with the empty retrovirus (C2.7 and LT.N2) and analyzed by Western blot with antibodies specific for the indicated proteins. Cell lysates were prepared either 48 h postinfection, from cells kept in growth medium (samples GM) or after an additional 16 h in differentiation medium (samples DM). (B) LT.N2 cells were infected with pBabe MyoD retrovirus and, 48 h later, shifted to differentiation medium. After another 8 h, attached (A) and detached (D) cells were separately collected and analyzed by Western blot against PyLT and MHC.

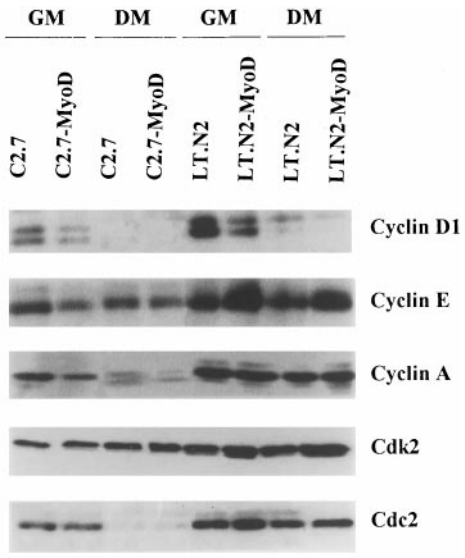


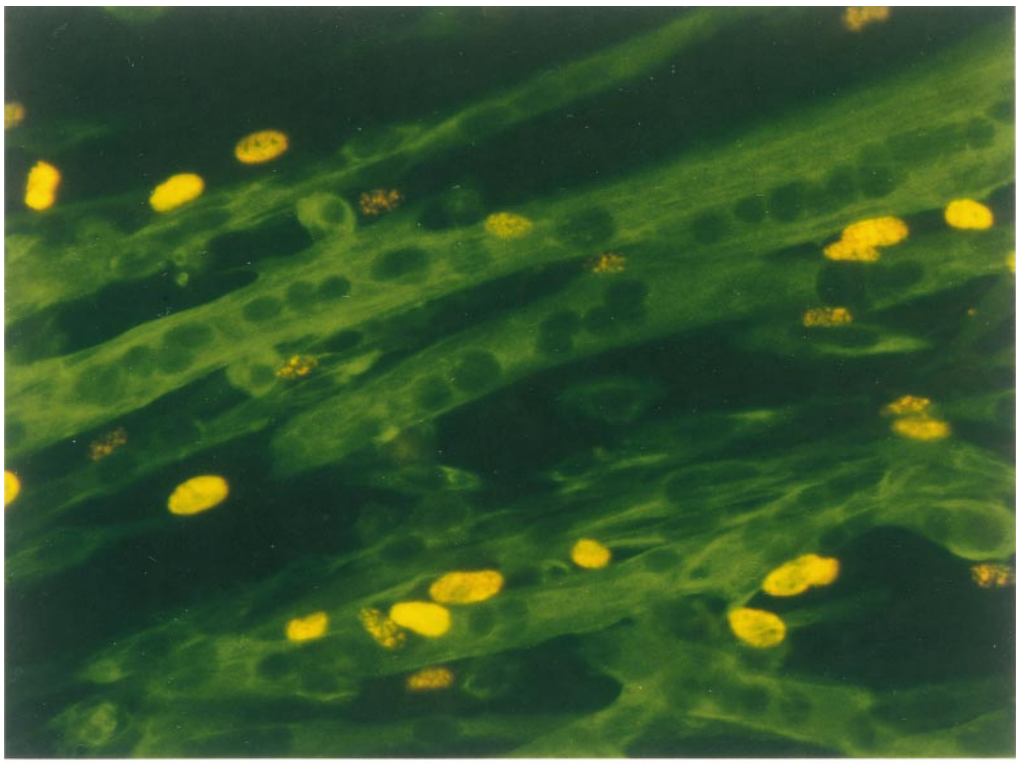
Figure 7. MyoD does not prevent the expression of cell cycle markers in PyLT-expressing cells. Cell extracts from infected cells were prepared as in Figure 6A and analyzed by Western blot against the indicated proteins.

is not inhibited by growth factors other than bFGF and TGFβ, known as strong inhibitors of both the expression and the functional activity of myogenic factors (Vaidya *et al.*, 1989). Our finding appears interesting when compared with those from other in vitro cell systems in which bFGF is much less efficient than IGF-I and PDGF in protecting cells from apoptosis (Barres *et al.*, 1992; Harrington *et al.*, 1994). In addition, TGFβ has seldom been described as a survival factor; rather, it is better known as an inducer of cell death in several epithelial cell types (Bursch *et al.*, 1993). Growth factors are known to activate multiple intracellular pathways, that affect, in a complex way, cell growth and differentiation. In LT.N2 cells the induction of proliferation does not correlate with the inhibition of apoptosis just as there is no simple correlation between the induction of proliferation and the inhibition of differentiation in muscle cells. In fact, IGF-I and insulin promote cell proliferation but do not protect from cell death, whereas TGFβ inhibits apoptosis without inducing proliferation. In a preliminary study we have also found that the overexpression of the cdk-inhibitor p16 does not impair the ability of bFGF to inhibit either C2 cell differentiation or LT.N2 cell death, although it does reduce the proliferative effect of this growth factor in both cell lines (our

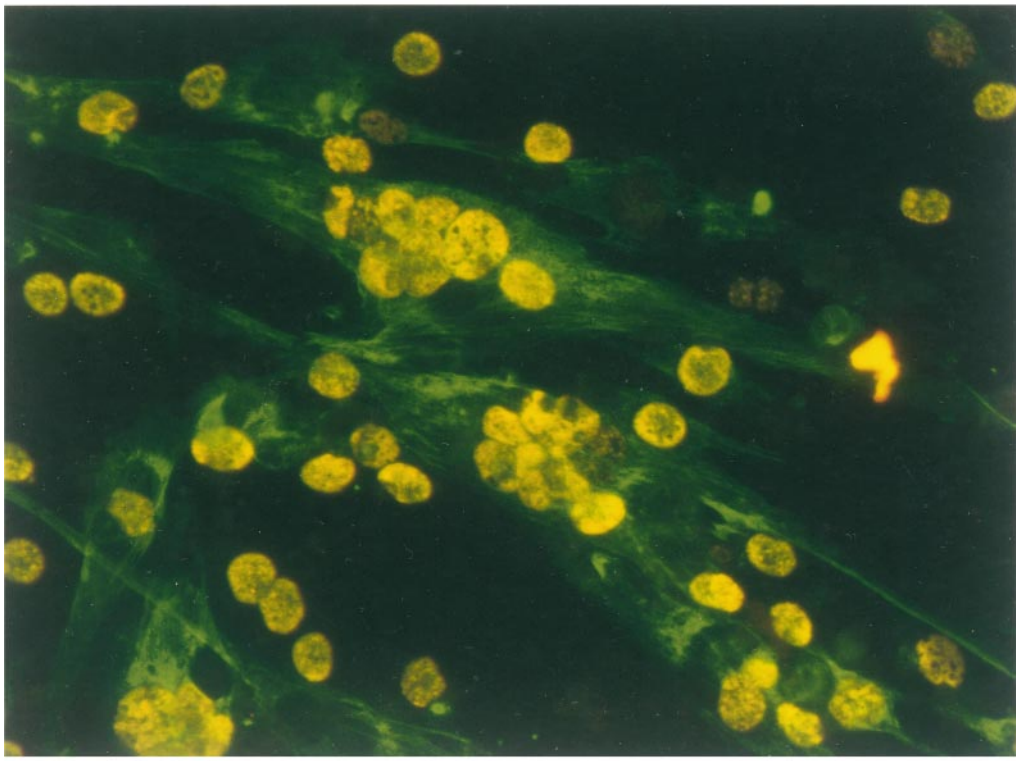
Table 3. The activation of differentiation does not prevent cell cycle progression in LT.N2 cells

	BrdU positive (%)	BrdU/MHC double positive (%)
C2.7	20 ± 2	0.2 ± 0.1
LT.N2	78 ± 6	35 ± 4

C2.7 and LT.N2 cells were infected with pBabeMyoD retrovirus and, 48 h later, shifted to DM. After another 24 h they were then analyzed by double immunofluorescence staining for MHC expression and incorporation of BrdU (added to the culture medium for the last 5 h). The percentages of BrdU-positive and BrdU/MHC double positive cells were calculated with respect to total nuclei, visualized by DAPI staining. At least 400 nuclei were counted for each sample, and the results are the mean of three independent experiments.



C2.7



LT.N2

Figure 8. DNA synthesis in differentiated LT.N2 cells. Fluorescence micrograph of a representative sample of data reported in Table 3. Green staining: MHC; orange staining: BrdU.

unpublished observations). These results support the idea that in this system growth factors inhibit cell death by preventing the initiation of differentiation.

The opposite effect has been observed upon increased differentiation stimuli: while MyoD overexpression forces growth arrest and myotube formation in C2 cells, even in growth medium, the same treatment accelerates the kinetics and increases the level of cell death in LT.N2 cells, concomitantly with the activation of both early and late markers of in vitro muscle differentiation. Furthermore, the overexpression of the MyoD mutant B2proB3, unable to induce myogenic differentiation, does not enhance LT.N2 cell death. All these findings are consistent with the hypothesis that the activation of muscle differentiation pathways is involved in the induction of apoptosis in PyLT-expressing myoblasts. The differences observed in the cell death of uninfected and MyoD-overexpressing LT.N2 cells are interpretable as a more efficient commitment to differentiation in the latter. This view is supported by the finding that in MyoD-infected cells, late markers of myogenesis, such as MHC, are also induced.

It has been recognized that the ability of MyoD to induce both differentiation and growth arrest requires the presence of functional RB (Gu *et al.*, 1993), but the exact points at which the two pathways are overlapped still need to be clarified. The two activities appear to be separately controlled in this system, as we found that MyoD, when overexpressed, can induce the expression of differentiation markers, whereas it does not prevent cell cycle progression. LT.N2 cells, when shifted to low serum medium, and even in the presence of MyoD-induced differentiation, fail to down-regulate the expression of several genes involved in cell cycle progression, such as cyclin E, cyclin A, and cdc2. Since these cell cycle regulatory genes are known to be E2F targets (DeGregori *et al.*, 1995), this is likely a consequence, at least in part, of deregulated E2F activity, as a result of RB inactivation by PyLT. The composition and the activity of cyclin–cdk complexes in this system have not yet been analyzed. However, even in the presence of high levels of p21, there is significant DNA synthesis: about 80% of LT.N2 cells, even after MyoD infection, incorporated BrdU in differentiation medium, a percentage comparable to that of asynchronously proliferating cells, if labeling is protracted for 5 h. The apparent discrepancy with the data from cytofluorometric analysis, which shows a relatively low percentage of cells with an S phase DNA content in differentiation medium, could reflect an abnormal and slow progression through the cell cycle. LT.N2 cells, after MyoD overexpression, do not appear to accumulate significantly in any phase of the cell cycle. Moreover, the combination of flow cytometry and nick-end labeling techniques shows an equal distribution of the apoptotic fraction throughout the cell cycle (our unpublished observations). The behavior of LT.N2 cells is different with respect to RB-deficient fibroblasts that, after ectopic MyoD expression, not only undergo poor differentiation, but also accumulate in S and G2/M phases of the cell cycle (Novitch *et al.*, 1996). This can be explained by obvious differences between the cell systems used. First, LT.N2 cells express a viral oncogene that is expected to affect cell growth control in a more complex manner than simply inactivating RB (for example, PyLT also interacts with the other RB family member p107 [Holman *et al.*, 1994] and has been found to bind cyclin-cdk complexes [our unpublished results]). Moreover, LT.N2 cells derive from a cell line already determined to the myoblast lineage, which shows a higher propensity to respond to differentiation signals and in which a broader spectrum of targets is expected to be transactivated by myogenic factors with respect to fibroblasts, thus likely resulting in a different mode of interference with the cell cycle progression. More work is required to define which MyoD-dependent biochemical events are involved in generating the conflict that leads to apoptosis.

It has been frequently reported that the attempt to reactivate the cell cycle in postmitotic cells or to arrest proliferation in oncogene-transformed cells results in a defective cell cycle and leads to apoptosis. Here we have shown that the attempt to induce differentiation causes cell death in PyLT-expressing myoblasts. Furthermore, our work brings the muscle-regulatory factor MyoD into the mechanism that triggers cell death in myoblast cells lacking RB function. A concomitant activation of differentiation and proliferation pathways occurs in these cells, even though the two processes do not appear accomplished, probably due to a reciprocal interference. This system is suitable not only to gain further insights into the mechanisms that render mutually exclusive cell cycle progression and muscle differentiation, but also to understand how defects in proliferation and/or differentiation are detected and converted into an apoptotic response. Our results also suggest that myogenic factors' activity can play a role in the control of muscle programmed cell death and support the idea that forcing differentiation of oncogene-transformed cells can potentiate their susceptibility to the induction of apoptosis.

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