CMLS, Cell. Mol. Life Sci. 58 (2001) 135–140
1420-682X/01/010135-06 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

T-antigen regulated expression reduces apoptosis of Tag-transformed human myoblasts

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Received 24 July 2000; received after revision 18 October 2000; accepted 9 November 2000

Abstract. The generation of human myogenic cell lines could potentially provide a valuable source for cell transplantation in myopathies. The dysregulation of proliferative-differentiative signals by viral oncogenes can result in the induction of apoptosis. Whether apoptosis occurred in myogenic cells expressing large T antigen (Tag) from SV40 upon differentiation was unknown. Human muscle satellite cells were transfected with two different constructs, containing either an origin-defective SV40 genome or Tag under vimentin promoter control. When differentiation was triggered, Tag expression reduced the formation of myotubes and dead cells showing apoptotic features were present. However, the cells expressing SV40 Tag under vimentin promoter control retained their capacity to form myotubes and expressed the myofibrillar proteins as myosin heavy chain and dystrophin when Tag expression was silent. Their apoptotic rate was similar to that of untransfected cells. The observation that apoptosis can be prevented by the down-regulation of Tag suggests that the programmed cell death induced in transformed cells can be reversed, and confirms the regulatory efficiency of the human vimentin promoter.

Key words. Myogenic cells; SV40 large T antigen; apoptosis; vimentin; myotube.

Myoblast transplantation has been proposed as possible therapy for reconstitution of new muscle tissue and also for the correction of genetic defects in muscular dystrophies [1]. Normal myoblasts injected in *mdx* mice, an animal model of Duchenne Muscular Dystrophy (DMD), fuse with dystrophic myoblasts to form hybrid myotubes, which leads to restoration of dystrophin expression [2]. Based on this observation, clinical trials were undertaken using myoblasts from immuno-compatibile and partially compatible donors in DMD patients [3]. The trial failed for several reasons, including the poor survival rate of injected myoblasts, probably related to immunorejection [4]. The immunological response might be preventable in an ex vivo approach, in which patient cells from muscle biopsy are expanded in vitro, transduced with a vector encoding the therapeutic gene, then reinjected into the diseased muscles.

Human satellite cells have a strictly limited growth potential and senesce after a defined number of replications, probably related to the progressive reduction in telomere length [5]. The amount of tissue derived from muscle biopsy is also limited, and a low number of cells

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are recovered. Dystrophic human myoblasts have decreased replicative capacity, due to the various cycles of degeneration and regeneration in which the cells exhaust their proliferative potential [6]. To overcome the limited life span of myogenic precursors, the generation of human myogenic cell lines with enhanced proliferative capacity without inhibiting myogenic differentiation could provide a valuable source for cell transplantation in myopathies.

Cell lines can be generated by the introduction of immortalizing oncogenes such as the large tumor antigen (Tag) of simian virus 40 (SV40) or polyoma virus [7]. To overcome the safety limitation due to the persistence of an oncogene, a vector in which SV40 Tag is excised by the transient expression of Cre recombinase was constructed [8]. Ectopic expression of telomerase was recently proposed to prevent telomere erosion and extend life span. However, telomerase did not significantly extend normal human myoblast survival, and immortalized only Tag-positive myoblasts [9].

The effects of this life expansion on cell behavior must be investigated if these modified cells are to be used in gene transfer. Tag has been previously described to interfere with the in vitro terminal differentiation of myoblasts by binding and inactivating the retinoblastoma antioncogene product (RB), which is implicated in suppression of cell proliferation [10]. Moreover, the polyomavirus large T antigen, an oncogene similar to SV40 Tag, has been shown to induce apoptosis in myoblasts upon differentiation [10]; this process was shown to be p53 independent [11].

The interference of Tag in myoblast differentiation can be bypassed with tight regulation of its expression and/or activity. The use of constructs containing a thermolabile Tag and promoter that can be down-regulated has already been shown to allow differentiation of myoblasts [12]. In these studies, a plasmid containing Tag under human vimentin promoter control was used [13]. Vimentin is an intermediate-filament protein expressed during proliferation of undifferentiated myoblasts and is naturally down-regulated as soon as myotubes form.

To investigate whether differentiation-associated apoptosis occurs in SV40-transformed human myoblasts, we transfected human myogenic cells with two different constructs containing the SV40 origin-defective genome or the SV40 large T antigen under human vimentin promoter control. Now we report that down-regulation of Tag during myotube formation indeed results in a drastic reduction in cell death.

Materials and methods

Plasmids. All nucleic acid manipulations were performed following standard protocols [14]. Vector pRVSneo contains the gene encoding geneticin resistance and permits selection of transfected clones. Vector pRNS (Ori$^-$SV40) was constructed by inserting the 5200 bp of the SV40 genome into pRSVneo; in this plasmid, 6 bp of the replicative origin of SV40 were deleted [15]. Vector pHuVim830$_{ts}$T/δt contains the large T antigen from SV40 plus the regulatory region (nucleotides -830 to $+93$) of the human vimentin gene [13, 16]. pHuVim830$_{ts}$T/δt was a gift of Prof. D. Paulin. All plasmids were purified by Plasmid Maxi Kit (Quiagen).

Cell culture and transfection. Human skeletal muscle cells were isolated from a diagnostic biopsy obtained from a 27-year-old man, who was ultimately deemed to be free of muscle disease, as previously described [17]. The cells were maintained at 37 °C, 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 20% fetal calf serum (FCS).

Myoblasts were seeded at 1×10^6 cells on 60-mm tissue culture dishes and transfected with LipofectAmine (Life Technologies-Gibco) according to the manufacturer's instructions.

In the first series of experiments, cells were co-transfected with 5 μg of pHuVim830$_{ts}$T/δt plasmid and 2 μg of pRSVneo. The second type of transfection was performed with 7 μg of pRNS (Ori$^-$SV40). In both cases, selective medium containing G418 (400 μg/ml) was supplied to the cells 48 h after transfection, and resistant colonies appeared after 15 days. Isolated colonies were picked, transferred to multi-well plates, and expanded. Only the clones containing more than 95% neural cell adhesion molecule (N-CAM)-positive cells were considered myoblast clones. Positive clones were further expanded in a proliferative medium (DMEM plus 15% FCS).

Growth curves were determined by plating three clones for each cell type at a density of 8×10^4 per 35-mm-diameter wells. The cells were counted after 24, 48, and 72 h, and on days 5 and 7. There were triplicate dishes for each day for each clone. Cells were counted in triplicate using a hemacytometer and numbers averaged.

Number of divisions is expressed as mean population doublings (MPD). MPD always refers to the total number of MPD from the time after the initial population was isolated including the division made before transfection for the transfected cells.

The percentage of fusion was estimated by counting the number of nuclei in multinucleated cells and expressed as a percentage of the total number of nuclei. Three clones for each cell type were counted in triplicate. To induce differentiation, cells were grown to confluence and then shifted to a medium containing a low serum concentration (DMEM plus 0.5% FCS). This determination was made after 8 days of differentiative medium.

Immunocytochemistry analysis. Cells were rinsed with phosphate-buffered saline (PBS) and fixed for 10 min in

90% methanol at −20 °C. They were then incubated overnight at 4 °C with the following primary antibodies: monoclonal desmin (at 1/40 dilution, DE-R-11; Novocastra, Newcastle-upon-Tyne, U.K.); anti-fast myosin heavy chain (1/40 dilution WB-MHCf; Novocastra), and anti-human dystrophin (1/40 dilution, NCLDys3; Novocastra). A monoclonal anti-Tag antibody was raised against purified Tag antigen (gift of Prof. D. Paulin).

After washing with PBS, cells were incubated with the secondary fluorochrome (FITC-conjugated) antibody diluted in PBS, washed twice with PBS (1/40) and mounted with 70% glycerol in PBS. Cells were observed under phase contrast and epifluorescence.

For N-CAM detection (anti-N-CAM clone H28-123-16; Boehringer Mannheim), immunoperoxidase staining was performed using an avidin biotin peroxidase complex as previously described [18]. The immunocytochemical results were evaluated in all selected clones, with triplicate determinations in three independent experiments.

Apoptosis analysis. Cell viability and chromatin condensation were examined by staining for 10 min with 1 μg/ml DNA-binding fluorochrome 4′,6′-diamine-2′-phenylindole dihydrochloride (DAPI; Boehringer Mannheim). The slides were washed and mounted with 50% glycerol in PBS. Samples were analyzed under phase contrast and appropriate fluorescent light. This analysis was performed in all selected clones, with triplicate determinations in three independent experiments 24 and 48 h after the shift to the lower-serum condition.

To examine nucleosome laddering, an equal number of cells was seeded in 60-mm-diameter culture dishes. At different times after starvation, both attached and floating cells were collected, centrifuged, and washed with PBS twice. Cells were lysed with cold Tris/EDTA buffer (5 mM Tris-HCl, pH 8, 10 mM EDTA) containing 0.5% Triton. The lysate was immediately centrifuged at 13,000 *g* for 20 min, and supernatant, containing fragmented DNA, was collected and incubated for 1 h at 37 °C in the presence of 20 μg/ml Rnase A. Proteinase K was then added to the mixture adjusted to 100 μg/ml in 1% SDS and incubated for 3 h at 50 °C. Proteins were removed by phenol-chloroform extraction, and DNA precipitated with 1/20 vol of 3 M sodium acetate and 2 vol of 96% ethanol at −20 °C. Samples were resuspended in distilled water and electrophoresis was performed in 1.5% agarose for 60 min at 80 V. DNA was visualized under ultraviolet light after staining with ethidium bromide.

Results

Myoblasts obtained from primary cultures were transfected with the plasmid containing Ori⁻SV40 or pHuVim830$_{ts}$T/δt. The transformed cells were then cloned and ten clones expressing desmin and N-CAM-positive staining (> 90%) for each plasmid were selected and further expanded.

Proliferative capacity was higher in Tag-expressing than in untransfected cells: up to 40 population doublings, the doubling time was 32 ± 1 h (mean ± SD) for Ori⁻SV40 cells and 36 ± 1 h for pHuVim830$_{ts}$T/δt cells, while the doubling time of untransfected cells was 40 ± 5 h at the earliest serial passage. After 40 MPD, the Tag-expressing clones had a doubling time of 42 ± 3 h, while the untransfected cells reached senescence with a doubling time of 100 ± 8 h.

Tag cellular expression was evaluated by immunocytochemistry. In permissive conditions (growth medium), all myoblasts transfected with Ori⁻SV40 and pHuVim830$_{ts}$T/δt expressed the large T antigen. After 48 h in a medium with low serum concentration, the Tag positivity of pHuVim830$_{ts}$T/δt-transfected cells was reduced to 60–70% of the initial level. All the newly formed myotubes, which do not express vimentin, were Tag negative. After 5 days in differentiative medium, only 10% non-fusing pHuVim830$_{ts}$T/δt myoblasts were Tag positive. Ori⁻SV40 Tag cells were Tag positive in both growth and differentiative medium (fig. 1a–d).

When cultured cells were switched to non-permissive conditions, the majority of cells stopped proliferating. In the Ori⁻SV40 clones, myotube formation began after 6 days in the differentiation medium. By day 8, the percentage of fusion was about 10–12% and myotubes contained 2–15 nuclei. Nonetheless, few myotubes were found to express dystrophin or fast myosin (< 0.1%), two proteins usually associated with late myoblast differentiation. In the pHuVim830$_{ts}$T/δt cells, the percentage of fusion was higher (28–32%); 5–6% pHuVim830$_{ts}$T/δt myotubes expressed dystrophin and 1% myotubes also expressed fast myosin (fig. 1e, f).

Tag antigen has previously been demonstrated to affect myotube fusion and interfere with adult myosin heavy chain expression, and here we show that in differentiative conditions, a fraction of Tag-expressing myoblasts undergoes cell death with apoptotic features (figs 1g, h, i, l, 2).

We observed that floating cells began to appear in Ori⁻SV40 cultures a few hours after reduction of serum and reached maximum levels between 24 and 48 h. pHuVim830$_{ts}$T/δt cells also underwent some cell death, but with a percentage similar to untransfected cells. These experiments were performed at 23–25 MPD. The typical apoptotic features of nuclear condensation and fragmentation were present in these cells, although at different proportions (figs 1g, h, i, l, 2). The percentage of apoptotic cells showing nuclear condensation with DAPI was 10–12% in normal and pHuVim830$_{ts}$T/δt cells, and 20–22% in Ori⁻SV40 clones after 48 h. The level of apoptosis correlates with the expression of Tag (Student t-test: $p < 0.01$).

Because the vimentin construct contains a sequence coding for a thermolabile Tag, to evaluate if temperature Tag inactivation further decreases the apoptosis rate, pHuVim830$_{ts}$T/δt cells grown at 34 °C were shifted to 39 °C and induced to differentiate. Under these conditions, we observed 10–12% of apoptotic cells, a degree of cell death comparable to that obtained at 37 °C by vimentin promoter Tag repression.

To further confirm the presence of an apoptotic process, we performed an electrophoretic analysis of DNA extracted from both Tag-expressing clones and from untransfected myoblasts as negative control. Internucleosomal DNA degradation was observed by a clear pattern of ladder formation in Ori$^-$SV40-transfected cells kept in differentiative medium for 24 h, while only faint bands were seen in normal and pHuVim830$_{ts}$T/δt cells (fig. 2). Two different clones for each type of transfection were analyzed.

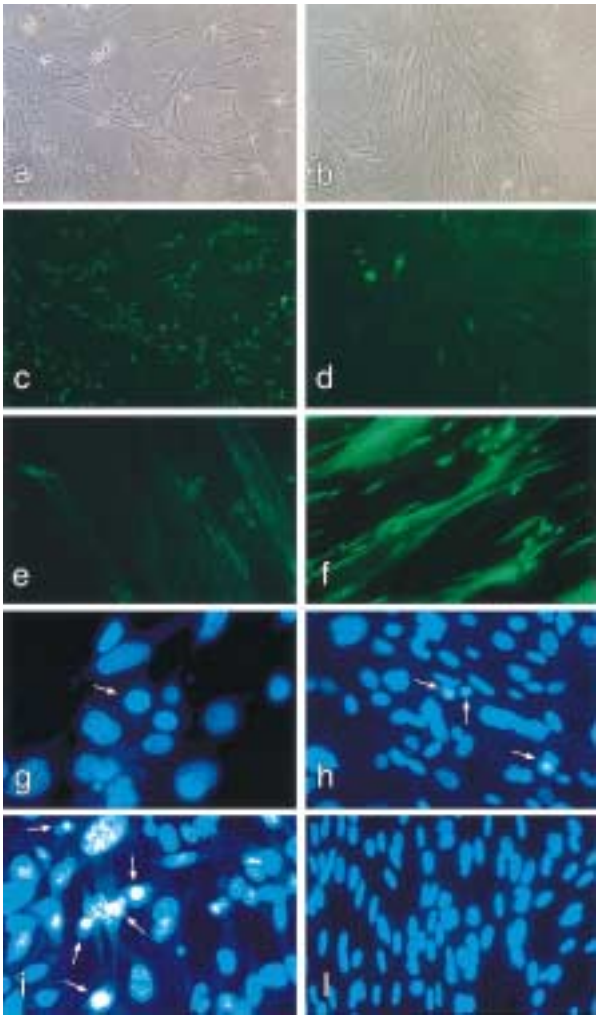

Figure 1. (*a*, *b*) Phase contrast images of *c*, *d*. (*c*–*i*) Immunocytochemistry analysis of pRNS(Ori$^-$SV40)-transfected cells (*c*, *e*, *g*, *i*) and of pHuVim830$_{ts}$T/δt-transfected cells (*d*, *f*, *h*, *l*). Cells were kept for 7 days in differentiative medium and stained with a monoclonal antibody against large T antigen (*c*, *d*) (× 20) and with an anti-fast myosin heavy chain antibody (*e*, *f*) (× 20). DAPI-stained myoblasts showing nuclear chromatin condensation after 48 h from serum reduction to induce differentiation (*g*, *h*, *i*, *l*); arrows show positive apoptotic cells.

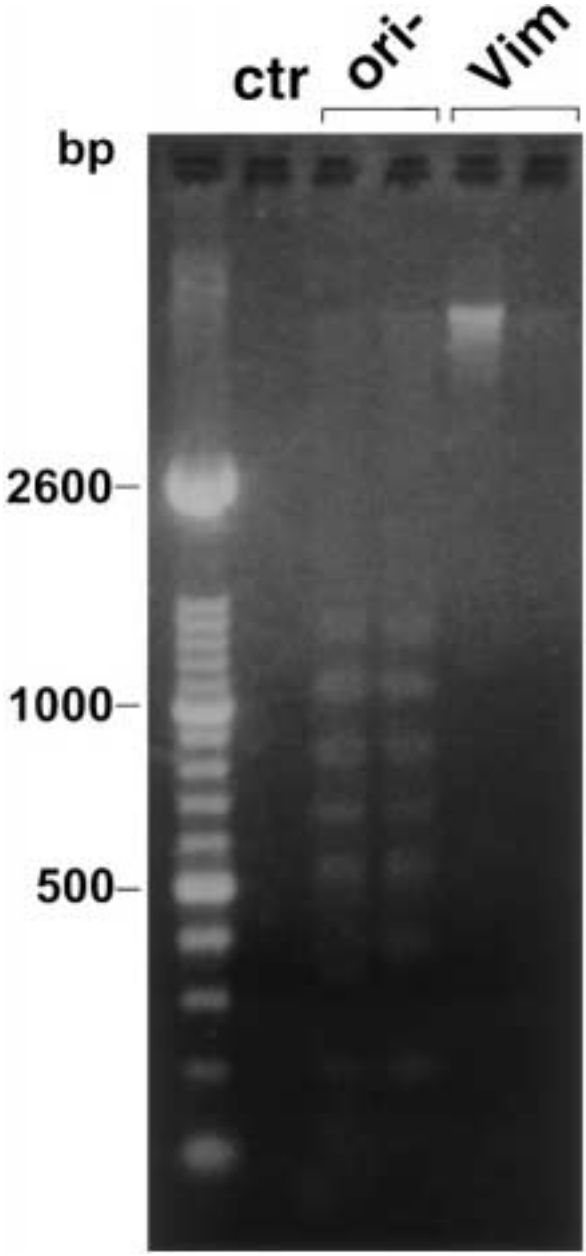

Figure 2. Profile of total DNA isolated from cells kept in differentiative medium for 48 h. Control (ctr) lane represents DNA from untransfected cells. Two different clones for each type of transfection are shown. Molecular-size markers are indicated on the left.

Discussion

Proteins from the retinoblastoma (RB) tumor suppressor family bind myogenic factors such as MyoD and myogenin, keeping differentiated myotubes out of the cell cycle [19] and promoting the establishment of a full myogenic program [20, 21]. SV40 Tag and other tumor virus oncoproteins such as adenovirus E1A disrupt this equilibrium by binding and inactivating the retinoblastoma tumor suppressors (RB, p107, p130) and the unrelated protein p300 [22], leading to impaired differentiation [23]. Dysregulation of proliferation/differentiation processes is supposed to lead to programmed cell death, resulting from an unbalanced cell cycle progression in the presence of a contrasting signal that compels differentiation. C2 mouse myoblast cells expressing the polyomavirus Tag (PyTag), a protein that resembles SV40 Tag, are unable to terminally differentiate, despite their ability to perform some early steps of differentiation [10]. This inability to differentiate was later shown to result in an apoptotic cell death [11]. Apoptosis in PyTAg mouse myoblast cells starts after growth factor removal, is promoted by cell confluence, and correlates with expression of early markers of myogenic differentiation. Moreover, the presence of growth factors was shown to prevent apoptosis, while the forced expression of myogenic factors such as MyoD increased cell death [10]. This process has been shown to be p53 independent [11].

Whether apoptosis occurred in SV40 Tag myogenic cells upon differentiation was unknown. We thus examined human myogenic cells transformed with two different plasmids containing the SV40 T antigen. A higher proliferative capacity and a shorter doubling time than in primary untransfected myoblasts were found in both cell lines. This proliferative advantage is probably due to Tag binding to the protein products of the retinoblastoma gene [22].

In fact, this binding also interferes with the terminal differentiation of skeletal myoblasts. In the immortalized cells, the ability to fuse, to differentiate into myotubes, and to express myofibrillar proteins is reduced [24]. Interestingly, apoptotic features were only observed in the cells that could not down-regulate Tag expression upon differentiation (Ori$^-$SV40 Tag cells). We thus confirm that down-regulation of Tag using the transcriptional control of the human vimentin promoter allows a partially normal myogenic behavior [24]. The pHuVim830$_{ts}$T/δt cells showed a lower percentage of fusion than the untransfected ones, probably because T antigen expression was not completely abolished. We also studied the expression of specific differentiation markers and observed that the expression of dystrophin and fast myosin heavy chain was almost absent in the Ori$^-$SV40 cells, and weak in the pHuVim830$_{ts}$T/δt cells, indicating that Tag had modified the differentiation properties of these cells. Our observations imply that low-level Tag expression slightly inhibits the myogenic differentiation program but fails to trigger apoptosis. The increased proliferative capacity obtained through Tag could be useful in cell transplantation studies. However, apoptosis could be a relevant limitation. In our study we observed that the extension in life span of pHuVim830$_{ts}$T/δt-transfected cells is compatible with a death rate similar to that of untransfected cells because of the down-regulation of Tag at the differentiative step. Thus the desired amount of cells can be obtained and then shifted to the non-permissive condition for almost normal differentiation and cell survival. We also demonstrated that the apoptosis in Tag cells is due to a direct effect of Tag in the apoptosis signal cascade and not to a collateral effect of enforced proliferation that induces early senescence and cell death, because apoptosis is strictly associated with the presence of Tag. The observation that apoptosis can be prevented by the down-regulation of Tag suggests that the programmed cell death induced in immortalized cells can be reversed, and confirms the regulatory efficiency of the human vimentin promoter. This procedure could be useful in the derivation of cell lines from diseased human muscles.

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