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# Defective Growth In Vitro of Duchenne Muscular Dystrophy Myoblasts: The Molecular and Biochemical Basis

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**Abstract** As the molecular basis of Duchenne Muscular Dystrophy (DMD) was being discovered, increasing focus was placed on the mechanisms of progressive failure of myoregeneration. In this study, we propose a pathogenesis model for DMD, where an autocrine growth factor release of TGF-β1—from necrotic myofibers—could contribute to the increasing loss of muscle regeneration. In fact, we report evidence that DMD myoblasts reduce their proliferation rate, in time and later cultures; in connection with this, we observed TGF-β1 increase in conditioned media of DMD myoblasts, able to control the myoblast growth by reducing fusion and differentiation of DMD satellite cells. J. Cell. Biochem. 76:118–132, 1999. © 1999 Wiley-Liss, Inc.

**Key words:** Duchenne muscular dystrophy; muscle regeneration; myoblasts; TGF-β1 autocrine release

Ten years ago, the identification of the DYS gene [Monaco et al., 1986] and of its protein product—the dystrophin—permitted a better understanding of the pathophysiology of Duchenne muscular dystrophy (DMD) [Ozawa et al., 1995; Bonnemann et al., 1996]. It is now clear that the deficiency of dystrophin, an important component of the cytoskeleton membrane, causes the membrane fragility leading to necrosis of myofibers, with abnormal passage of biological molecules in and out of the myofiber [Campbell and Kahl, 1989]. On the other hand, it appears quite convincingly that the progressive muscular weakness of patients suffering from dystrophinopathies, most severe in DMD, directly correlates with the progressive loss of myofibers, accompanied by connective and adipous tissue hypertrophy. The mechanism underlying this unceasing progression, however, until now has remained so far a mystery. Among current theories is one maintaining that in human dystrophinopathies there is an alteration of the inherent repair capacity potential of skeletal muscles based on satellite cell recruitment in the proliferation cycle. The extent of necrosis does not appear to be the cause, since many examples demonstrate that large areas of necrosis—as occurs in mdx mice—or even entire muscle necrosis (ischemized extensor digitorum longus [EDL] of rat) can be completely repaired in a short time. Some in vitro studies are consistent with the assumption that the progressive loss of muscle fibers in DMD results from proliferative senescence of satellite cells, but this has not been critically evaluated. In particular, these investigations do not provide important information on the developmental pathobiology of the disease and do not establish conclusively whether, and to what extent, the genetic-epigenetic interactions ultimately result in the failure of myorigeneration and in the excessive proliferation of fibroblasts.

Early in the 1990s, searching for the myotrophic factors released by ischemized small muscles of control rat—which regenerate entirely in 2 weeks—we found that C2 cell (mouse muscle satellite cells), and 3T3 fibroblasts can

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be recruited into the cell cycle by exposure to saline muscular extracts of rats, obtained by previously described methods [Bischoff, 1986; Melone et al., 1991]. Subsequently, we found that saline extracts from gently crushed human control muscles contain potent mitogenic activities affecting C2 cells, as well as primary human myoblast cultures. This human muscle growth factor (which may happen to be more than one) has not yet been purified. It acts as competence factors to commit C2 quiescent satellite cells to enter the cell cycle, but also as progression factor, because it is constantly required, to sustain proliferation [Melone et al., 1995; Cotrufo et al., 1995, Congress communications]. Fibroblast growth factor (FGF) and insulin growth factor (IGF) are found to be candidates as competence and progression factors, respectively; in fact, the growth activity in saline muscular extracts is partially reduced by neutralizing antibodies to FGF and/or IGF [Melone et al., unpublished data]. Obviously, saline muscular extracts may contain many other factors modulating satellite cell growth [Chenn et al, 1994; Haugk et al., 1995; Hodgkinson et al., 1995]; in particular, recent studies have identified several factors inhibiting satellite cell proliferation. The best-studied negative polypeptide growth factor is transforming growth factor-beta1 (TGF-β1), which displays wide arrays of biological activities, depending on the cell type involved. In fact, TGF-β1 can suppress division and totally block fusion of rat satellite cells in serum-medium [Allen and Boxhorn, 1987] perhaps, via suppression of c-myc protoncogene expression [Moses et al.,1990; Pietenpol et al., 1990, 1991]; in other myogenic cell lines, TGF-β1 can inhibit myogenic differentiation, without effect on cell proliferation [Massague et al., 1986]. Furthemore, TGF-β1 can stimulate fibroblasts to proliferate, migrate and form an extracellular matrix, all critical events for wound healing [Moses et al., 1990].

First, the aim of the our research was to verify whether extracts from DMD muscles showed the same mitogenic activity as already observed with extracts from human control muscles.

In this study, the behaviour in vitro of DMD myoblasts and control myoblasts, grown in primary cultures in presence of "growth factors" obtained from DMD muscle specimens, was investigated.

Second, in the complex pathway ranging from growth factors and their receptors to transcription factors, we studied the proteins encoded by proto-oncogenes c-myc and c-Ha-ras, that are activated in response to mitogenic stimuli.

Previous studies have demonstrated that TGF-β1 can inhibit both proliferation and differentiation of myoblasts, as mentioned above. Moreover, TGF-β1 has been shown to be secreted, at least under specialized conditions, by cultured myoblasts. For this reason, we have suggested that TGF-β1 might be a good candidate as an autocrine factor able to modulate myoblast proliferation and differentiation; here we presented evidences confirming the hypothesis.

Furthermore, isolated myoblasts were used to produce "conditioned" media to determine the contribution of autocrine production of TGF-β1 to myoblasts undergoing proliferation and differentiation (by activating muscle-specific regulatory factors of the family of basic-helix-loop-helix DNA binding proteins).

We report here evidence that TGF-β1 released from DMD myoblasts, in the later stages of cultures, might control the growth by reducing fusion and differentiation of DMD satellite cells, thus playing a role in DMD muscle regeneration.

## MATERIALS AND METHODS

### Human Primary Cultures

Muscle samples were obtained from control donors (n = 10) and DMD patients (n = 9) matched by age, ranging from 4.2 to 5.5 years, undergoing with corrective orthopedic surgery; genetic diagnoses were acquired for all patients. Primary cell cultures were prepared from all donors, both control and DMD patients. Myoblasts and fibroblasts were cultured from muscle explants; to effect dissociation, fragments smaller than 3 $mm^3$ were taken from the vastus lateralis or medialis or sartorius surgically operated on and freed of excess connective tissue. The muscles of DMD patients were macroscopically free of fibrosis and the histological findings microscopically confirmed this evaluation. The fragments were seeded onto Falcon dish in a small volume of Dulbecco's modified Eagle's medium (DMEM) with a high concentration (40–50%) of foetal calf serum (FCS)—the same batch (from GIBCO BRL, Life Technologies) was employed for all the experiments.

Once the fragments had adhered to the plastic surface, the first outgrowth of fibroblasts usually followed. When the cells covered at least 50% of the growth surface, the explants were picked off from the center of the outgrowth, minced to obtain fragments smaller than 1 $mm^3$, and transferred to fresh 60-mm plates, coated with either 0.01% human type I collagen or 1% gelatin. Myoblasts—plated at a density approximating the expected satellite cell number in human muscle ($10^7/cm^3$)—were allowed to grow to 60–70% (~3 weeks) in a serum mitogen-rich growth medium, containing DMEM nutrient mixture supplemented with 3.7 g/l sodium bicarbonate, 20% foetal calf serum (FCS), 2 mM glutamine, 0.5% chick embryo extract (CEE), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. The 20% FCS contained 2 nM insulin and 5 mM glucose. To subculture, cells were trypsinized and passaged at density of $1 \times 10^4$ cells in 35-mm coated culture plates. The original monolayer was designated by the first cell population doubling. Subsequent population doublings were calculated using the formula: $Nh/Np = 2^x$ where Nh is the number of cells harvested, Np is the cell plating number, and x is the number of population doublings.

### Cytofluorimetry

Cultures obtained from muscle explants, were washed three times in a warm medium, well drained, and incubated with 5 ml of 0.6 mM EDTA (1/5,000 Versene, GIBCO, Grand Island) at 37°C for 13–15 min. The adherent cells were then vigorously washed off the plates, resedimented, washed in medium, and resuspended to $10^6$ cells/ml in cold PBS containing 2% (w/v) bovine serum albumin (PBS-BSA) for FACS analysis, as previously described [Webster et al., 1988] with some modifications. Briefly, cells were examined in a Becton Dickinson cytofluorograph (FACScan, Mountain View, CA), using the 488-nm emission of an argon laser—to determine the gating parameters for the final sorting procedure; an aliquot of the cell suspension was analyzed for forward light scatter (size), perpendicular light scatter (nucleocytoplasmic ratio), and propidium iodide staining (dead cells). Live cells were those without bright propidium iodide staining and with sufficient forward scatter to exclude remaining cellular debris. Thereafter, the viable cells were displayed on a graph combining forward versus perpendicular light scatter. Isodensity lines of the contour plot defined two populations of cells—small cells with a lower nucleocytoplasmic ratio, and large cells with a higher nucleocytoplasmic ratio.

### Immunofluorescent Labeling

In order to determine whether one of these two populations of cells—small and large—might correspond to muscle satellite cells, 25 μl of fixed and permeabilized cells and 10 μl of FITC-conjugated human monoclonal antibody Leu19 (MoAb-Leu19; Becton Dickinson, Milano, Italy) were incubated for 30 min at 4°C, washed three times in PBS-BSA at 4°C and resuspended in 100 μl of the same buffer. The specificity of Leu19 for an antigen of satellite cells, which are the stem cells of muscle regeneration, has already been described [Schubert et al., 1989].

The fluorescence was calibrated to standard sensitivity using fluorescent polystyrene microspheres. A flow rate of 1,000 cells/sec was optimal to our purposes. Control cell samples not stained with specific antibody allowed determination of the proper FACScan gates to distinguish positive from negative cells in each staining procedure. The percentages of fluorescence-positive cells and the mean fluorescence and scatter values of the fluorescence-positive cells were determined using Lysis II software. The proportion of each cell type obtained was determined by integration over selected regions of the multiparameter data space. We concluded, from this method, that small cells corresponded to muscle satellite cells, whereas large cells were fibroblasts, according to previous data [Baroffio et al., 1993].

### Preparation of DMD Muscular Extracts (DMDMEs)

The samples of DMD muscles, weighing from 500 mg to 2.2 g, were pressed gently seven times with a pair of blunt forceps in 10 volumes of cold Dulbecco's PBS and incubated at 4°C with gentle shaking for 90 min. Released soluble substances were collected in the supernatant after centrifugation of crushed muscles at 5,000 rpm × 10 min and filtration. The resulting supernatant was inactivated at 60°C × 30 min, centrifuged at 20,000 rpm × 30 min and filtered through a 0.22 μm Millex-GS filter (Millipore). The protein concentration in the DMD muscle extracts (DMDMEs) was determined by Bradford's method [Bradford, 1976] with BSA

(Sigma) as a standard, before testing their biological activity in cultures. The protein yield in the crude extracts was about 2.4 mg/g tissue. To determine the dose-response growth curve of myoblast cultures—both control and DMD—the DMD muscle extracts were used in 0.5% FCS DMEM, at concentrations ranging from 50 μg to 500 μg of total proteins/ml. The saturating amount of DMD muscle extracts was 250 μg/ml.

### Conditioned Medium Collection

The small mononucleated myoblasts—both control and DMD—which correspond to satellite cells, were made mitotically quiescent by serum-starvation (0.5% FCS), or grown in high serum (20% FCS) or in 0.5% FCS DMEM containing a saturating amount of DMD muscle extracts (250 μg/ml). To obtain conditioned medium (CoMe), at subconfluence, cells were maintained in 0.5% FCS DMEM for 48 h; and then stimulated with 20% FCS or with DMD muscle extracts. After 48 h, media were collected, filtered (0.45 μm), and stored at -20°C.

### DMDMEs and CoMe Myoblast Stimulation

Cells were plated in 24-well plates (Falcon) at 1–3 $\times$ $10^5$ cells/well in medium containing DMEM nutrient mixture supplemented with high serum (20% FCS) and allowed to achieve a density of 2–4 $\times$ $10^5$ cells/well. On days -2 to 0, the cells were made mitotically quiescent by serum-starvation (0.5 % FCS). On day 0, cells were introduced to DMD muscle extracts, conditioned medium (in 0.5% FCS DMEM), or 20% FCS, and harvested on different days after seeding. For testing biological activity of DMD myoblast-derived conditioned medium, control myoblasts were plated as described, in serum-starved (0.5% FCS) medium supplemented with 0.1% bovine serum albumin, for 18 h. In a lot of experiments, conditioned media were incubated (37°C, 30 min) in presence of TGF-β1 neutralizing antibodies or control antibodies, before addition to the cells.

### [$^3$H]Thymidine Incorporation

0.1 ml of quiescent cell suspension (5 $\times$ $10^4$ cells/ml) was inoculated into each well of a 96-well microtest plate (Falcon) and incubated at 37°C. During the 24 h culture period in the DMD muscle extracts, in conditioned medium ± Ab or in 20% FCS, cells were exposed to 1 μCi $^3$H-TdR (2 Ci/mmol Amersham) with 2 h pulses. Finally, cells were harvested and washed on glass fiber filters, and radioactivity was determined.

### DNA Content by Flow Cytometry

To confirm results of [$^3$H]Thd uptake, DNA content per cell was assessed by flow cytometric measurement of propidium iodide (PI) fluorescence. Fractions of cells in S phase were then analysed, estimating DNA contents [Gray et al., 1987]. Cells were passaged and harvested as described above for growth assays. At a cell density approximating 80–85%, quiescence was induced (= day -2) by serum starvation (0.5% FCS). On day 0, medium was changed to either 0.5% FCS plus DMD muscle extracts or 20% FCS. Treated cells were placed into test tubes at a concentration of 5 $\times$ $10^5$ cells/ml. Chicken erythrocytes (CRBC, Whittaker Bioproducts) were added in amounts equal to 20% of the tested cells, as internal biological standards. Samples were centrifuged at 200*g* for 5 min and the cell pellets resuspended in PBS, gently vortexed, and washed twice. Finally, 1 ml of staining solution, (0.01 M Trisma base, 700 units/liter RNAse, 0.1% Nonidet P40, 10 mM NaCl and 7.5 $\times$ $10^{-5}$ M Propidium Iodide), was added per test tube and vortexed gently. After 30 min incubation on ice water, the nuclear suspensions were fixed by the addition of 100 μl of 2% paraformaldehyde per test tube, vortexed, and stored in the dark at 4°C overnight until flow cytometric analysis. The CRBC also served as a threshold for acceptable fluorescence intensity; events having less fluorescence than CRBC nuclei were considered debris. In all samples, a total of 10,000–20,000 nuclei were recorded, and results were analysed using LYSYS software.

### Determination of Myogenic Cell Fusion Rate and of Creatine Kinase (CK) Activity

Fusion of myogenic cells was assessed by the formation of plurinucleated myotubes, and expressed as the number of nuclei included in each myotube per square centimeter. The entire dish area was counted from day 4 to 8 and only half the dish area on day 10. So, at least 2,000 nuclei from myotubes were counted at each determination on day 10.

For Creatine Kinase (CK) activity, cells were collected and sonicated in 15 mM NaCl and

1 mM EDTA, pH 7.5. CK activity was determined by CK N-acetylcystein-activated monotest.

### RNA Extraction and Semiquantitative RT-PCR

Total RNA was extracted from the cell cultures using RNAzol reagent (Biotech Lab.Inc.) according to the protocol of the manufacturer. The mRNA sequences of c-myc, MyoD, Myogenin, Id1, and Ras retrieved from the databank "Lasergene" (DNAstar Inc.), were used to select primer pairs for RT-PCR amplification, using "OLIGO.4" software (National Biosciences Inc.). The mRNA levels of the genes under analysis were then measured by RT-PCR amplification, as already reported [Galderisi et al., 1996]. Appropriate regions of hypoxantine guanosine phosphorybosyl transferase (HPRT) were used as control in RT-PCR experiments. Amplifications, carried out for 28–30 cycles, were as follows: 94°C for 1′, 57°C for 1′ and 72°C for 1′. Each RT-PCR experiment was repeated at least three times. Amplification products were electrophoresed on 2% agarose gel in 1× TAE buffer. Semiquantitative analysis of mRNA levels was carried out by "GEL DOC 1000 UV FLUORESCENT GEL DOCUMENTATION SYSTEM" (Biorad Company).

### Western Blotting

For identification of TGF-β1 in the myoblast, control myoblast and DMD myoblast aliquots were lysed in a buffer containing 1% Nonidet P40 for 30′ at 4°C. The lysates were then centrifuged for 10′ at 10,000*g* at 4°C. After centrifugation, 40 μg of each sample was loaded, electrophoresed in a 10% polyacrilamide gel, and electroblotted onto a nitrocellulose membrane. Further incubation was carried out with TGF-β1 antibody (Santa Cruz Biotechnology Inc.). Blots were then incubated with phosphatase alkaline secondary antibodies and developed in substrate solution. The relative amounts of protein bands were determined by "GEL DOC 1000 UV FLUORESCENT GEL DOCUMENTATION SYSTEM" (BioRad Company).

### TGF-β1 Assay

TGF-β1 levels were measured in medium conditioned by quiescent cells or by DMD muscle extract stimulated-DMD myoblasts and DMD muscle extract stimulated-control myoblasts, using Biotrak human TGF-β1 ELISA system of Amersham, according to the protocol of the manufacter. Owing to the possible presence of TGF-β1 in DMD muscle extracts, basal levels of TGF-β1 were determined in DMD muscle extracts. The TGF-β1 amount in the conditioned media was expressed as ng/$10^6$ cells; the sensitivity of the assay was 4 pg/ml.

## RESULTS

### Growth Activity of DMD Muscle Extracts

In the experiments listed here, we used "mitogens" and growth factors obtained with saline extraction from DMD muscles [Melone et al., 1992], according to Bischoff's procedure [Bischoff, 1986, 1990]. We tested their activity on cultures of myoblasts from control and DMD muscles, matched according to the age of the donors, versus FCS as "positive" control of growth.

Initially, experiments were performed on DMD myoblasts (DMDm) and control myoblasts (Cm) derived from the first subculture and directly stimulated with DMD muscle extracts (DMDMEs) or FCS. Both primary cells showed a practically comparable proliferation rate with each other, without any significant difference on [$^3$H]thymidine incorporation in cells stimulated with DMD muscle extracts (DMDMEs) in comparison with FCS stimulated cells (Fig. 1).

All the subsequent experiments concerning DMD myoblast and control myoblast proliferative assay were carried out on cultures allowed to proliferate for several days to obtain the semiconfluence and then cultured in 0.5% FCS medium for 48 h to induce quiescence.

In this way, GO/G1 arrest was obtained, since cell cycle analysis revealed that 95% of the cells had a GO/G1 DNA content. Semiconfluent quiescent myoblasts were replated to obtain a new subculture, and complete cell attachment was observed after 8 h. At this time, (zero time for the treatment), 90% of the cells had still a GO/G1 DNA content and this population was considered pseudosynchronized.

In these conditions, we evaluated the effects of FCS or DMD muscle extracts (DMDMEs) added to culture medium on cell proliferation and on cell cycle progression of both control myoblasts and DMD myoblasts. As shown in Figure 2, control myoblast (Cm) proliferation, in terms of [$^3$H] thymidine incorporation and cell number, was markedly affected by FCS or DMDMEs supplementation, since an increase

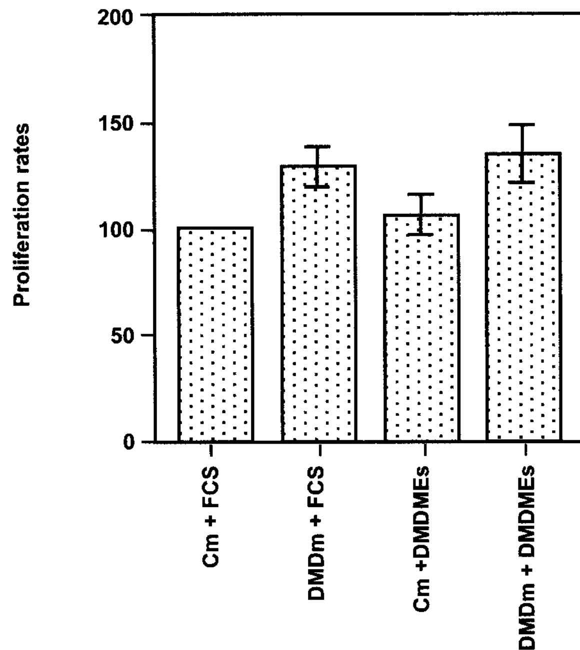



**Fig. 1.** [3H] thymidine incorporation into the first subculture of control myoblasts (Cm) or DMD myoblasts (DMDm) stimulated with 20% FCS, or DMD muscle extracts (DMDMEs). [3H] thymidine incorporation in FCS stimulated control myoblasts was 460 ± 25 dpm. Data are expressed as the mean ± SEM (each point in quadruplicate).

of both parameters was observed as early as 24 h after stimulation; the increase in the rate of proliferation was not significantly higher when DMDMEs were used instead of FCS. At 20% FCS, the calculated doubling time of the control myoblasts (Cm) was 11.7–12.2 h. The doubling time of the DMD muscle extract (DMDMEs)-stimulated control myoblasts (Cm) was shortened to 10.2–10.7 h.

When measuring the effects of FCS and DMD muscle extracts (DMDMEs) on quiescent DMD myoblasts (DMDm) (Fig. 2), an increase of DNA synthesis in DMD myoblasts (DMDm)—measured as increase of [3H] thymidine incorporation—was observed, though at a level significantly lower than in control myoblasts (Cm). Under our culture conditions with medium containing FCS, DMD myoblasts (DMDm) exponentially proliferated in a doubling time of 17.5 h. In the presence of DMD muscle extracts (DMDMEs), the apparent doubling time of DMD myoblasts (DMDm), after reaching semiconfluence,were 20.1 h.

The DMD muscle extract (DMDMEs) mitogenic effect was also assessed by flow cytometry. As shown in Figure 3, 95% of quiescent control myoblasts (Cm) was in the growth-arrested (G0/G1) phase of the cell cycle, whereas 24 h after stimulation with DMDMEs, 43% of the cells were in the S phase and 12% in the G2 + M phase of the cell cycle (Fig. 3). On the contrary, flow cytometry analysis revealed that after DMD muscle extract (DMDMEs) addition, a significant decrease of the proportion of DMD myoblasts (DMDm) with a S or G2 + M DNA content was observed ($P < 0.01$ by Student's-$t$ test).

## Effect of DMD Muscle Extracts on Myoblast Differentiation

We examined also the effect of DMD muscle extract (DMDMEs) stimulation on the terminal differentiation of the control myoblasts (Cm) and DMD myoblasts (DMDm) (Fig. 4A–C). When cultured in DMDMEs, the morphology of control myoblasts (Cm) was essentially the same as FCS-stimulated cells; after 8 days' culture, control myoblasts (Cm) stopped proliferating and formed highly multinucleated myotubes (Fig. 4A,C). On the contrary, the majority of DMD myoblasts (DMDm), cultured in medium additioned with DMDMEs, after 8 days' culture remained as "pauci"-nucleated cells and scarcely formed myotubes (Fig. 4B,C). These findings suggest that the terminal differentiation of DMD myoblasts (DMDm) into myotubes was significantly inhibited.

To define the terminal differentiation in control myoblasts (Cm) and DMD myoblasts (DMDm) biochemically, we analyzed the accumulation of CK a terminal differentiation marker enzyme—in myotubes. In control myoblasts (Cm) cultured with DMD muscle extracts (DMDMEs), the CK levels greatly increased with the progression of myogenesis. However, low CK level was detected in DMD myotube cultures (Fig. 4C).

We examined the expression levels of MyoD mRNA in the control myoblasts (Cm) cultured in presence of DMD muscle extracts (DMDMEs). The MyoD mRNA level in confluent control myoblasts (Cm) was ~7-fold over that in exponentially growing myoblasts. Three days after the induction of myogenesis, the expression level of MyoD mRNA in control myotubes was maximal (Fig. 5; lane 2). In DMD muscle extract (DMDMEs)-stimulated DMD myoblasts (DMDm), the MyoD mRNA level decreased to half of that in the control myoblasts at confluence. After the induction of differentiation, the MyoD mRNA slowly peaked on the 3rd day,

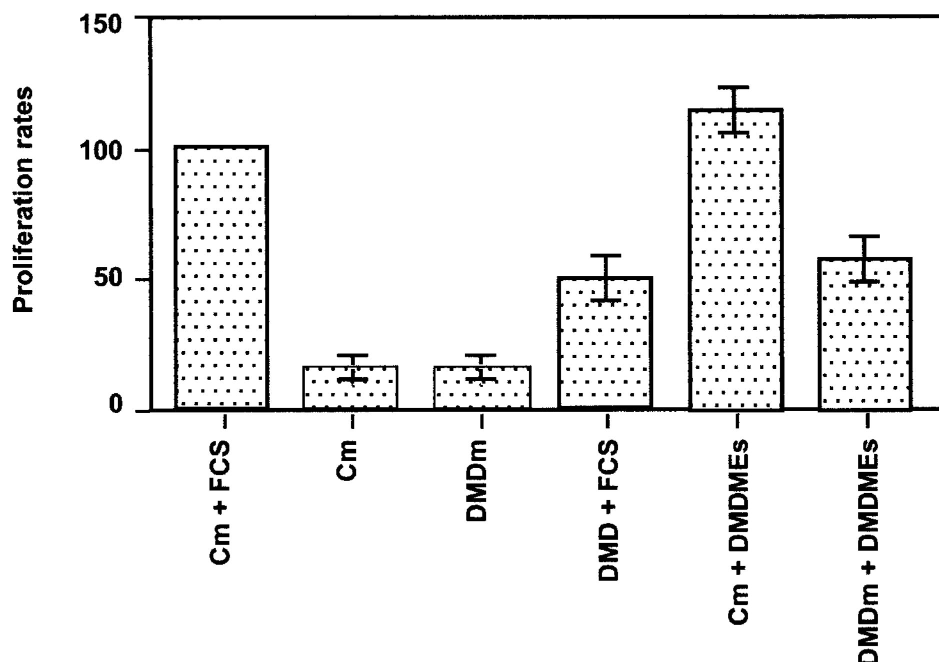



Fig. 2. [$^3$H] thymidine incorporation into the late cultures of control myoblast (Cm) or DMD myoblast (DMDm) unstimulated cells or stimulated with 20% FCS, or DMD muscle extracts (DMDMEs). [$^3$H] thymidine was added 22 h after the exposure of quiescent cells to FCS, DMD muscle extracts (DMDMEs) and incubation was continued for 2 h longer. [$^3$H] thymidine incorporation in FCS stimulated control myoblasts (Cm) was 410 ± 21 dpm. Data are expressed as the mean ± SEM (each point in quadruplicate).

reaching only 60% of the level in the control myotubes (Cm) (Fig 5; lane 1).

Next, we examined the mRNA levels for myogenin (Figs. 5, 6). The expression level of myogenin during myoblast status was extremely low in each cell culture. In control myoblasts (Cm)—stimulated by DMD muscle extracts (DMDMEs)—mRNA levels were rapidly induced and reached maximal levels on the 3rd day after the induction of differentiation (Fig. 5; lane 6). Myogenin mRNA levels in DMD myoblasts (DMDm) were also increased with myogenesis, but the level on the 3rd day after the induction of differentiation was only 65% of that in control myotubes (Cm) (Fig. 5; lane 5). As expected, Id mRNA showed, in all DMD muscle extract (DMDMEs)-exposed myoblast cultures, a level practically complementary to myogenin mRNA. In this case, Id mRNA expression on the 3rd day following the beginning of differentiation was at low level and the difference between control myotubes and DMD myotubes was not comparable to that shown in the other probes (Fig. 5; lanes 3,4; Fig. 6).

The steady-state levels of c-myc and c-Ha-ras mRNAs were first determined in control myoblasts (Cm) in the various phases of the cell cycle.

In confluent control myoblast-GO/G1 arrested cells, c-myc transcript was not detectable and c-Ha-ras was very low. Eighteen hours after seeding and DMD muscle extract (DMDMEs) stimulation (time O), c-myc and c-Ha-ras mRNA levels in control myoblasts (Cm) increased and reached a maximum 2 h later (Fig. 7A,B; lanes 1, 3). Then c-myc mRNA level decreased and remained stable up to h 10 and c-Ha-ras decreased progressively up to h 10 . According to densitometric analysis, at h 10 the c-myc mRNA level was about 35%, compared to the maximum level observed at h 2, and 20% in the c-Ha-ras transcript (Fig. 7A). This time corresponded to the entry of the cells in the S phase since 17% of the cells were in S phase at h 10 compared to 3% at h 6. At h 24, a second transcript peak for both genes was detectable (about 80% of the maximum level) and the cell population was asynchronous (40% of cells in GO/G1, 40% in S, and 20% in G2 + M phases). These results suggest that c-myc and c-Ha-ras mRNA are induced early in the G1 phase of

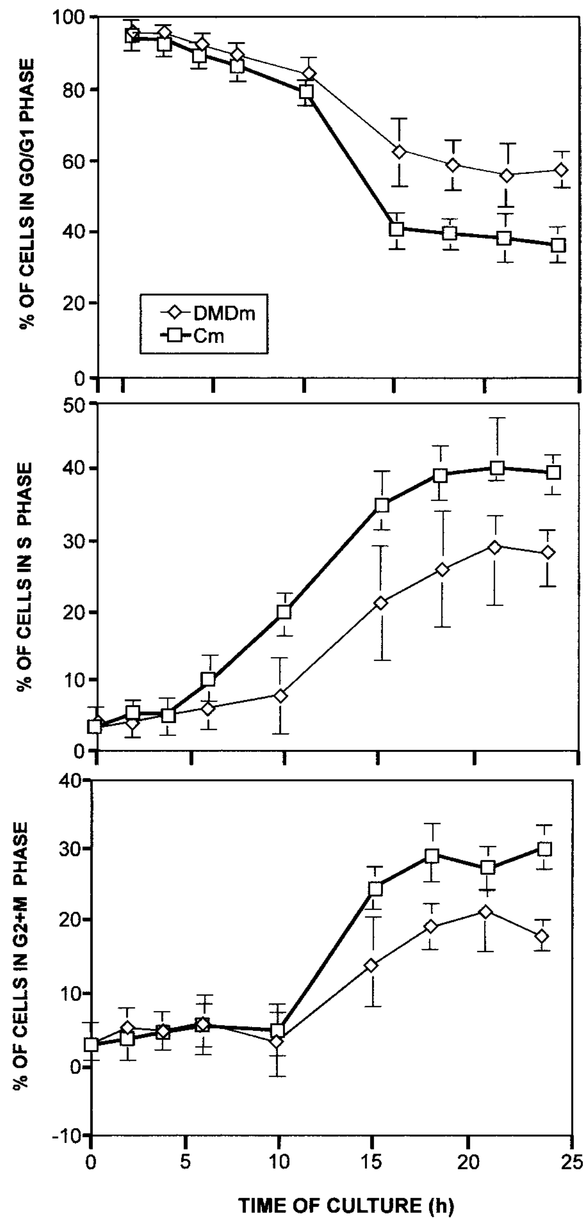



Fig. 3. Cell cycle analysis of control myoblasts (Cm) and DMD myoblasts (DMDm) stimulated by DMD muscle extracts. Cell cycle distribuition was analyzed by flow cytometric measurement of propidium iodide fluorescence. Each value is the mean of three separate experiments ± S.D.

control myoblasts (Cm) and persist during the cell cycle.

Compared to control myoblasts (Cm), DMD myoblasts (DMDm) showed a decrease in c-myc mRNA level as early as h 1, after DMD muscle extracts (DMDMEs) treatment initiation (Fig. 7A). Two hours after the stimulant addition, this decrease was even more substantial: densitometric analysis revealed a 60% decrease of c-myc mRNA level. Subsequently, this level remained low and the difference in c-myc content, between control (Cm) and DMD myoblasts (DMDm) was significant at all times considered. Twenty-four hours after the stimulant addition, the c-myc mRNA level was 30% lower in DMD myoblasts (DMDm) compared to control myoblasts (Cm). As for c-myc mRNA, Figure 7A,B (lane 4) shows that the c-Ha-ras mRNA level decreased after treatment initiation; the decrease was at its highest at h 2 (c-Ha-ras mRNA level was approximately 40% of control cell level). During the following hours (h 4 and 10), c-Ha-ras mRNA decreased progressively. At h 24, the c-Ha-ras mRNA level remained 30% lower than in control.

To determine whether FCS or DMD muscle extracts (DMDMEs) could modify c-myc and c-Ha-ras mRNA stability in DMD myoblasts (DMDm), the HTPR gene transcript was chosen, since its mRNA possesses a very high stability. Figure 7B (lanes 5, 6) revealed that the HTPR mRNA level was not modified by stimulant treatment. Taken together, these results suggest that the decrease in c-myc and c-HA-ras mRNA levels after the stimulant addition could be the consequence of changes in their transcription rates.

## Conditioned Medium Activity

To determine whether DMD myoblast (DMDm) activation by FCS or DMD muscle extracts (DMDMEs) induced the secretion of factors able to modulate cell proliferation, matched conditioned media—obtained either from serum-starved DMD myoblasts (unstimulated control cells), or from cells stimulated by FCS or DMD muscle extracts (DMDMEs)—at various times, were added to quiescent cultures of control myoblasts (Cm), and [$^3$H] thymidine incorporation was measured after 22 h. As positive controls, some of the control myoblasts were stimulated directly with either 20% FCS or DMD muscle extracts (DMDMEs). As shown in Figure 8, FCS or DMD muscle extracts (DMDMEs) stimulated significant DNA synthesis in the control myoblasts (Cm), while conditioned media obtained from DMD myoblasts (DMDm) (DMDCoMe)—stimulated with either FCS or DMDMEs (for 24 h)—showed decreased mitogenic activity compared with conditioned medium samples from unstimulated cells. Moreover, DMDCoMe seems to inhibit not only the proliferation of control myoblasts, but also the

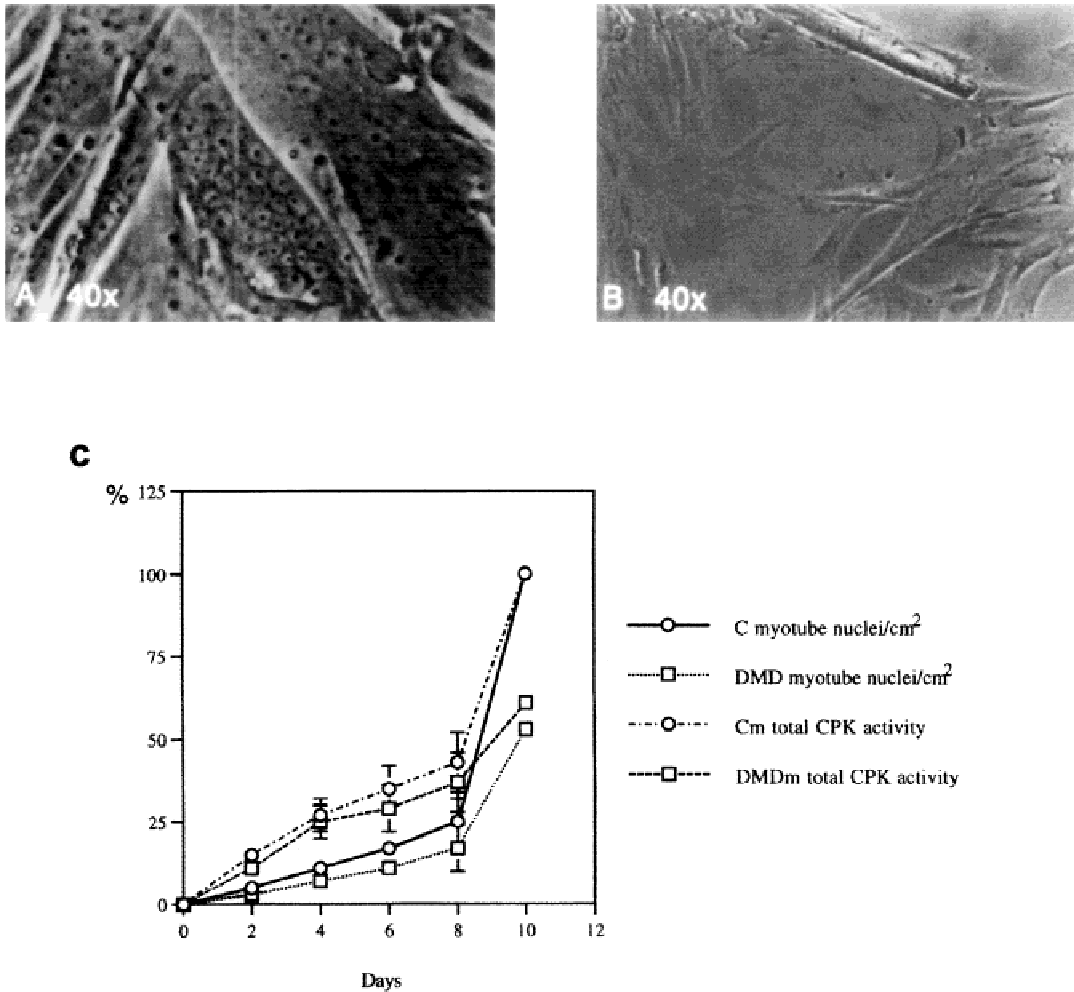



**Fig. 4.** Morphological characteristic of control myoblasts (**A**) and DMD myoblasts (**B**) at day 10 of culture. **C**: Rate of fusion expressed as myotube nuclei/$cm^2$ (both control [C] and DMD) and total CPK activity in control myoblast (Cm) and DMD myoblast (DMDm) cultures stimulated by DMD muscle extracts (DMDMEs). Results are expressed as a percentage (mean ± SEM) of the result in FCS-stimulated control myoblasts (at least eight parameter) at day 10 (100%).

differentiation of the same cells, as shown in Figure 9. This finding supports the assumption that stimulation of DMD myoblasts (DMDm) by FCS or by DMD muscle extracts (DMDMEs) induces the release of autocrine inhibiting factors from the DMD myoblasts.

### Role of TGF-β1

Since the data—above described—imply that FCS and DMDMEs seem to stimulate the secretion of inhibitory molecules from DMD myoblasts, it was appropriate to identify the factor(s) whose "de novo" expression by stimulated DMD myoblasts, contribute to the inhibition of myoblast proliferation. Therefore, according to the hypothesis that TGF-β1 could contribute as

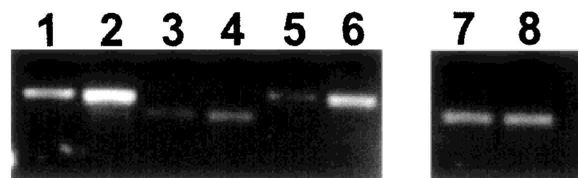



**Fig. 5.** Agarose gel electrophoresis analysis of RT-PCR products in DMD muscle extract-stimulated control myoblasts (Cm) and DMD myoblasts (DMDm) after three days from the beginning of differentiation. **Lane1:** DMD myoblast (DMDm) MyoD mRNA; **Lane 2:** Control myoblast (Cm) MyoD mRNA; **Lane 3:** DMD myoblast (DMDm) Id mRNA; **Lane 4:** Control myoblast (Cm) Id mRNA; **Lane 5:** DMD myoblast (DMDm) Myogenin mRNA; **Lane 6:** Control myoblast (Cm) Myogenin mRNA; **Lane 7:** DMD myoblast (DMDm) HPRT mRNA; **Lane 8:** Control myoblast (Cm) HPRT mRNA. Each PCR amplification was performed for 28–30 cycles.

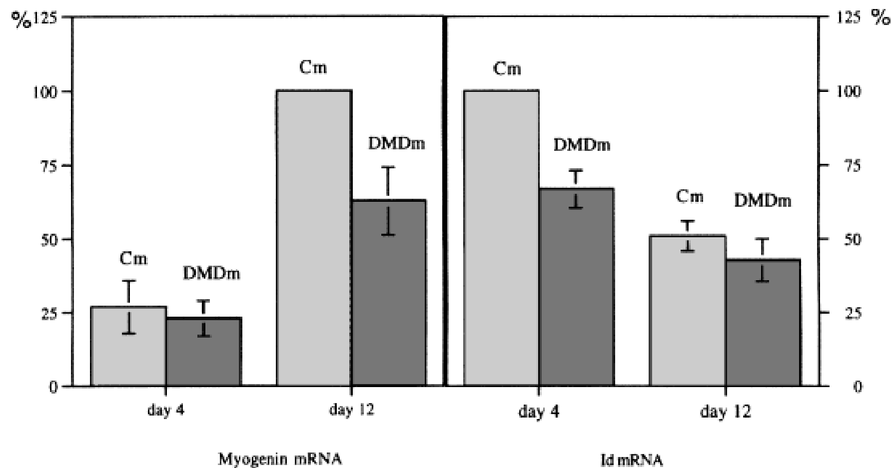


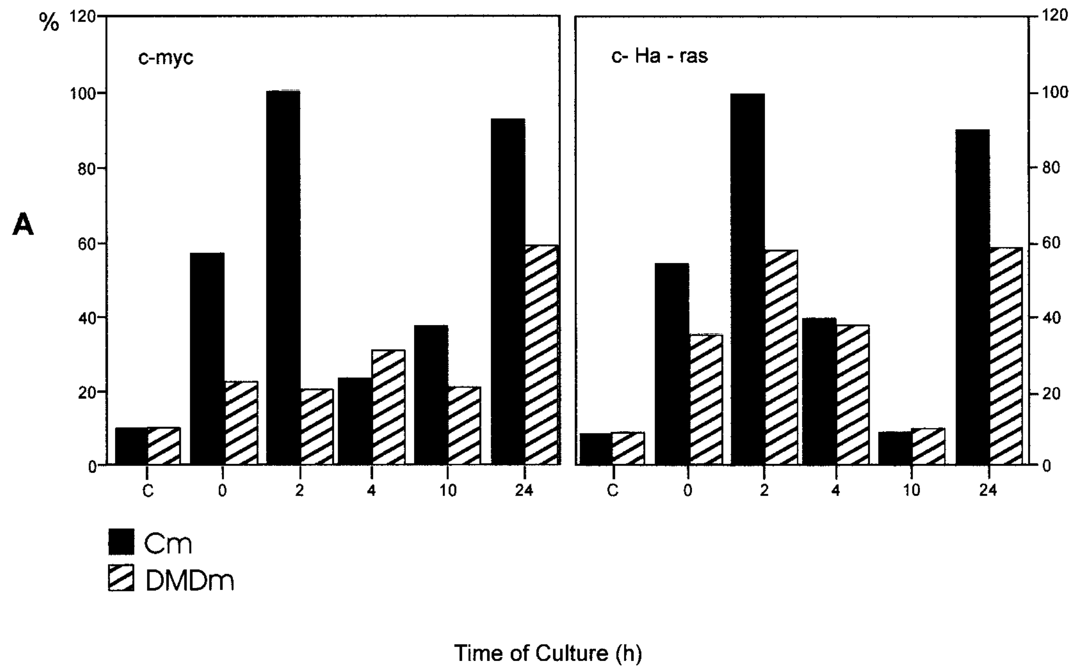

**Fig. 6.** Myogenin/HPRT and Id/HPRT mRNA ratio in control myoblast (Cm) and DMD myoblast (DMDm) stimulated by DMD muscle extracts, at day 4 and 12 of culture. Results are compared to the data found in paired FCS stimulated control myoblasts evaluated, respectively, at day 4 (100%) and day 12 (100%) for myogenin and Id.

possible inhibitory component in conditioned medium samples obtained from stimulated DMD myoblasts, TGF-β1 neutralizing specific antibodies were used. As shown in Figure 8, DNA synthesis decreased by DMD muscle extracts (DMDMEs) in control myoblasts (Cm) was effectively restored by preincubation with the proper specific TGF-β1 neutralizing antibodies. In contrast, when samples of stimulated-DMD myoblast (DMDm) conditioned media (DMDCoMe) were pretreated with control antibodies, we failed to observe any significant effect on the inhibitory activity of the control myoblasts (Cm). Again, whereas the addition of anti TGF-β1 antibody to DMD myoblast conditioned media affected the antiproliferative properties of the conditioned media, it did not reduce the antidifferentiative effects of DMD myoblast conditioned media.

The above observation—namely that the effect of DMD myoblast conditioned media (DMDCoMe) was blocked by anti-human TGF-β1 antibody—suggested that TGF-β1 might be involved. Thus, the kinetics of TGF-β1 production was measured (Fig. 10B). During the first 1-day subculture, conditioned media derived from DMD myoblasts actively proliferating (Fig. 1), did not show any detectable amount of TGF-β1. On the contrary, the TGF-β1 contents in the conditioned medium derived from resting DMD myoblasts as well as in DMD muscle extracts and control myoblast (Cm) conditioned medium were ranging from 0.10 to $0.15 \pm 0.05$ ng/$10^6$ cells. The addition of DMD muscle extracts (DMDMEs) to DMD myoblasts seemed to enhance the release of TGF-β1. The amount was significantly increased in 6 h after stimulation and reached a peak at h 24. In addition, by Western blot analysis, it was possible to identify high level of TGF-β1 in lysate of DMD myoblasts stimulated by DMD muscle extract (DMDMEs) (lane 3), following a 24 h culture, whereas in DMDMEs-stimulated control myoblasts (Cm) (lane 1) or in quiescent DMD myoblasts (DMDm) (lane 2) the level of TGF-β1 was very low (Fig. 10A).

## DISCUSSION

Extracts from gently crushed adult skeletal muscles contain potent myoblast mitogens [Bischoff, 1986, 1990; Chen et al., 1994; Haugk et al., 1995] and may used as a source of "growth factors." In fact, our study demonstrates that the DMD muscle extracts, also, contain many growth factors able to activate the proliferation both of DMD myoblasts—in initial stage of cultures—and of control myoblasts, practically comparable to FCS; the activation in vitro of DMD myoblasts by DMD muscle extracts or FCS results in a time-related secretion of factor(s) affecting DNA synthesis, cell proliferation, and differentiation of control myoblasts. These findings support the concept that DMD myoblasts may directly regulate muscle cell proliferation and potentially reduce the muscle regenerative capacity instead of potentiating it. Moreover, these data are consistent with recent studies reporting an expression of TGF-β1 in DMD patient muscles, and suggesting that this factor is critical in initiating muscle fibrosis at



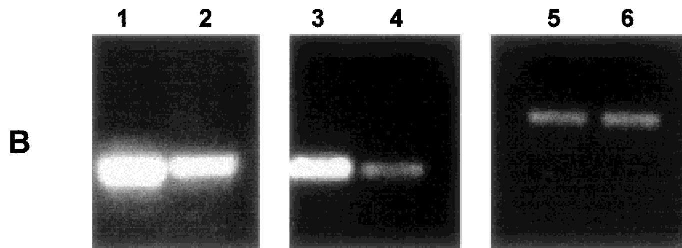




Fig. 7. **A**: C-myc/HPRT and c-HA-ras/HPRT mRNA ratio in control myoblasts (Cm) (black bars) and DMD myoblasts (DMDm) (hatched bars) at different times after DMD muscle extracts (DMDMEs) addition to the culture medium. Results are compared to the data found in FCS stimulated control myoblasts evaluated at h 2 (100%) for c-myc and c-HA-ras, respectively. **B**: Agarose gel electrophoresis analysis of RT-PCR products in control myoblasts (Cm) and DMD myoblasts at different times following DMD muscle extracts addition to the culture medium. **Lane 1:** Control myoblast (Cm) c-myc mRNA; **Lane 2:** DMD myoblast (DMDm) c-myc mRNA; **Lane 3:** Control myoblast (Cm) c-Ha-ras mRNA; **Lane 4:** DMD myoblast (DMDm) c-HA-ras mRNA; **Lane 5:** Control myoblast (Cm) HPRT mRNA; **Lane 6:** DMD myoblast (DMDm) HPRT mRNA.

least in the early stages of DMD disease [Yamazaki et al., 1994; Bernasconi et al., 1995]. On the other hand, it has not yet been established whether the TGF-β1 synthesis is sustained by other molecules present in the specific DMD microenvironment or whether it is the result of a peculiar secretory phenotype shown by DMD myoblasts.

In this paper we analyze at first, the biological consequences of DMD muscle extract addition to control and DMD myoblast "pure" cultures. An interesting approach to the study of the DMD muscle extract biological properties is to investigate whether its administration to myoblasts modifies the levels, or the function, of cell cycle or differentiation controlling factors. In our model, we observed an initial and subststantial proliferation rate of both DMD myoblasts and control myoblasts in the first subpopulation cultures, at h 24; but when the subsequent later populations of cells were grown in vitro, in presence of DMD muscle extracts or FCS, decreased mitogenesis only of DMD myoblasts was evident, while control myoblasts, intriguingly, showed a normal proliferative behaviour, as depicted by proliferation assay and FACS analysis.

Because of this delayed decrease in the rate of DMD myoblast proliferation, studies were performed to determine whether the expres-

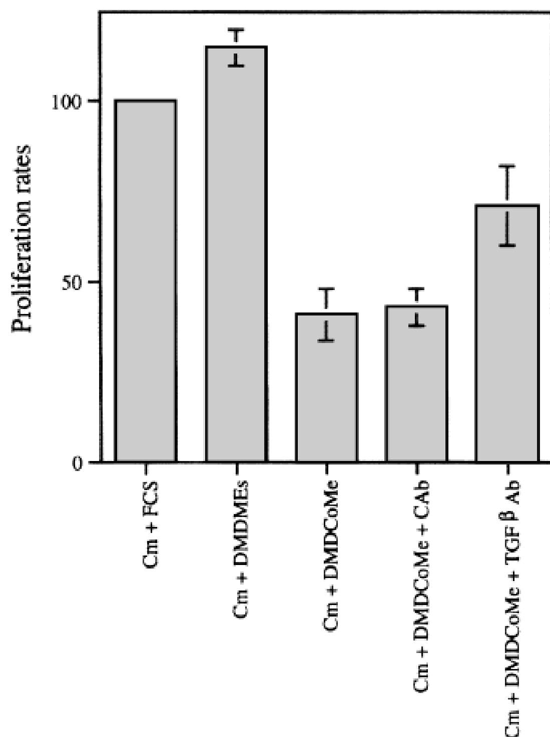



Fig. 8. [$^3$H] thymidine incorporation into control myoblasts (Cm) stimulated with DMD muscle extracts (DMDMEs) or DMD myoblast conditioned medium (DMDCoMe) in absence or presence of anti-human TGF-β1 antibody (TGFβAb1 or control antibody (CAb1). Results are compared to the data found in FCS-stimulated control myoblasts (Cm).

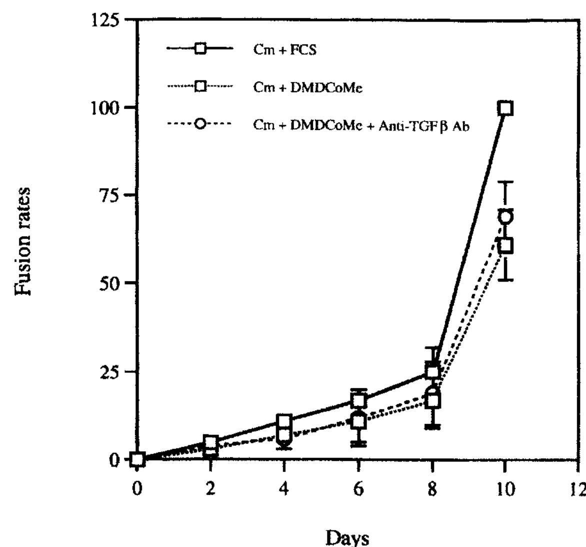



Fig. 9. Control myoblast (Cm) fusion rates in cultures incubated, beginning from day 4, with DMD myoblast conditioned medium (DMDCoMe) in presence or absence of anti-human TGF-β1 antibody (TGFβAb). Results are expressed as the percentage (mean ± SEM) of the result in FCS stimulated control myoblasts (Cm).

sion of oncogenes associated with cell growth, such as c-*myc* [Packham and Cleveland, 1995] and c-Ha-*ras* [Lowy and Willumsen, 1993], was modified in DMD myoblasts in comparison with control myoblasts. C-*myc* and c-Ha-*ras* mRNA steady-state levels during the DMD myoblast cycle were unknown; first, we measured their levels in pseudosynchronized cells of the first subculture. Variations in the c-*myc* and c-Ha-*ras* mRNA levels were observed during the cell cycle in contrast with confluent DMD myoblast arrested in GO/G1 phase. In particular, c-Ha-*ras* and c-*myc* mRNA levels were barely detectable in DMD myoblasts after stimulation with DMD muscle extracts or FCS, whereas both mRNAs were increased in stimulated control myoblasts in the early G1 phase, which agrees with our findings concerning the different proliferation rate between control myoblasts and DMD myoblasts, in later cultures.

Moreover, we found that c-*myc* mRNA persisted only in control myoblasts thereafter in the cell cycle and even when the cell population was asynchronized (40% of cells were in GO/G1, 40% in S, and 20% in G2 phase). At this time, a second peak of the c-*myc* mRNA was observed as the gene was transcribed.

C-Ha-*ras* expression in control myoblasts appears similar to that of c-*myc*, with a peak early in G1; c-Ha-*ras* transcript was detected in control myoblasts thereafter in the cell cycle, particularly at h 24. In contrast, c-Ha-*ras* in DMD myoblasts was induced at low level, late in G1 and early in S phases.

Next, the consequences of DMD myoblast conditioned media both on control myoblast proliferation and on the expression of the genes implicated in cell growth were established. Compared to control cells, we observed a two- to three-fold decrease of c-*myc* and c-Ha-*ras* mRNA levels in control myoblasts treated with DMD myoblast conditioned media as early as 1 h after the treatment, and this effect was maintained for at least 24 h. Moreover, in the conditioned medium of DMD myoblasts stimulated by DMD muscle extracts, high levels of TGF-β1 were detected; whereas conditioned medium of DMD myoblasts—actively proliferating in early cultures—failed to show similar levels. These results also imply that the anti-mitogenic effect of DMD myoblast conditioned media is mediated by a late release of TGF-β1 like activity; as a matter of fact, the anti-human TGF-β1 antibody inhibits conditioned medium-induced phe-

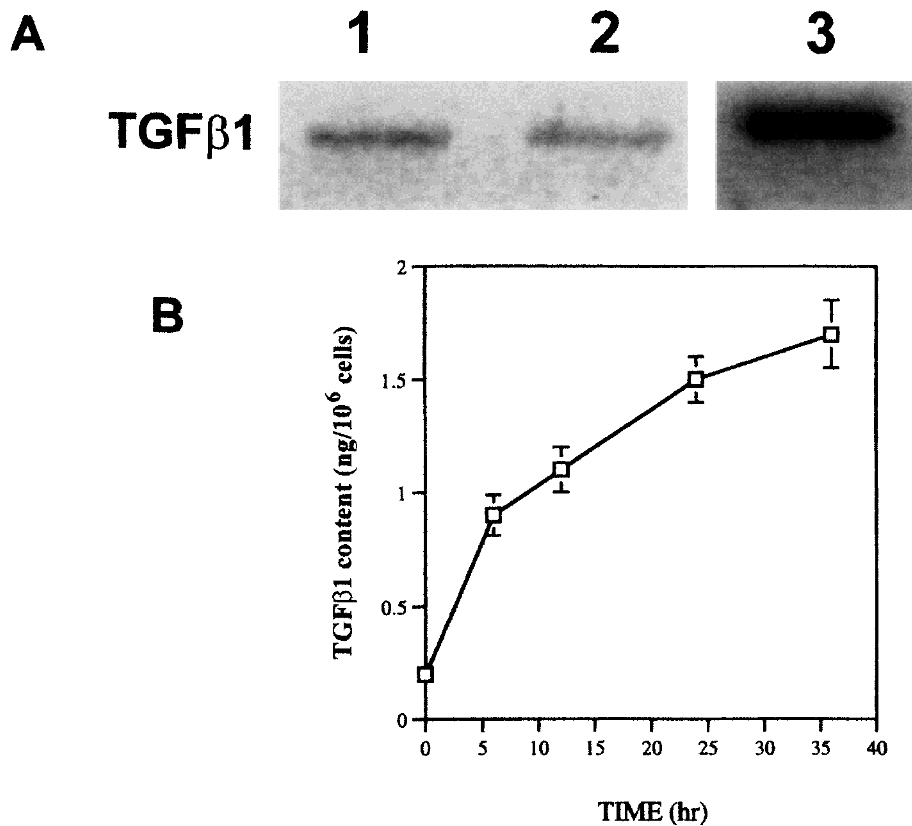



**Fig. 10.** **A**: Identification of TGF-β1 by Western blot assay in cell lysates from DMD muscle extract-treated control myoblasts (**lane 1**), quiescent DMD myoblasts (**lane 2**), and DMD muscle extract-treated DMD myoblasts (**lane 3**). **B**: Stimulation of TGF-β1 release in DMD myoblasts treated with DMD muscle extracts for the indicate times. TGF-β1 content in the conditioned medium from resting DMD myoblasts or from control myoblasts treated with DMD muscle extracts, were 0.15 ± 0.03 and, 0.17 ± 0.06 ng/$10^6$ cells, respectively. The TGF-β1 amount in DMD muscle extracts was undetectable. Data are presented as the mean ± SEM.

nomena of aged DMD myoblasts cultured in DMD muscle extracts. Indeed, a direct measurement in the cells indicates high content of TGF-β1 protein only in DMD muscle extract-challenged DMD myoblasts. Previous studies have demonstrated that TGF-β1 suppress division and totally block fusion of rat myoblasts [Allen and Boxhorn, 1989]. Since c-myc expression is induced rapidly and remains high throughout G1 and early into S phases [Coffey et al., 1988], c-myc is a canditate for mediation of TGF-β1 growth inhibition. In effect, other studies showed that TGF-β1 blocked transcriptional initiation of the c-myc gene and that probably, pRB is a necessary component in the pathway for TGF-β1 suppressor of c-myc [Pietenpol et al., 1990, 1991]. Furthermore, anti-TGF-β1 antibody addition prevented this effect indicating that TGF-β1 could be a good candidate as an autocrine factor, able to modulate myoblast proliferation.

Since a complex sequence of metabolic and genetic events is initiated when cells are stimulated to divide in response to several factors, and since proteins encoded by different protooncogenes—particularly ras and myc—have been shown to be implicated in these processes, we can postulate that the c-*myc* and c-Ha-*ras* mRNA low levels observed in DMD myoblast resting cells and in control myoblasts, following DMD myoblast-conditioned medium treatment, can be related to the inhibition of cell proliferation and to the accumulation of cells in the G2 phase of the cell cycle.

In the second step of experiments we have evaluated the effects of DMD muscle extracts and DMD myoblast-conditioned medium on the differentiation of control myoblasts and DMD myoblasts. Terminal differentiation of muscle cells, both in vivo and in vitro, is dependent upon the functions of the myogenic regulatory factors (MRFs) [Edmondson and Olson, 1993].

These include factors of the basic helix-loop-helix family MyoD, Myf5, and myogenin [Winter et al., 1993]; expression of any one of these MRFs is sufficient to steer a variety of cell types to the myogenic differentiation pathway. As matter of fact, recent study on expression of MRFs in human neuromuscular disorders demonstrated that same MRF immunoreactivity was observed in the nuclei of small regenerating fibers [Olive et al., 1997]. On the contrary, the Id protein family functions as negative regulators of myoblast cell differentiation [Benezra et al., 1990] and as positive regulators of G1 cell cycle control. For this reason, we have evaluated the expression of specific mRNAs coding for these genes in differently treated control myoblasts and DMD myoblasts.

We show that downregulation of Id in control myoblasts stimulated with DMD muscle extracts occurs as early as the onset of differentiation, when cells become myogenin-positive. Indeed, our observations are consistent with the conclusion that, in case of DMD muscle extract stimulation, strong expression of Id (in contrast to that of MyoD) is restricted to myoblasts in the proliferative state. This conclusion raises the possibility that the downregulation of Id could be a prerequisite for the progress of differentiation.

We also demonstrate that in DMD myoblasts and DMD myoblast-conditioned medium-treated control myoblasts, Id as well as MyoD and myogenin are partially depleted both during the mitotic and the differentiation phase, respectively. With ovine satellite cells, TGF-β1 has been shown to inhibit differentiation [Hathaway et al., 1991]. In the case of DMD myoblasts the addition of anti-TGF-β1 antibodies is only partially able to restore the differentiation pathway, suggesting that secreted TGF-β1 from DMD myoblasts, may also play a role in preventing differentiation of satellite cells but that is not remarkable and other secretory factors are involved in mediating the inhibition of myoblast differentiation. In fact, TGF-β1 may act to prevent muscle differentiation by both depressing endogenous IGF secretion and modulating IGF binding proteins (IGFBPs) [McCusker and Clemmons, 1994]; contemporaneously the TGF-β1 action on connective cells, where it stimulates cell proliferation and extracellular matrix synthesis, can accompany an increase in IGFBP fibroblast secretion; in particular we have found an increase in IGFBP-5 levels in media conditioned by DMD fibroblasts proliferating in presence of growth factor muscle extracts [Melone et al., 1998, Congress communication]. So, the exact role of these autocrine/paracrine loops in determining the response of different cell populations—myoblasts and fibroblasts—to milieu growth factors in Duchenne's disease, remains to be elucitated.

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