Terminal differentiation of Sol 8 myoblasts is retarded by a transforming growth factor- β autocrine regulatory loop

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In DM (differentiation medium), Sol 8 myoblasts spontaneously form myotubes and express the β MHC (β -myosin heavy chain), their main marker of terminal differentiation. This marker is detectable at 24 h, and increases up to 72 h. Our aim was to define temporal effects of TGF β (transforming growth factor β) on β MHC expression in Sol 8 cells. TGF β 1 (1 ng/ml) added at time zero to DM decreased MyoD expression and completely inhibited β MHC expression in Sol 8 cells. This inhibition of β MHC expression was progressively lost when TGF β 1 was added from 8 to 34 h. After 34 h, the cells were irreversibly differentiated, and TGF β 1 did not inhibit β MHC accumulation any longer. Two independent approaches showed that a TGF β autocrine regulatory loop retarded and partially impaired Sol 8 cell terminal differentiation. First, permanent immunoneutralization of the active TGF β s released by the cells into DM increased β MHC

levels at 72 h compared with controls. Secondly, a dominant-negative mutant of the TGF β type II receptor was overexpressed in Sol 8 cells under the control of the β MHC promoter. Both the dominant-negative receptor and the β MHC gene were expressed after 24 h in DM. The delayed blocking of the TGF β signalling pathway by the dominant-negative receptor was as effective as permanent immunoneutralization to promote β MHC expression. To conclude, TGF β inhibits Sol 8 cell terminal differentiation within a narrow time interval (24–34 h) that coincides with the onset of β MHC expression.

Key words: autocrine regulatory loop, immunoneutralization, Sol 8 myoblasts, terminal differentiation, transforming growth factor β (TGF β), dominant-negative TGF β type II receptor.

INTRODUCTION

Transforming growth factor β (TGF β) is a member of a wide family of multifunctional regulatory peptides that are secreted by a large variety of cells and regulate cell proliferation, differentiation, apoptosis, migration and extracellular matrix production [1–4]. Expression of TGF β 1, 2 and 3 is active from embryonic development to adulthood. Studies on skeletal muscle differentiation demonstrate that TGF β has profound effects on myogenic cell differentiation *in vitro* [5,6]. There have been conflicting reports on the role of TGF β in skeletal muscle differentiation. TGF β has been reported to be a potent inhibitor of myoblast differentiation in cells cultured in low serum concentration [5–9]. On the other hand, in the presence of higher serum concentrations, TGF β can induce myoblast differentiation [10]. Myoblasts differentiate in response to a number of environmental signals that induce cells to elongate, fuse and form multinucleate myotubes. The differentiation process is accompanied by an increase in the levels of muscle-specific proteins such as creatine phosphokinase, myosin (heavy and light chains) and actin [11].

TGF β exerts its numerous biological effects through a set of specific receptors [12]. Among these, the type I (T β RI) and the type II (T β RII) TGF β receptors are transmembrane serine/threonine kinases. They mediate most of the multiple effects of TGF β . The intracellular domain of T β RII has a constitutive serine/threonine kinase activity that is essential for signal transduction. Functional T β RII is required to activate T β RI through transphosphorylation. Active T β RI phosphorylates downstream targets: the Smad2 and Smad3 proteins [13]. Overexpression of kinase-deficient T β RII mutants, which prevent the phosphorylation

cascade of T β RI, Smad2 and Smad3 proteins, blocks the TGF β signalling pathway [14–16].

The aim of the present study was to analyse whether TGF β has an inhibitory or stimulatory effect on the differentiation of Sol 8 cells. Sol 8 cells are mouse slow-twitch skeletal muscle myoblasts that express the β MHC (β -myosin heavy chain) when they differentiate [17–19]. First, we show that WT (wild-type) Sol 8 cells respond to exogenous TGF β 1. TGF β 1 totally inhibits the terminal differentiation of these cells up to 34 h, with no effect on their proliferation. In addition, we report for the first time that a TGF β autocrine loop negatively regulates myogenic differentiation in Sol 8 cells. In these cells, the effects of endogenous TGF β s are blocked by a neutralizing antibody against the three TGF β isoforms and by the overexpression of a dominant-negative T β RII. Finally, we define the time interval during which the TGF β autocrine loop is functional by showing that endogenous TGF β inhibits myotube formation and β MHC accumulation in Sol 8 cells, along with the onset of the activation of the β MHC gene.

EXPERIMENTAL

Cell line and transfection

The Sol 8 mouse muscle cell line derived from the soleus of a C3H mouse was obtained from Dr C. Pinset (Institut Pasteur, Paris, France) [20]. These myoblasts proliferated in GM (growth medium), high-glucose (4.5 g/l) DMEM (Dulbecco's modified Eagle's medium) supplemented with 20 % FBS (fetal bovine serum). Fusion into myotubes was induced by DM (differentiation

Abbreviations used: CMV, cytomegalovirus; DM, differentiation medium; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; GM, growth medium; HS, horse serum; β MHC, β -myosin heavy chain; MRF, myogenic regulatory factor; TGF β , transforming growth factor β ; T β R(I/II), type I/II TGF β receptor; WT, wild-type.

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medium), DMEM supplemented with 5 % HS (horse serum) or by the confluence of cells cultured for several days in GM. Cells were stably transfected by using LipofECTAMINE™ Plus according to the manufacturer's instructions (Invitrogen, Cergy-Pontoise, France). Briefly, 24 h after plating (7.14×10^4 cells/cm² in six-well plates), cells were co-transfected for 5 h with either p- β MHC-T β RII-KR-EGFP (where 'EGFP' represents 'enhanced green fluorescent protein') or p- β MHC-EGFP plasmid and a plasmid conferring hygromycin resistance (pHygro, Clontech; 1 μ g of DNA/well, ratio 10:1), 6 μ l of LipofECTAMINE™ Plus reagent and 0.4 % LipofECTAMINE™ in the medium without antibiotics and serum (total volume of 1 ml/well). Stable transfectants were selected in GM with 600 μ g/ml hygromycin for 10 days, and then maintained with 300 μ g/ml hygromycin. Stable Sol 8 clones expressing EGFP or the fusion protein were defined as p- β MHC-EGFP or p- β MHC-T β RII-KR-EGFP respectively.

Immunoneutralization experiments

WT Sol 8 cells (5×10^5 cells/well in six-well plates) were cultured for 64 h in DM with or without anti-TGF β antibody (Pan-specific TGF β antibody; R&D systems, Minneapolis, MN, U.S.A.). Preliminary data (results not shown) indicated that a concentration of 10 μ g/ml was sufficient to immunoneutralize the biological activity of the TGF β isoforms produced by Sol 8 cells.

[³H]Thymidine incorporation

WT Sol 8 cells were seeded in GM (5.68×10^4 cells/cm² in 24-well plates). On the following day, cells were changed to DMEM 1 % (v/v) FBS supplemented or not with 1 ng/ml TGF β 1 for 20 h. Cells were then pulse-labelled for 4 h with 1 μ Ci/ml of [³H] thymidine. Cells were fixed with methanol/acetic acid (3:1, v/v) for 15 min at room temperature, washed once with the fixative and twice with 80 % (v/v) methanol. Cells were then solubilized with 0.4 % (w/v) sodium deoxycholate in 0.5 M NaOH, and the radioactivity was measured.

Tissue-specific expression vectors

A fragment of 1200 bp of human T β RII cDNA without the last 639 bp of its coding region, containing a Lys²⁷⁷ → Arg mutation and fused in-frame with the EGFP sequence [21], was inserted into the *Hpa*I site of the plasmid containing the β MHC promoter to generate p- β MHC-T β RII-KR-EGFP. EGFP cDNA (737 bp) was also inserted into the *Hpa*I site to generate p- β MHC-EGFP. The plasmid p- β MHC was provided by Dr J. Gulick and Dr J. Robbins (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, U.S.A.) [17]. This plasmid contains, in the multiple cloning site of pBluescript2 SK⁺, a 7143 bp promoter sequence comprising the 5'-flanking region, the non-coding exons 1–3 and the start of exon 4 with the first three introns of the mouse cardiac β -myosin heavy-chain gene, and 624 bp of the polyadenylation signal of the human growth hormone gene. EGFP fluorescence was observed in living cells by epifluorescence using standard filters for fluorescein. Y-1 cells stably transfected with the p-CMV-T β RII-KR-EGFP construct (where 'CMV' represents 'cytomegalovirus') [21] were used as positive controls (see Figures 4 and 7B).

Luciferase reporter assay

WT Sol 8 cells and stable clones were transiently transfected by LipofECTAMINE™ Plus with the TGF β -responsive construct p(CAGA)₁₂MPL-Luc, provided by Dr J. M. Gauthier [22]. This construct contains 12 copies of the CAGA box (a TGF β -responsive element of the human plasminogen activator in-

hibitor-1 promoter) controlling the expression of luciferase. Briefly, each clone (1×10^5 cells/well in 24-well plates) was transfected with 0.4 μ g of p(CAGA)₁₂MPL-Luc/well for 5 h. At 1 day after transfection, cells were treated with or without 1 ng/ml TGF β 1 in DMEM containing 1 % FBS. Cells were harvested 20 h later, and assayed with the luciferase assay system (Promega, Charbonnières, France). Total light emission was measured during the initial 15 s of the reaction using a luminometer.

RNA extraction and Northern blot analysis

Total RNAs were extracted [23] and subjected (20 μ g/lane) to Northern analysis [24]. The cDNA probes for EGFP (whole coding sequence of 743 bp), T β RII (816 bp of the coding sequence; obtained from Dr J. Massagué, Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.) and TGF β 1, 2 and 3 (obtained from Dr R. Derynck, University of California, San Francisco, CA, U.S.A.) [25] were labelled with [α -³²P]dCTP using Ready-to-go DNA-labelling beads (-dCTP; Amersham, Little Chalfont, Bucks., U.K.). Equal loading of RNA in each lane was confirmed by the amount of ethidium bromide-stained ribosomal 28 S RNA.

Protein extraction and Western blot analysis

Cell lysis was performed in 10 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 0.5 % (w/v) SDS with a protease inhibitor cocktail (Roche-Boehringer, Meylan, France). Proteins (20–50 μ g) were analysed under denaturing conditions by electrophoresis, using 7.5 % or 10 % polyacrylamide gels. Western blot analysis was performed using the following primary antibodies: mouse monoclonal MF20 antibody against all sarcomeric myosins in striated muscles (Developmental Studies Hybridoma Bank, University of Iowa, IA, U.S.A.) (1/1000), mouse monoclonal antibody against MyoD (Hybridoma Bank; 1:500 dilution), mouse monoclonal antibody F5D against myogenin (Hybridoma Bank; 1:500 dilution), rabbit polyclonal antibody against EGFP (1:10 000 dilution) [21] and goat polyclonal AF-241-NA antibody against TGF β -RII (R&D systems; 1:500 dilution). The secondary antibodies (1:2000 dilution) were conjugated with horseradish peroxidase. For detection, the ECL[®] Western blotting detection reagent (Amersham) was used.

Immunocytochemistry

Sol 8 cells were plated (1×10^4 cells/chamber) in eight-chamber tissue-culture slides. After 3 days, cells were fixed for 30 min in 3 % (w/v) paraformaldehyde, washed in PBS containing 10 mM glycine, permeabilized for 5 min with PBS/1 % Triton X-100, washed with PBS/10 mM glycine and incubated for 30 min with PBS/25 mM glycine. Cells were then incubated for 30 min with PBS/3 % FBS to block non-specific binding sites. The MF20 antibody was used as primary antibody overnight at 4 °C at a 1:500 dilution. After three washes with PBS, the secondary antibody raised against mouse IgGs conjugated with horseradish peroxidase was applied at a 1:100 dilution for 90 min at room temperature. After three washes in PBS, cells were incubated for 2 min with 0.05 % 3,3'-diaminobenzidine tetrahydrochloride and 0.01 % H₂O₂ to visualize the antigen–antibody complexes.

RESULTS

Response of WT Sol 8 cells to exogenous TGF β 1

The effects of TGF β 1 on WT Sol 8 cell proliferation and differentiation were investigated. WT Sol 8 cells were transiently

Figure 1 Effects of TGF β 1 on WT Sol 8 cell differentiation

(A) Effect of TGF β 1 on myotube formation. Cells were photographed after culture in DM (5% HS) with or without 1 ng/ml TGF β 1 for 72 h. Cells were observed with an $\times 10$ or a $\times 40$ objective. (B) Sol 8 cells were cultured in GM (20% FBS) or in DM with or without TGF β 1 (1 ng/ml). M, myosin marker. (C) Time course of β MHC expression in WT Sol 8 cells. Cells were changed to DM at 0 h, and were harvested at the times indicated. (D) Time course of the inhibiting effects of TGF β 1 after the initiation of differentiation. Cells were changed to DM at 0 h. TGF β 1 (1 ng/ml) was added at the times indicated, and renewed every 24 h until cells were harvested at 72 h. Protein extracts analysed by Western blotting using the MF20 antibody in (C) and (D) were run on the same gel. These experiments were repeated twice.

transfected with the TGF β -responsive construct p(CAGA)₁₂MLP-Luc. This construct is exclusively stimulated by Smad3 activation [22]. Luciferase activity was measured in cell lysates from untreated cells or cells treated with various concentrations of TGF β 1 (0.2–5 ng/ml) for 20 h (results not shown). The maximal luciferase activity (50–100-fold) was obtained with 1 ng/ml TGF β 1. [³H]Thymidine incorporation was measured in TGF β 1-treated and control cells. TGF β 1 had no significant effect on proliferation of WT Sol 8 cells (results not shown). In DM, Sol 8 myoblasts spontaneously form myotubes (Figure 1A) and express β MHC (Figure 1B). The results in Figure 1(B) show that β MHC is undetectable in subconfluent WT Sol 8 cells grown in GM, but is strongly expressed in cells cultured in DM. Time-course studies in WT Sol 8 cells cultured in DM (Figure 1C) show that the amounts of β MHC were barely detectable after 8–10 h of culture, but steadily increased after 24 h of culture. WT Sol 8 myoblasts in DM did not differentiate in the presence of TGF β 1 (1 ng/ml). No myotubes were formed in these cells compared with

Figure 2 TGF β mRNA expression and TGF β effects in WT Sol 8 cells

(A) TGF β mRNA expression. Northern blot analyses were performed using a mix of human cDNA probes for TGF β 1, 2 and 3 on RNAs from WT Sol 8 cells and a β MHC-T β R11-KR/EGFP clone cultured for 64 h in GM (20% FBS) or in DM (5% HS). In the bottom panel, 28 S signals indicate RNA loading. (B) Immunoneutralization of TGF β activity. WT Sol 8 cells were cultured for 64 h in DM with or without TGF β 1 (1 ng/ml) and in the presence or the absence of anti-TGF β antibody (10 μ g/ml). Protein extracts were analysed by Western blotting using the MF20 antibody. Either two or four independent repetitions of these experiments, respectively, were performed.

untreated cells (Figure 1A). Moreover, no β MHC was detected in WT Sol 8 cells treated with TGF β 1 when cultured in DM. We defined whether TGF β 1 inhibition on β MHC expression had a limited time effect. TGF β 1 (1 ng/ml) was added at different times to cells in DM, and the cells were harvested at 72 h of culture. The level of β MHC (Figure 1D) was partially blocked when TGF β 1 was added up to 34 h after the cells were changed to DM (Figure 1C).

Endogenous secretion of biologically active TGF β s by WT Sol 8 cells and stable clones

Northern analysis showed that WT Sol 8 cells and p- β MHC-T β R11-KR-EGFP clones, cultured in GM or DM, expressed TGF β 1 and TGF β 3 mRNAs (Figure 2A). Moreover, the secretion of bioactive TGF β s by WT Sol 8 cells was demonstrated by immunoneutralization experiments. WT Sol 8 cells were treated with the anti-TGF β antibody (10 μ g/ml) as soon as they were changed to DM (Figure 2B). Western blotting with the MF20 antibody showed, at 64 h of culture in DM, higher β MHC levels in these cells compared with untreated cells. In WT Sol 8 cells treated with both TGF β 1 (1 ng/ml) and anti-TGF β antibody, the expression of β MHC was lower than in cells treated with the antibody alone.

Sol 8 cell stable clones overexpress EGFP or T β R11-KR-EGFP mRNA and protein

We analysed the expression of EGFP and T β R11-KR-EGFP in Sol 8 cell stable clones transfected with the constructs p- β MHC-EGFP and p- β MHC-T β R11-KR-EGFP. Fluorescence

Figure 3 Expression of p- β MHC constructs in Sol 8 cells

WT Sol 8 cells were transfected with p- β MHC-EGFP or with p- β MHC-T β RII-KR-EGFP, and stable clones were obtained. EGFP fluorescence was observed with a microscope equipped with epifluorescence. Homologous fields were observed for each clone: top, phase contrast; bottom, fluorescence. Two myotube nuclei are labelled with black asterisks in the lower centre panel, and white arrows in all the panels show the delineation of the cell membrane of the most representative myotube.

with the EGFP and T β RII-KR-EGFP constructs was only observed in the myotubes formed in clones cultured in DM (Figure 3). Myoblasts present in DM were not fluorescent, and provided support for myotube formation. In p- β MHC-EGFP stable clones, EGFP fluorescence was uniform throughout the cytoplasm and seemed to accumulate in the nuclei of the myotubes. In contrast, in

Figure 4 Overexpression of T β RII-KR-EGFP in Sol 8 stable clones

(A) Total RNAs extracted from WT Sol 8 cells and from Sol 8 clones stably transfected with p- β MHC-T β RII-KR-EGFP were hybridized with human T β RII cDNA (T β RII probe) or with EGFP cDNA (EGFP probe). Cells were cultured for 64 h in GM (20% FBS) and in DM (5% HS). In the lanes 1, Y-1 cells stably transfected with p-CMV-T β RII-KR-EGFP were used as a positive control. (B) Protein analysis from WT and p- β MHC-T β RII-KR-EGFP clones. Proteins were detected with both the anti-T β RII and anti-EGFP antibodies. In the lanes 1, Y-1 cells stably transfected with p-CMV-T β RII-KR-EGFP were used as a positive control. Each experiment was performed three times.

p- β MHC-T β RII-KR-EGFP clones, the fluorescence was localized at the level of the plasma membrane and in cytoplasmic organelles of the myotubes.

Northern blot analysis was performed with the T β RII or EGFP probes (Figure 4A). In two Sol 8 cell p- β MHC-T β RII-KR-EGFP stable clones cultured in DM, the T β RII probe revealed a major transcript of approx. 2.6 kb in size, which corresponded to T β RII-KR-EGFP mRNA. This transcript was absent in the same clones cultured in GM and in WT Sol 8 cells taken as negative controls. A minor transcript approx. 5 kb in size was identified as the endogenous T β RII mRNA in comparison with HepG2 cell mRNA, which was used as a positive control. HepG2 cells are known to heavily express the endogenous T β RII mRNA [21]. The endogenous T β RII mRNA was observed in WT cells and in the p- β MHC-T β RII-KR-EGFP clones. The levels of endogenous T β RII mRNA in cells cultured in GM or DM were the same. Transcripts that hybridized to the EGFP probe were only observed in clones cultured in DM (Figure 4A). This probe hybridized strongly with a transcript about 1.8 kb in size corresponding to EGFP mRNA in p- β MHC-EGFP clones (results not shown). Moreover, this probe hybridized with the 2.6 kb T β RII-KR-EGFP transcript (Figure 4A).

Northern blot results were confirmed by Western blot analysis with the anti-EGFP and anti-T β RII antibodies. A 27 kDa signal for the EGFP protein was observed in p- β MHC-EGFP stable clones cultured in DM (results not shown). In p- β MHC-T β RII-KR-EGFP stable clones cultured in DM, both the anti-EGFP and anti-T β RII antibodies detected the same doublet of proteins (Figure 4B). Their estimated sizes were approx. 73 kDa, which

Figure 5 Inhibition of the effect of TGF β 1 in Sol 8 cells stably transfected with p- β MHC-T β RII-KR-EGFP

WT Sol 8 cells and stable clones expressing EGFP or T β RII-KR-EGFP were transiently transfected with p(CAGA)₁₂ MLP-Luc, a luciferase reporter plasmid. After 24 h of culture in DM (5% HS), cells were incubated with or without TGF β 1 (1 ng/ml). After a further 20 h, cells were lysed and luciferase activity was determined. Luciferase activity (means $\pm$ S.D.) is shown as the ratio of luciferase activity measured in the presence of TGF β 1 to luciferase activity measured in the absence of TGF β 1. Each assay was performed in quadruplicate, in at least three independent experiments. The bars, from left to right, represent WT, and the clones p- β MHC-EGFP, p- β MHC-T β RII-KR-EGFP C16 and p- β MHC-T β RII-KR-EGFP C22, respectively.

corresponds to the calculated molecular mass of the T β RII-KR-EGFP protein.

Dominant-negative effects of the T β RII-KR-EGFP protein on the TGF β signalling pathway

The TGF β -responsive construct p(CAGA)₁₂MLP-Luc was used to determine whether T β RII-KR-EGFP blocked the effects of exogenous TGF β 1. In WT Sol 8 cells and in a p- β MHC-EGFP stable clone (Figure 5), the ratio of luciferase activity in TGF β 1 treated cells to untreated cells was high (approx. 40–45). In contrast, in two p- β MHC-T β RII-KR-EGFP stable clones, this ratio was low (between 8 and 15), showing that in these clones actions of exogenous TGF β 1 were markedly blocked.

The respective efficiency of either the anti-TGF β antibody or the dominant-negative fusion protein to block the effects of endogenous TGF β on β MHC expression was analysed. In p- β MHC-T β RII-KR-EGFP clones cultured for 64 h in DM (Figure 6A), the level of β MHC was much higher than in WT Sol 8 cells. In addition, the level of β MHC in p- β MHC-T β RII-KR-EGFP clones was similar to that observed in WT Sol 8 cells treated with the anti-TGF β antibody. No further increase in β MHC expression was noted when p- β MHC-T β RII-KR-EGFP clones were cultured for 64 h in DM with the anti-TGF β antibody. In WT Sol 8 cells cultured for 64 h in DM, TGF β 1 (1 ng/ml) inhibited MyoD expression (Figure 6B) and myogenin (results not shown). MyoD expression increased by immunoneutralization of endogenous TGF β s and T β RII-KR-EGFP overexpression (Figure 6B).

T β RII-KR-EGFP expression is parallel to the time course of β MHC gene activation

To investigate the time-course activation of either endogenous or transfected β MHC promoter, the kinetics of the expression of β MHC (Figure 7A) and T β RII-KR-EGFP protein (Figure 7B) were analysed in WT Sol 8 cells and in a p- β MHC-T β RII-KR-EGFP clone. In WT Sol 8 cells cultured in DM, the β MHC

Figure 6 Dominant-negative effect of T β RII-KR-EGFP on β MHC and MyoD expression

(A) WT Sol 8 cells and clones expressing T β RII-KR-EGFP (C16, C22) seeded at the same density were cultured for 64 h in DM with or without anti-TGF β antibody (10 μ g/ml). Western blot analyses were performed using the MF20 antibody. (B) WT Sol 8 cells and clone C16. Cells seeded at the same density were cultured for 64 h in DM with or without TGF β 1 (1 ng/ml) and in the presence or the absence of anti-TGF β antibody (10 μ g/ml). Western blot analyses were performed using the anti-MyoD antibody. Each experiment was performed twice for each clone.

Figure 7 Parallel increase of both β MHC and T β RII-KR-EGFP expression in p- β MHC-T β RII-KR-EGFP clones

(A) Time-course comparison between WT Sol 8 cells and p- β MHC-T β RII-KR-EGFP stable clone C22 cells (lanes labelled 'C'). Cells were seeded at the same density, and then cultured in DM (5% HS) and harvested at the times indicated. Protein extracts were then analysed by Western blotting using the MF20 antibody. Below the Western blot, a histogram represents the quantification of β MHC expression of four independent experiments (open bars, WT; closed bars, clone). Statistical analysis showed a significant increase in β MHC expression from 48 h (* P < 0.05). (B) The same protein extracts were used for Western blot analysis with the anti-T β RII antibody. M, myosin marker; T+, Y-1 cells stably transfected with p-CMV-T β RII-KR-EGFP used as a positive control.

level displayed a time-dependent increase from 30 h to 72 h (Figure 7A). In contrast, in the stable p- β MHC-T β RII-KR-EGFP clone, the β MHC level increased very rapidly to reach its maximal stabilized value at 64 h. The expression of the fusion protein was parallel with that of β MHC (Figure 7B).

Figure 8 Myotube formation and β MHC expression in p- β MHC-T β RII-KR-EGFP clones treated with exogenous TGF β 1

(A) WT Sol 8 cells and the p- β MHC-T β RII-KR-EGFP clone C22 were seeded at the same density and cultured for 72 h in DM (5%) with or without TGF β 1 (1 ng/ml). Then, immunocytochemical staining was performed using the MF20 antibody. Nuclei were stained with haematoxylin. (B) A time-course comparison between WT Sol 8 cells and the p- β MHC-T β RII-KR-EGFP stable clones C16 and C22. Cells were seeded at the same density, then cultured in DM and harvested at the times indicated. TGF β 1 (1 ng/ml) was added 24 h after the change to DM, and renewed every 24 h. Western blots were performed using the MF20 antibody. M, myosin marker. Below the Western blot, a histogram represents the quantification of seven independent experiments. Statistical analysis showed a significant increase in β MHC expression from 64 h (* $P < 0.05$).

T β RII-KR-EGFP prevents the inhibition of Sol 8 cell differentiation by exogenous TGF β 1

WT Sol 8 cells and two p- β MHC-T β RII-KR-EGFP stable clones were cultured for 64 h in DM to determine whether T β RII-KR-EGFP blocked the effects of exogenous TGF β 1. After 24 h of culture, TGF β 1 (1 ng/ml) was added every 24 h until the end of the experiment (64 h). Immunocytochemistry with the MF20 antibody shows that, in WT Sol 8 cells as well as in p- β MHC-EGFP clones (results not shown) treated with exogenous TGF β 1, myotube formation was fully inhibited, and β MHC was only expressed in a few globular cells (Figure 8A). In contrast, in p- β MHC-T β RII-KR-EGFP clones, numerous myotubes were formed in spite of TGF β 1 treatment, and their immunoreactivity for β MHC was intense (Figure 8A). Western blot analysis was performed with WT Sol 8 cells or p- β MHC-T β RII-KR-EGFP clones treated with TGF β 1 (as described above). In WT

Sol 8 cells, TGF β 1 treatment maintained β MHC at constant low levels (Figure 8B). In contrast, owing to the dominant-negative effect of T β RII-KR-EGFP in the two clones, TGF β 1 lost its inhibitory effect on β MHC expression. In these clones, β MHC levels kept on increasing during the course of the experiment.

DISCUSSION

TGF β effects in Sol 8 cells have never been studied. Our results show that exogenous TGF β 1, as in other muscle cells tested [5,7,26,27], had no significant effect on Sol 8 cell proliferation. However, TGF β 1 added to Sol 8 cells from the start of culture in DM decreased MyoD expression and fully inhibited β MHC expression. Interestingly, this effect was restricted in time. TGF β 1 progressively lost its ability to inhibit Sol 8 cell terminal differentiation when its addition to DM was delayed from 8 to 34 h. After 34 h of culture in DM, all the cells that were capable in engaging in terminal differentiation will have reached an irreversible state of differentiation. In these cells, TGF β 1 will not inhibit β MHC accumulation. This lack of inhibition did not seem to be due to the down-regulation of the T β RII in Sol 8 cells (shown in Figure 4A). However, a down-regulation of this receptor was reported in two other myoblast cell lines [27]. Furthermore, TGF β has been shown to inhibit terminal myogenic differentiation through Smad3 activation [28]. Smad3, but not Smad2, acts downstream of TGF β to repress the MyoD family of MRFs (myogenic regulatory factors) in C2C12 myoblasts. These MRFs are already expressed in undifferentiated myoblasts. Smad3 inhibits the activation of specific muscle genes by interacting with the bHLH (basic helix-loop-helix) domain of MyoD. This will prevent MyoD binding to the E-box sequences in the promoters [28]. Such a mechanism of inhibition is likely to occur in Sol 8 cells which, like C2C12 cells, express MyoD and myogenin, as well as Smad3.

We investigated further whether Sol 8 cells were able, as are other myoblast cell lines [29], to produce endogenous TGF β s that will control their differentiation. TGF β 3 and TGF β 1 transcripts were detected in Sol 8 cells. TGF β 3 is highly expressed in the embryonic heart [30], in skeletal muscles [6] and C2C12 myoblasts. TGF β 3 secretion and transcription is regulated during myogenic differentiation [6]. TGF β 1 is also known to play a part in the differentiation of myoblasts [5,7,26].

Two independent approaches have shown that a TGF β autocrine regulatory loop retarded and partially impaired Sol 8 cell terminal differentiation. First, permanent immunoneutralization of the active TGF β s released by the cells into the DM increased the number of myotubes, as well as β MHC and MyoD levels, compared with controls. Secondly, a dominant-negative mutant of T β RII was overexpressed under the control of the β MHC promoter. Owing to the blocking of the endogenous TGF β s, the terminal differentiation of Sol 8 cells overexpressing the mutated T β RII was promoted, as shown by the rapid increase of β MHC accumulation and myotube formation.

Both immunoneutralization and dominant-negative receptor approaches used in the present study underline the biological relevance of the TGF β autocrine regulatory loop in the inhibitory control of the terminal differentiation of Sol 8 cells. However, these two different approaches had similar results in promoting terminal differentiation in Sol 8 cells. Addition of the antibodies as soon as Sol 8 cells were cultured in DM permanently blocked endogenous TGF β signalling. In contrast, 24 h in DM was required for the β MHC promoter to induce detectable levels of the dominant-negative receptor in the p- β MHC-T β RII-KR-EGFP stable clones. Such a temporal pattern of activation of

the β MHC promoter allowed us to investigate when the TGF β autocrine regulatory loop exerts its inhibition on the terminal differentiation of Sol 8 cells. Inhibition of Sol 8 cell terminal differentiation by endogenous TGF β s is concomitant with the onset of activity of the β MHC promoter. Blocking endogenous TGF β s in the early phase of differentiation (the first 24 h in DM) with the neutralizing antibodies does not promote further Sol 8 cell terminal differentiation.

Our results demonstrate that a kinase-deficient mutant T β RII receptor, expressed under the control of the β MHC promoter, promotes Sol 8 cell terminal differentiation. A seemingly similar approach was used in C2C12 myoblasts [31], and gave results that were opposite to ours. Like Sol 8 cells, C2C12 myoblasts differentiate into myotubes in DM, and this process is inhibited by TGF β 1 [5,26]. In contrast, C2C12 clones, overexpressing a dominant-negative mutated T β RII under the control of the constitutive CMV promoter, did not differentiate in DM. The constitutive overexpression of the dominant-negative T β RII blocked the TGF β autocrine loop in these C2C12 clones in GM. Blocking the TGF β autocrine loop during the growth phase inhibited MyoD and myogenin expression. These clones were unable to differentiate when they were later cultured in DM [31]. On the other hand, in our model, the TGF β autocrine loop was only blocked after 24 h in DM as soon as the β MHC promoter was activated. The dominant-negative effect of the mutant receptor did not interfere with the commitment of the cells as myoblasts in GM. However, the induction of the dominant-negative effect concomitantly with the activity of the β MHC promoter prevented the inhibition by endogenous TGF β s of terminal differentiation. As stated above, after 34 h in DM, terminally differentiated Sol 8 cells were insensitive to the inhibition by exogenous TGF β of β MHC accumulation in the cells. In short, endogenous TGF β partially inhibits Sol 8 cell terminal differentiation between 24 h and 34 h in DM. This time interval coincides with the onset of β MHC expression in the Sol 8 cells engaged in terminal differentiation.

The study of the TGF β autocrine regulatory loop in myoblasts may clarify the conflicting reports on the inhibitory as compared with stimulatory effects of TGF β on muscle cell differentiation. The TGF β autocrine regulatory loop in myoblasts has opposite effects, depending on the conditions of culture. In mitogen-rich media, endogenous TGF β s act positively on the expression of the MRFs and maintain myoblast commitment to muscle cells. In low-mitogen media, endogenous TGF β s have opposite effects. They slow down terminal differentiation by negatively interfering with the activity of the MRFs. Using the β MHC promoter to express a mutant dominant-negative T β RII in transgenic animals could provide more information on the *in vivo* role of TGF β in fetal ventricular myocardium and slow-twitch skeletal muscles [32–34].

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