Transforming growth factor β decreases the rate of proliferation of rat vascular smooth muscle cells by extending the G₂ phase of the cell cycle and delays the rise in cyclic AMP before entry into M phase

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Transforming growth factor β 1 (TGF- β 1) decreased the rate of proliferation of rat aortic vascular smooth muscle cells (VSMCs) stimulated with serum showing a maximal effect at > 5 ng/ml (200 pM). However, it did not reduce the proportion of cells which passed through S phase ($> 90\%$) and entry into S phase was delayed by less than 3 h. The proportion of cells passing through M phase ($> 90\%$) was also unaffected, but entry into mitosis was delayed by approx. 24 h. This increase in cell cycle time was therefore due mainly to an increase in the G₂ to mitotic metaphase period. Addition of TGF- β 1 late in G₁ or late in S phase failed to delay the onset of mitosis, but the presence of TGF- β 1 between 0 and 12 h after the addition of serum to quiescent cells was sufficient to cause the maximal delay in mitosis of approx. 24 h. The role of cyclic AMP in the mechanism of the TGF- β 1 effects on the cell cycle was examined. Entry into mitosis was preceded by a transient 2-fold increase in cyclic AMP concentration and TGF- β 1 delayed both this increase in cyclic AMP and entry into mitosis to the same extent. Addition of

forskolin or 8-(4-chlorophenylthio)-cyclic AMP to cells 30 h after stimulation with serum completely reversed the increase in duration of G₂ in the presence of TGF- β 1, suggesting that the rise in cyclic AMP levels which precedes mitosis might trigger entry of the VSMCs into M phase. Addition of forskolin late in S phase (26 h after stimulation with serum) advanced the entry of the cells into M phase and they divided prematurely. This effect was unaffected by the addition of cycloheximide with the forskolin; however, the effect of forskolin on cell division was completely inhibited when cycloheximide was added late in G₁. TGF- β 1 prevented the loss of smooth-muscle-specific myosin heavy chain (SM-MHC), which occurs in primary VSMC cultures in the presence or absence of serum, and the cells proliferated while maintaining a differentiated phenotype. However, TGF- β 1 did not cause re-differentiation of subcultured VSMCs which contained very low amounts of SM-MHC and the effect of TGF- β 1 in extending the G₂ phase of the cell cycle is exerted independently of its effect on differentiation.

INTRODUCTION

The proliferation of intimal vascular smooth muscle cells (VSMCs) in vascular disease is considered to result from endothelial dysfunction or injury [1,2]. One consequence of the absence of a functional endothelium is increased adhesion of leucocytes and platelets at the site of injury [3]. The VSMCs migrate to form a neointima in response to release by platelets of chemotactic substances, in particular platelet-derived growth factor (PDGF) [4]. These intimal VSMCs proliferate in response to a complex mixture of growth-promoting substances released by adherent leucocytes, macrophages, platelets [5–7], endothelial cells [8] and by the intimal VSMCs themselves [9,10]. The establishment of a neointima is assumed to depend on the balance between growth-promoting agents and factors which inhibit the de-differentiation, migration and/or proliferation of the medial VSMCs. One such potential growth-industry substance is transforming growth factor β (TGF- β) [11–13].

The TGF- β isoforms are a family of multifunctional dimeric polypeptides which are known to have diverse roles, affecting proliferation and differentiation of various cell types ([14–17] and reviewed in [18]). The best-studied member of the family, TGF- β 1, is a disulphide-linked dimer of two identical chains of 112 amino acids and is contained in the alpha granules of

platelets [19]. It is released in a latent form from platelets at sites of injury and appears to be involved in repair of many tissues. Active TGF- β 1 is thought to be produced by the action of plasmin [20] on latent TGF- β . As tissue-type plasminogen activator is produced at sites of vascular injury [21], active TGF- β would be expected to be present at these sites. The TGF- β type-II receptor has intrinsic serine/threonine kinase activity [22] and there is evidence that some of its effects are mediated by a decrease in cellular cyclic AMP concentration [23–26]. However, little is known about the coupling between the receptor kinase activity and the regulation of cyclic AMP.

Active TGF- β 1 can either promote or inhibit cell proliferation in culture depending on the cell type and the conditions. TGF- β 1 is reported to reduce the rate of proliferation of VSMCs stimulated by serum [27–29]. In the presence of serum, TGF- β 1 caused hypertrophy of normotensive rat VSMCs in addition to reducing their rate of proliferation. The hypertrophic effect was not dependent on growth arrest in the G₁- or G₂-phases of the cell cycle, or withdrawal of the cells from the cell cycle [27]. In the absence of serum, TGF- β 1 inhibited DNA synthesis stimulated by PDGF in rabbit [28] and human [29] arterial VSMCs.

TGF- β 1 is known to affect the state of differentiation of various cell types (reviewed in [18]), including chondroblasts [30], ROS osteosarcoma cells [31] and epithelial cells [32,33]. It also

Abbreviations used: aChGM, antiserum to chicken gizzard myosin; DMEM, Dulbecco's modification of Eagle's medium; EGF, epidermal growth factor; FCS, fetal-calf serum; PDGF, platelet-derived growth factor; SM-MHC, smooth-muscle-specific myosin heavy chain; $t_{1/2}$ M, time after restimulation when 50% of the cells have divided; TGF- β 1, transforming growth factor β 1; VSMC, vascular smooth muscle cell.

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promotes the differentiated phenotype of human VSMCs, assayed by the expression of smooth-muscle-specific α -actin [29]. It has been suggested that de-differentiation is required for proliferation in VSMCs [34–36]. However, we have found that VSMC clones of several distinct phenotypes can be derived from freshly dispersed rat aortic VSMCs and that cells of three of the clonal phenotypes will proliferate normally while retaining a complement of smooth-muscle-specific myosin heavy chain (SM-MHC) and smooth-muscle α -actin similar to that in the freshly dispersed cells (D. J. Grainger, C. M. Witchell, C. M. Shanahan, J. C. Metcalfe and P. L. Weissberg, unpublished work). This is in marked contrast with the behaviour of the freshly dispersed VSMCs in primary cultures at densities in which proliferation and de-differentiation, marked by the loss of the smooth-muscle-cell-specific proteins, occur over the same time course [38,39].

We have examined the effects of TGF- β 1 on the proliferation of rat aortic VSMCs stimulated by serum or defined growth factors in serum-free medium with the aim of determining how TGF- β inhibits VSMC proliferation. There is accumulating evidence that VSMCs are heterogeneous and we therefore used assays to establish whether the whole cell population responded uniformly to TGF- β 1 at S- and M-phases or whether the cells were heterogeneous in their responses. We have also examined the effects of TGF- β 1 on the differentiation of VSMCs to determine whether the differentiated status of the cells affected their ability to proliferate.

MATERIALS AND METHODS

Cell culture and counting

The aortae were removed from 12–17-week-old Wistar rats killed by CO₂ asphyxiation. VSMCs from the tunica media were dispersed as previously described [38,40]. Hanks' balanced-salts solution (ICN/Flow) supplemented with 10% (v/v) fetal-calf serum (FCS; ICN/Flow) was used throughout the preparation. The cells (4×10^5 /ml) were suspended in medium M199 (ICN/Flow) supplemented with 12.5 mM sodium bicarbonate, 2 mM glutamine and penicillin (100 units/ml), and plated on to sterile culture dishes (ICN/Flow) at 8.3×10^4 cells/cm². Cells cultured in the absence of serum were prepared as above except: (1) aortae were removed from 7–9-week-old Wistar rats; (2) serum-free Hanks' balanced-salt solution was used during the preparation so that the cells never came into contact with serum; and (3) the culture dishes were coated ($5 \mu\text{g}/\text{cm}^2$) with bovine plasma fibronectin (Sigma Chemical Co.) in sterile phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 1.5 mM KH₂PO₄, pH 7.4) for 45 min, then aspirated. TGF- β 1 purified from pig platelets (Peninsula Labs) was dissolved in 5 mM HCl to give a $5 \mu\text{g}/\text{ml}$ stock which was diluted in 25 mM Tris/HCl, pH 7.4, containing 0.2% BSA and filtered through a $0.22 \mu\text{m}$ pore diam. filter to give a $1 \mu\text{g}/\text{ml}$ stock. The cells were incubated in 5% CO₂ in air (pH 7.2) at 37 °C; the start of this incubation was defined as 0 h in all time-course experiments and the medium was replaced every 48 h. At 48 h more than 99% of the cells stained positively for SM-MHC isoforms using an antiserum raised to chicken gizzard smooth muscle myosin (aChGM) as described [41].

Subcultured cells were obtained by treatment of confluent stationary-phase cultures with trypsin/EDTA (Gibco) for 3–4 min and dilution of the released cells at a 1:2 ratio in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS. The subcultured cells were used between passages 7 and 13 and the cells were allowed to grow for 24 h after subculture in DMEM plus 10% (v/v) FCS before performing the

experiments. Quiescent cells were obtained by washing the cultures three times in serum-free DMEM before incubation in serum-free DMEM for 48 h. Swiss 3T3 fibroblasts were subcultured, at a 1:3 dilution in DMEM containing 10% (v/v) FCS, every 3 days. Fibroblasts were used between passages 11 and 17 and were made quiescent in serum-free DMEM for 24 h. Recombinant epidermal growth factor (EGF; Gibco) and recombinant PDGF BB homodimer (PDGF-BB; Bachem) were dissolved in 25 mM Tris/HCl, pH 8.0, containing 0.2% fatty acid-free BSA (Sigma) to give $10 \mu\text{g}/\text{ml}$ stocks.

Cells were prepared for counting by haemocytometer from triplicate culture dishes as described [38]. Cells were also counted by direct microscopic observation of gridded culture dishes. The grids were scored into the plastic on the inner surface so that cells could not migrate into or out of the area being counted during the time-course experiment. Cells in each of four squares in two separate wells were counted at each time point and photographed. All cell-counting experiments were repeated on at least three separate cultures as indicated and cells numbers are plotted as means $\pm$ S.E.M.

DNA synthesis

[³H]Thymidine incorporation into DNA was assayed by the addition of $5 \mu\text{Ci}/\text{ml}$ of [6-³H]thymidine (Amersham International) to the culture medium for 6 h before harvesting. At the end of the pulse, the medium was aspirated and the cells washed twice in ice-cold PBS, then lysed in 0.5 ml of 1.0% (w/v) SDS. The lysate was treated with an equal volume of 15% (w/v) trichloroacetic acid on ice for 20 min before filtering through Whatman GF/C paper and washing with 4 ml of 7.5% (w/v) trichloroacetic acid then 4 ml of 96% (v/v) ethanol. The filters were dried in air and scintillation-counted. DNA synthesis was also assayed by bromodeoxyuridine incorporation for the periods indicated using a cell-proliferation kit (Amersham International).

Where entry into DNA synthesis was assayed by [³H]thymidine incorporation during successive pulses in replicate sets of cells under different conditions (e.g. control cells and cells in the presence of heparin), the delay in entry into S phase was calculated from the formula:

$$D = \{\sum_{x=2 \text{ to } n} [(C_x - H_x)/(C_x - C_{x-1})] \times T\} / (n - 1)$$

where D is the delay in h, n is the total number of successive [³H]thymidine pulses of equal duration, C_x is the counts (c.p.m.) incorporated in the control cells during pulse $n = x$, H_x is the counts incorporated in the test cells during pulse x , C_{x-1} is the counts incorporated in the control cells during pulse $x - 1$, and T is the duration in h of each pulse. If DNA synthesis is inhibited in the test cells but not delayed, the formula will give a delay of 0 h. If the DNA synthesis time course is broadened (because the cell population is less synchronous) the formula will give a delay of 0 h. If DNA synthesis is delayed and inhibited the formula will give the same delay value as if DNA synthesis is delayed but not inhibited. Comparative estimates of the time taken for cell populations to pass through S phase are obtained from the width (in h) of the pulsed [³H]thymidine incorporation peak at half-peak height.

Analysis of myosin heavy chain (MHC) isoforms

Samples of 4×10^5 cells were prepared for MHC isoform analysis as described for cell counting, centrifuged (900 g ; 4 min) and lysed in gel sample buffer [42] after removing the supernatant. The samples were analysed by SDS/PAGE and Western blotting as described previously [38] using aChGM [43] to detect SM-

MHC and a peroxidase-linked second antibody. Labelling was visualized with diaminobenzidine and H_2O_2 in the presence of nickel ions.

Determination of cyclic AMP level

The medium was aspirated and the cells washed three times in ice-cold PBS. The cells were lysed in 6% (w/v) perchloric acid and the lysate neutralized with 1 M KOH/2 M K_2CO_3 . Protein and solid KClO_4 were precipitated (14000 g; 4 min) and the supernatant lyophilized and stored at -80°C . The cyclic AMP content of the lyophilized cell lysate was determined using a cyclic AMP determination kit (Amersham International). Cyclic AMP levels were determined in duplicate for each of six sample dishes at each time point. Values are plotted as means $\pm$ S.E.M.

RESULTS

Effects of TGF- β 1 on the cell cycle of VSMCs

We have shown previously that subcultured rat aortic VSMCs proliferate with a cell cycle time of approx. 35 h in 10% (v/v) FCS [38,44]. When TGF- β 1 was present, the rate of proliferation decreased, with maximal inhibition at concentrations above 5 ng/ml (Figure 1). In the presence of 10 ng/ml TGF- β 1 the increase in cell number at 96 h after the addition of serum was reduced by $76\% \pm 4.1\%$ ($n = 3$). The lower rate of proliferation in the presence of TGF- β 1 might be due to a complete block on the proliferation of a proportion of the cells or to an increase in the cell cycle time of all the cells. To distinguish between these possibilities, the proportions of cells passing through S- and M-phases and the time courses for these transitions were determined.

The effect of TGF- β 1 on the proportion of quiescent, subcultured VSMCs which passed through S phase was examined 36 h after stimulating the cells by addition of 10% (v/v) FCS in the presence of bromodeoxyuridine (10 μM). In the absence of TGF- β 1, $93\% \pm 2\%$ ($n = 3$) of the cells had entered, or passed

through, S phase by 36 h. Concentrations of TGF- β 1 up to 10 ng/ml had no significant effect on the proportion of nuclei which had entered, or passed through, S phase by 36 h [$94\% \pm 3\%$ ($n = 3$) at 1 ng/ml TGF- β 1; $90\% \pm 2\%$ ($n = 3$) at 10 ng/ml TGF- β 1]. In the absence of FCS, only $6.9\% \pm 1.7\%$ ($n = 3$) of the nuclei stained positively for bromodeoxyuridine. These results were confirmed by separate experiments in which the total incorporation of [^3H]thymidine into cells over 36 h was unaffected ($\pm 5\%$) by the presence of TGF- β 1 at concentrations up to 10 ng/ml.

The time course of S phase for subcultured VSMCs was determined by pulse-labelling for 3 h with [^3H]thymidine between 9 h and 27 h after stimulation of quiescent cells with 10% (v/v) FCS. TGF- β 1 (10 ng/ml) caused only a small delay (< 3 h) in re-entry into S phase (Figure 2a) when calculated from the [^3H]thymidine pulse data using the formula given in the Materials and methods section.

To determine the time course of M phase after stimulating quiescent cells with 10% (v/v) FCS in the presence and absence of 10 ng/ml TGF- β 1, time-lapse photomicroscopy was used to count the cells at 8 h intervals (Figure 2b). In the absence of TGF- β 1, $> 95\%$ of the subcultured cells had divided by 36 h, whereas there was no significant increase in cell number in the presence of TGF- β 1 before 54 h. However, by 72 h more than 95% of the cells had divided in the presence of TGF- β 1 (Figure 2b). The time taken for 50% of the cells to divide after stimulation by serum (defined as $t_{1/2}$ M) was increased from 35 ± 2 h ($n = 4$) to 58 ± 2 h ($n = 4$) by TGF- β 1.

As TGF- β 1 did not reduce significantly the proportion of cells either entering or completing the cell cycle, the data implied that the inhibition of proliferation was due to an increase in the cell cycle time of nearly all ($> 90\%$) of the proliferating cells. The delay in the entry into S phase (< 3 h) was much too small to account for the inhibition of proliferation of VSMCs by 10 ng/ml of TGF- β 1 (Figure 1). It was clear that the increase in time taken to traverse the G_2 phase of the cell cycle was mainly responsible for the decrease caused by TGF- β 1 in the rate of proliferation of the serum-stimulated VSMCs.

To determine when TGF- β 1 must be present to extend the G_2 phase of the cell cycle, quiescent VSMCs were stimulated with 10% (v/v) FCS and 10 ng/ml TGF- β 1 was added at various times during the first cell cycle. The presence of TGF- β 1 in the medium for the first 12 h after stimulation was sufficient to delay mitosis as effectively ($t_{1/2}$ M = 53 ± 5 h; $n = 3$) as when TGF- β 1 was present throughout the cell cycle. Addition of TGF- β 1 (10 ng/ml) at 12 h after stimulation (i.e. at the G_1 /S boundary; see Figure 2a) or at 26 h after stimulation (immediately after S phase was completed) had no significant effect on the duration of the cell cycle [$t_{1/2}$ M = 34 ± 2 h ($n = 4$) and 33 ± 3 h ($n = 4$) respectively] (Figure 2c). These results indicate that TGF- β 1 must act on the cells during G_0 and/or G_1 to increase the duration of the G_2 phase of the cell cycle.

Effects of TGF- β 1 on the proliferation and differentiation of primary VSMCs

We have previously shown that primary rat VSMC cultures take longer to reach mitosis in the first cell cycle after plating of the freshly dispersed cells than subcultured cells [38,45]. We therefore examined the effect of TGF- β 1 (10 ng/ml) on primary VSMC cultures and found that it extended the G_2 phase of all of the cells in the first cell cycle by a similar extent to that observed in the subcultured cells. The $t_{1/2}$ M of primary VSMCs for the first cell cycle was increased from 56 ± 3 h ($n = 4$) to 82 ± 4 h ($n = 4$) by 10 ng/ml TGF- β 1 (Figure 2d).

Figure 1 Rate of proliferation of subcultured rat VSMCs in the presence of TGF- β 1

Subcultured VSMCs (passage 5–9) were made quiescent in serum-free DMEM for 48 h and restimulated with 10% (v/v) FCS in the absence (○) or presence (●) of TGF- β 1 at concentrations between 0.1 and 10 ng/ml. Cell number was determined at 24 h intervals by removing the cells from triplicate wells with trypsin and counting on a haemocytometer as described in the Materials and methods section. Values are means from three separate cultures; error bars are S.E.M.s.

Figure 2 Effect of TGF-β1 on entry into S phase and M phase in VSMCs

(a) Effect of TGF-β1 on DNA synthesis. Subcultured (passage 7–10) VSMCs were made quiescent in serum-free DMEM for 48 h and restimulated with 10% (v/v) FCS in the absence (○) or presence (●) of 10 ng/ml TGF-β1. DNA synthesis was assayed by measuring incorporation of [³H]thymidine in cells in triplicate wells during 6 h pulses between 12 and 42 h after restimulation. Values are means from four separate cultures; error bars are S.E.M.s. (b) Effect of TGF-β1 on cell division in subcultured VSMCs. Subcultured (passage 6–9) VSMCs were made quiescent in serum-free DMEM for 48 h and restimulated with 10% (v/v) FCS in the absence (○) or presence (●) of 10 ng/ml TGF-β1. Cells were counted in each of four grid squares in two separate wells from time-lapse photomicrographs at 8 h intervals and cell numbers are expressed relative to the cell number present at restimulation with serum (0 h). Values are means from six separate cultures; error bars are S.E.M.s. (c) Effect of time of addition of TGF-β1 on cell division. Subcultured (passage 5–12) VSMCs were made quiescent in serum-free DMEM for 48 h and restimulated with 10% (v/v) FCS. Cells were restimulated in the absence (○) of TGF-β1 or with 10 ng/ml TGF-β1 in the medium for different periods of the first cell cycle: (●) TGF-β1 present throughout (0–36 h); (▲) TGF-β1 present during G₂ (26–36 h); (■) TGF-β1 present from the end of G₁ onwards (12–36 h); and (△) TGF-β1 present only during G₁ (0–12 h). Cell numbers were determined by time-lapse photomicroscopy as for (b). Values are means from two separate cultures; error bars are S.E.M.s. (d) Effect of TGF-β1 on cell division in primary VSMC cultures. Primary cultures of VSMCs were plated out in DMEM plus 10% FCS in the absence (○) or presence (●) of 10 ng/ml TGF-β1 from 0 h and cell numbers were determined by time-lapse photomicroscopy as for (b). Values are means from two separate cultures; error bars are S.E.M.s.

TGF-β1 is known to affect differentiation in several types of cells, including chondroblasts, ROS osteosarcoma cells and endothelial cells [30–33,46,47]. We have shown previously that in primary cultures, VSMCs lose both the 204 kDa (SM-1) and the 200 kDa (SM-2) SM-MHC isoforms whether they are plated out in FCS [38] or in serum-free medium on to fibronectin [45]. The presence of TGF-β1 (10 ng/ml) substantially inhibited the loss of both SM-1 and SM-2 in the presence or absence of FCS (Figure 3a). However, TGF-β1 did not induce re-expression of SM-MHC in subcultured cells which have very low levels of the protein (Figure 3b). It was concluded that TGF-β1 maintains the differentiated state (as defined by SM-MHC content) in primary VSMCs, but cannot induce re-differentiation in the de-differentiated proliferating cells.

Changes in cyclic AMP levels at the G₂/M transition

Agents which increase the concentration of cyclic AMP have been shown to inhibit the action of TGF-β on several types of cell [23–26]. The effect of TGF-β1 on cyclic AMP concentration during the VSMC cell cycle was therefore determined. Addition of 10% (v/v) FCS to quiescent subcultured VSMCs in the

presence or absence of TGF-β1 (10 ng/ml), did not affect the cyclic AMP concentration significantly for the first 24 h of the cell cycle. In the absence of TGF-β1, the amount of cyclic AMP/cell rose by approx. 2-fold between 24 and 33 h, immediately before cell division ($t_{1/2}$ M = 35 h in the experiment shown in Figure 4a), and started to fall by 36 h, when the cells were beginning to re-enter the G₁ phase of the cell cycle. In the presence of TGF-β1 (10 ng/ml) the increase in cyclic AMP concentration was delayed by approx. 24 h (Figure 4b) and again occurred immediately before M phase ($t_{1/2}$ M = 52 h). These results show that a small (approx. 2-fold) rise in intracellular cyclic AMP precedes mitosis in VSMCs in the presence or absence of TGF-β1.

Further experiments were performed to examine the correlation between cyclic AMP concentration and the duration of the G₂ phase of the cell cycle in the presence or absence of TGF-β1. Quiescent, subcultured VSMCs were stimulated with 10% (v/v) FCS for 30 h, various concentrations of forskolin were added and the time course of the cyclic AMP response was determined. The increase in cyclic AMP was transient, with maximal increases after 30 min of approx. 2-fold and 15-fold in response to 0.33 μM and 5 μM forskolin respectively (Figures 5a

Figure 3 The effect of TGF- β 1 on SM-MHC content of primary and subcultured VSMCs

(a) SM-MHC content of 2×10^5 VSMCs in primary culture determined by Western blotting. Lane 1, freshly dispersed VSMCs (0 h); lane 2, cells plated out in the presence of 10% (v/v) FCS and harvested at 144 h after plating; lane 3, cells plated out in the presence of 10% (v/v) FCS and 10 ng/ml TGF- β 1 and harvested 144 h after plating; lane 4, cells plated out in the absence of serum on to fibronectin and harvested 144 h after plating; and lane 5, cells plated out in the absence of serum on to fibronectin in the presence of 10 ng/ml TGF- β 1 and harvested 144 h after plating. (b) SM-MHC content of 2×10^5 subcultured VSMCs (passage 7) determined by Western blotting. Lane 1, cells in exponential growth in 10% (v/v) FCS, 48 h after subculturing in the absence of TGF- β 1; lane 2, cells in exponential growth in 10% (v/v) FCS, 48 h after subculturing in the presence of 10 ng/ml TGF- β 1 from 24–48 h. The SM-MHC content of 2×10^5 freshly dispersed primary VSMCs is shown for comparison (lane P).

Figure 4 Comparison of the effect of TGF- β 1 on cyclic AMP and mitosis in VSMCs

(a) Subcultured VSMCs (passage 11) were made quiescent in serum-free DMEM for 48 h and restimulated with 10% (v/v) FCS at 0 h. The cyclic AMP concentration ($\bullet$) per cell was determined in duplicate on each of six dishes at various time points during the cell cycle as described in the Materials and methods section (error bars are S.E.M.s). Cell number ($\square$) was determined at each time point for cells in triplicate wells by removing the cells with trypsin and counting by haemocytometer and plotted relative to the cell number at 0 h [$1.1 \pm 0.1 \times 10^6$ cells/cm ^{2} ($n = 3$)]. The data shown are typical of three similar experiments. (b) Cyclic AMP concentration ($\bullet$) and relative cell number ($\square$) during the cell cycle were determined for subcultured (passage 11) VSMCs in the presence of 10 ng/ml TGF- β 1 from restimulation at 0 h as for (a). The cell number at 0 h was $1.1 \pm 0.1 \times 10^6$ cells/cm ^{2} ($n = 3$) and the data shown are typical of three experiments.

and 5b). Various concentrations of forskolin were added to the VSMCs in the presence of TGF- β 1 (10 ng/ml) at 30 h after stimulation by 10% (v/v) FCS (i.e. when the cells started to

Figure 5 Effect of forskolin on VSMCs in the presence of TGF- β

(a) Increase in intracellular cyclic AMP concentration with time after forskolin addition. Subcultured (passage 8) VSMCs were made quiescent in serum-free DMEM for 48 h and restimulated with 10% (v/v) FCS in the presence of 10 ng/ml TGF- β 1. At 30 h after restimulation forskolin (1 μ M) was added, and six dishes of cells were harvested at various times between 5 and 120 min after forskolin addition. [Cyclic AMP] per 10^6 cells was determined as described in the Materials and methods section. The experiment shown is typical of four similar experiments. (b) Dose-response curve showing effect of forskolin concentration on cyclic AMP concentration per cell 30 min after forskolin addition under the conditions described in (a). (c) Effect of forskolin addition on entry into M phase by VSMCs in the presence of TGF- β . Subcultured (passage 6–10) VSMCs were plated out on to gridded dishes, made quiescent in serum-free DMEM for 48 h and stimulated at 0 h with 10% (v/v) FCS in the absence ($\circ$) or presence (all other symbols) of 10 ng/ml TGF- β . Various concentrations of forskolin ($\triangle$, 5 μ M; $\bullet$, 1 μ M; $\blacktriangle$, 500 nM; $\blacksquare$, 100 nM; $\square$, no forskolin) were added at 30 h and cell number was determined at various times thereafter.

divide in the absence of TGF- β 1). As the concentration of forskolin increased the extension of G_2 caused by TGF- β 1 was reversed (Figure 5c). In response to 0.5 μ M forskolin, which caused an approx. 5-fold increase in maximal cyclic AMP

Figure 6 Effect of forskolin and 8-(4-chlorophenylthio)-cyclic AMP on VSMCs in the absence of TGF- β

(a) Effect of forskolin on entry into mitosis in VSMCs. Subcultured (passage 7–11) VSMCs were plated on to gridded dishes, made quiescent in serum-free DMEM for 48 h and stimulated at 0 h with DMEM plus 10% (v/v) FCS. Entry into M phase was monitored by counting the cells at various times in the absence (○) or presence of forskolin (▲, 5 μ M; ■, 1 μ M; □, 500 nM; ●, 100 nM). (b) Effect of 8-(4-chlorophenylthio)-cyclic AMP on entry into mitosis in VSMCs. Subcultured (passage 7–11) VSMCs were plated on to gridded dishes, made quiescent in serum-free DMEM for 48 h and stimulated at 0 h with DMEM plus 10% (v/v) FCS. The cells were counted at various times in the absence (○) or presence of 8-(4-chlorophenylthio)-cyclic AMP (▲, 100 μ M; ■, 10 μ M; ●, 5 μ M; □, 1 μ M). (c) Effect of forskolin on entry into mitosis in VSMCs in the presence of cycloheximide. Subcultured (passage 7–11) VSMCs were plated on to gridded dishes, made quiescent in serum-free DMEM for 48 h and stimulated at 0 h with DMEM plus 10% (v/v) FCS. The cells were counted after no further addition (○), after addition of forskolin (5 μ M) and cycloheximide (35 μ M) at 24 h (■), after addition of forskolin (5 μ M) alone at 25 h (●) and after addition of cycloheximide (35 μ M) at 15 h (□).

concentration (Figure 5b), the VSMCs divided slightly faster than cells in the absence of TGF- β . Higher concentrations of forskolin caused over 80% of the cells to divide within 3 h of addition, and division was nearly complete before the control

cells started to divide. In contrast with the effect of forskolin, the addition of the phosphodiesterase inhibitors theophylline (250 μ M) and RO 20-174 (1 μ M), either separately or together, to quiescent cells with 10% (v/v) FCS and TGF- β (10 ng/ml) had no effect on the delay of entry into M phase induced by TGF- β (results not shown).

The very rapid entry into M phase of the cells treated with concentrations of forskolin (> 5 μ M) that produced a maximal increase in cyclic AMP concentration prompted examination of the effect of forskolin addition late in S phase. Forskolin was added at various concentrations to VSMCs 24 h after stimulation with 10% (v/v) FCS, when over 70% of the cells have completed S phase by fluorescence-activated cell sorting analysis (results not shown). Concentrations of forskolin of 5 μ M or higher caused over 80% of the cells to divide within 4 h of addition (Figure 6a). The results indicate that cyclic AMP can induce the cells to divide before division occurs in control cells. However, a higher concentration of forskolin was required to achieve premature division than that required to reverse the effect of TGF- β 1 on G₂.

To test whether the effect of forskolin was due to the increase in cyclic AMP concentration it induces, various membrane-permanent analogues of cyclic AMP were tested for their ability to increase the rate of proliferation of subcultured VSMCs, of which 8-(4-chlorophenylthio)-cyclic AMP was the most effective (results not shown). The effect of various concentrations of 8-(4-chlorophenylthio)-cyclic AMP added to the cells 24 h after stimulation by 10% (v/v) FCS was similar to the effect of forskolin in advancing the entry of the cells into mitosis (Figure 6b).

The rapid entry of the cells into M phase immediately after S phase was complete, in response to forskolin, raised the question of whether any protein synthesis was required in G₂ for the cells to divide. When cycloheximide (35 μ M) was added together with forskolin (5 μ M) to cells late in S phase at 24 h, there was little effect on the rate at which the cells subsequently divided (Figure 6c). However, if cycloheximide was added before S phase, at 15 h after stimulation with 10% (v/v) FCS (see Figure 2a), there was no subsequent division of the cells. This is consistent with evidence that entry into S phase is dependent on protein synthesis until late in G₁.

Effects of TGF- β 1 on stimulation by PDGF and EGF

The effect of TGF- β 1 on the entry into S phase of quiescent, subcultured VSMCs stimulated by PDGF or EGF in the absence of serum was determined. The time course of entry into S phase, assayed by incorporation of [³H]thymidine during 6 h pulses, in response to 100 ng/ml EGF or 100 ng/ml PDGF-BB was similar to the time course in response to 10% (v/v) FCS (Figures 7a and 7b). Both EGF and PDGF-BB stimulated much less DNA synthesis than 10% (v/v) FCS and a lower proportion of the cells entered S phase, as assayed by bromodeoxyuridine incorporation. At 36 h, 94 $\pm$ 2% (*n* = 3) of cells had entered S phase in response to 10% (v/v) FCS, 36 $\pm$ 3% (*n* = 3) in response to 100 ng/ml EGF and 54 $\pm$ 3% (*n* = 3) in response to 100 ng/ml PDGF-BB. TGF- β 1 (10 ng/ml) inhibited DNA synthesis in response to EGF and PDGF-BB by 80% and 51% respectively (Figures 7a and 7b), in contrast with the absence of inhibition of DNA synthesis in VSMCs stimulated with 10% (v/v) FCS (Figure 2a).

It was unclear why TGF- β 1 did not inhibit entry into S phase of VSMCs stimulated with serum. One possibility was that serum components bind TGF- β 1 and prevent the free concentration of TGF- β reaching the level required for inhibition of S phase.

Figure 7 Effect of TGF- β 1 on DNA synthesis in VSMCs stimulated with EGF or PDGF

(a) Subcultured VSMCs (passage 7–9) were made quiescent in serum-free DMEM for 48 h and stimulated at 0 h with 10% (v/v) FCS (○) or with 100 ng/ml EGF alone (□) or with 100 ng/ml EGF and 10 ng/ml TGF- β 1 (●). DNA synthesis was assayed by measuring incorporation of [^{3} H]thymidine in cells in triplicate wells during 6 h pulses between 12 and 36 h after stimulation. Values are means from two separate cultures; error bars are S.E.M.s. (b) Subcultured VSMCs (passage 7–9) were made quiescent in serum-free DMEM for 48 h and stimulated at 0 h with 10% (v/v) FCS (○) or with 100 ng/ml PDGF-BB alone (□) or with 100 ng/ml PDGF-BB and 10 ng/ml TGF- β 1 (●). DNA synthesis was assayed as in (a). (c) Effect of high concentrations of TGF- β 1 on DNA synthesis in rat VSMCs. Subcultured (passage 7–11) VSMCs were plated on to gridded dishes, made quiescent in serum-free DMEM for 48 h and stimulated at 0 h with DMEM plus 10% (v/v) FCS in the presence of various concentrations of TGF- β 1. Entry into DNA synthesis was measured by [^{3} H]thymidine incorporation between 12 and 36 h after stimulation.

Raising the concentration of TGF- β to 100 ng/ml was sufficient to inhibit DNA synthesis in response to serum by 55% (Figure 7c). These results suggest that high free concentrations of TGF-

β 1 are able to inhibit the proliferation of VSMCs by inhibiting entry into S phase, irrespective of the activating mitogen.

DISCUSSION

The role of cyclic AMP in determining the duration of G_2

Our data are consistent with the hypothesis that an increase in cyclic AMP concentration of approx. 2-fold is part of the physiological mechanism triggering entry of VSMCs into M phase. An increase in cyclic AMP level of this magnitude precedes entry into mitosis in the presence or absence of TGF- β 1 and an increase in cyclic AMP concentration of less than 5-fold induced by forskolin is sufficient to reverse completely the effect of TGF- β in delaying the entry of the cells into mitosis by approx. 24 h. Furthermore, addition of forskolin or 8-(4-chlorophenylthio)-cyclic AMP late in S phase is sufficient to cause rapid entry of the cells into mitosis.

The biochemical events which are necessary for entry into mitosis are only partially defined in terms of the activation of complexes of cdk(s) with cyclins [48]. Entry into mitosis is not advanced by inhibitors of phosphodiesterases, either in the presence or absence of TGF- β 1, suggesting that activation of adenylate cyclase, rather than inhibition of phosphodiesterases, is the main mechanism by which the cyclic AMP pulse before mitosis is generated. The observation that increases in cyclic AMP induced by forskolin added late in S phase will drive the cells into M phase without requiring further protein synthesis was surprising. The results suggest that the appropriate concentrations of cyclins and cdk(s) for mitosis are already present in the cells at, or very soon after, the end of S phase and that the phosphorylation cascade required for entry into mitosis can be triggered by cyclic AMP-dependent protein kinases. It is unlikely, although not inconceivable, that protein kinase A acts downstream of cdk1, as activation of cdk1 is thought to be the key step in entry into M phase and mitosis can be induced by mimicking the cyclic AMP pulse which occurs before mitosis. However, the results provide no indication of the separation in the phosphorylation cascade between the activation of cyclic AMP-dependent protein kinase(s) and the activation of the cdk1 complex on entry into mitosis. Phosphorylation studies *in vitro* and *in vivo* will be required to analyse the coupling in this pathway.

Effect of TGF- β 1 on the cell cycle

There are few precedents for a decrease in the rate of cell proliferation in response to growth factors by lengthening the G_2 phase of the cell cycle. Previous studies have suggested that activation of the GTP-binding protein, G_i , which antagonizes the activation of adenylate cyclase, may be involved in mediating some of the effects of TGF- β [23–26]. If, as we hypothesize, cyclic AMP regulates entry into M phase of VSMCs, the mechanism by which TGF- β delays entry into mitosis may be to delay the cyclic AMP pulse. However, no significant reduction in cyclic AMP concentration was detected in G_2 in the presence of TGF- β compared with control cells. The postulated effect of TGF- β in regulating the cyclic AMP pulse before mitosis is presumably exerted on the cells in G_0 and/or in G_1 , as TGF- β did not extend the duration of the G_2 phase when added immediately before S phase (12 h after stimulation) or at the end of S phase (26 h after stimulation). The presence of TGF- β 1 at low concentrations (< 10 ng/ml) throughout G_1 had only a small effect on the entry of the cells into S phase: no decrease in the proportion of cells (> 90%) entering S phase in response to serum was detected and the delay of entry into S phase measured by [^{3} H]thymidine pulses

was less than 3 h. However, the delay was reproducible and provides evidence for a functional interaction of TGF- β 1 with the cells in G₁ at concentrations which do not inhibit DNA synthesis.

The results presented for the effect of TGF- β 1 on the duration of G₂ are consistent with previous reports on the inhibition of proliferation of rat and bovine VSMCs stimulated by serum [27–29]. The observations by Owens et al. [27] were qualitatively similar to ours: TGF- β 1 caused a marked increase (approx. 2-fold) in the cell cycle transit time of subcultured VSMCs and most, if not all of the cells, continued to cycle in the presence of TGF- β 1. There was also a gradual accumulation of the cells in the G₂ phase of the cell cycle which was associated with hypertrophy of the cells, without growth arrest or withdrawal from the cell cycle in either the G₁ or G₂ phases. It was suggested that the hypertrophy of the cells might result directly from the increase in cell cycle time by allowing the cells to increase in size before division. However, the ED₅₀ for the effect of TGF- β 1 on the rate of proliferation of VSMCs was 2 pM using human TGF- β 1 [27] compared with 40 pM in our study using pig TGF- β 1. This difference in effective TGF- β 1 concentrations can be attributed, at least in part, to the higher concentration of FCS (10%, v/v) used here, as it was shown by Owens et al. [27] that the potency of TGF- β was strongly dependent on serum concentration. TGF- β 1 is known to bind to serum components including α_2 -macroglobulin [44,49] and variation in the binding capacity of different sera would substantially affect the free concentration of TGF- β 1 and therefore the concentration of TGF- β 1 required to increase the duration of G₂.

A relatively high binding capacity of the FCS for TGF- β 1 used in this study would also account for the difference in ED₅₀ for the inhibition of DNA synthesis by TGF- β 1 compared with the data of Morisaki et al. [28]. They showed a partial inhibition by TGF- β , at concentrations about 10 ng/ml, of entry into S phase of rabbit medial and intimal VSMCs stimulated by 10% (v/v) FCS. A partial inhibition of *c-myc* gene expression by TGF- β 1 was consistent with its effect on DNA synthesis. In our study, a much higher concentration of TGF- β 1 (100 ng/ml) was required to cause 50% inhibition of DNA synthesis in cells stimulated by 10% (v/v) FCS. In contrast, our results for the effect of TGF- β 1 on DNA synthesis in response to the growth factors in the absence of serum are quantitatively consistent with similar previous studies. These include a study of human arterial smooth muscle cells by Björkerud [29] who showed that TGF- β 1 inhibited PDGF-induced DNA synthesis and a study by Morisaki et al. [28] who showed substantial inhibition of [³H]thymidine incorporation into DNA in rabbit VSMCs stimulated with PDGF or basic fibroblast growth factor. The latter study also showed that TGF- β 1 was effective in inhibiting proliferation only when added to the culture early in G₁ within 2 h of stimulation by PDGF.

The possibility raised by these observations is that there are distinct effects of TGF- β 1 on VSMC proliferation in G₂ and on S phase at low and high free TGF- β 1 concentrations respectively, with both effects mediated by interaction of TGF- β 1 with the cells in G₀ and/or G₁. The mechanisms involved and the role of the various TGF- β receptors will require further examination.

Effect of TGF- β 1 on differentiation

TGF- β 1 has been shown to have diverse effects on differentiation as well as proliferation in many types of cells. Several groups, most notably the Campbells and colleagues [1,34,35,40] and Thyberg et al. [36], have proposed that de-differentiation is

essential for the proliferation of VSMCs. TGF- β 1 completely inhibited the de-differentiation of primary rat aortic VSMCs, marked by loss of SM-MHC which occurs as the cells enter the first cell cycle after plating. In contrast, TGF- β 1 had no effect on SM-MHC content in exponentially growing subcultured cells which contain very small amounts of the protein. As the duration of the G₂ phase of the cell cycle is extended to a similar extent in both primary and passaged VSMC cultures it is clear that the pathways which mediate proliferation and differentiation are regulated independently. Furthermore, all of the primary VSMCs treated with TGF- β 1 proliferated with an extended cell cycle time while maintaining a fully differentiated complement of the SM-MHC protein. It is concluded that, consistent with our previous suggestions ([38,50], D. J. Grainger, C. M. Witchell, C. M. Shanahan, J. C. Metcalfe and P. L. Weissberg, unpublished work) and that of Björkerud [29] for human smooth muscle cells, de-differentiation, as marked by the loss of smooth-muscle-specific proteins, is not obligatory for proliferation of the aortic VSMCs.

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