Tamoxifen decreases the rate of proliferation of rat vascular smooth-muscle cells in culture by inducing production of transforming growth factor β

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Tamoxifen selectively and reversibly decreased the rate of proliferation of adult rat aortic vascular smooth-muscle cells (VSMCs). Half-maximal inhibition of proliferation occurred at 2–5 μM tamoxifen for VSMCs and at >50 μM for adventitial fibroblasts. The cell cycle time for all the VSMCs in the population was increased from 35±2 h to 54±4 h in the presence of 33 μM tamoxifen. Tamoxifen did not affect the time of entry into DNA synthesis, but delayed arrival at mitosis by >24 h. It therefore extended the duration of the G_2-to-M phase of the cell cycle. However, the rate of proliferation of VSMCs was not decreased by tamoxifen (at concentrations up to 50 μM) in the presence of neutralizing antibody to transforming growth factor β (TGF-β). The level of mRNA for TGF-β1 in VSMCs was strongly induced by 10 μM tamoxifen, and TGF-β activity in conditioned medium from tamoxifen-treated cells was more than 50-fold higher than from control cells. Tamoxifen therefore extended the G_2-to-M phase of the cell cycle in VSMCs by increasing TGF-β activity in the culture.

INTRODUCTION

Abnormal proliferation of vascular smooth-muscle cells (VSMCs) is a major component of vascular disease, including atherosclerosis, vascular rejection and restenosis following angioplasty [1,2]. Agents which selectively inhibit VSMC proliferation might therefore be expected to prevent or inhibit the progress of these diseases.

The mechanisms which maintain VSMCs in a non-proliferative state in the media of the healthy vessel remain unclear. It has been proposed that heparin or heparan sulphate proteoglycan is necessary for maintaining the medial VSMCs in a quiescent state [3]. This action of heparin may involve the release of transforming growth factor β (TGF-β) activity from inactive complexes with α_2-macroglobulin or the extracellular matrix [4]. TGF-β is known both to decrease the rate of proliferation and to promote the differentiated phenotype [5–8] of VSMCs in culture. TGF-β activity may therefore play a significant role in maintaining the vessel wall in a non-proliferating state [9].

TGF-β is produced as a latent propeptide which does not bind to TGF-β receptors and has no biological activity described [10]. In order to be active, the propeptide must be cleaved, for example by the serine protease plasmin [11–13]. Lipoprotein (a), a serum lipoprotein complex whose serum levels have been correlated with atherosclerosis, myocardial infarction and stroke [14,15], inhibits activation of TGF-β in culture models by preventing plasmin activation [13,16]. These observations further suggest that decreased TGF-β activity *in vivo* may contribute to the onset of vascular disease [11,13,16]. We have therefore proposed that agents which elevate TGF-β may be beneficial in reducing inappropriate VSMC proliferation [9,16].

Several studies have shown that the anti-oestrogenic drug tamoxifen induces expression of TGF-β mRNA and protein in breast carcinoma cells in cell culture [17,18]. We have therefore examined the effects of tamoxifen on VSMCs in culture to determine whether it induces the production of TGF-β and extends the G_2-to-M phase of the VSMC cell cycle, consistent with previous studies using purified TGF-β1 [6,7].

MATERIALS AND METHODS

Cell culture, assay of DNA synthesis and cell counting

Rat VSMCs were cultured after enzymic dispersion of the aortic media from 12–17-week-old Wistar rats as described previously [19]. When the cells reached confluence (after about 6 days) the cells were released with trypsin/EDTA (Gibco) and diluted 1:2 in Dulbecco's modification of Eagle's medium (DMEM; ICN/Flow) supplemented with 100 units/ml penicillin and 10% foetal-calf serum (FCS). The cells were then re-plated on tissue-culture plastic (ICN/Flow) at approx. 1×10^4 cells/cm^2. The cells were subcultured repeatedly in this way at confluence (about every 4 days) and the cells were used between passages 6 and 12.

Rat adventitial fibroblasts were cultured as described previously [20]. Briefly, the aortae were treated with collagenase (3 mg/ml) for 30 min at 37 °C. The tunica adventitia was stripped away from the media. The adventitia was dispersed for 2 h in elastase (1 mg/ml) and collagenase (3 mg/ml) dissolved in medium M199 (ICN/Flow). The cells were then spun out (900 *g*, 3 min), resuspended in DMEM + 10% FCS and plated out at 8×10^4 cells/cm^2 on tissue culture plastic. When the cells reached confluence (after about 10 days) they were subcultured as described for VSMCs. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DNA synthesis was assayed by [^{3}H]thymidine incorporation as previously described [19]. VSMCs were subcultured, grown in DMEM + 10% FCS for 24 h, made quiescent in serum-free DMEM for 48 h and re-stimulated with 10% FCS at 0 h. [^{3}H]Thymidine (5 μCi/ml; Amersham International) was added 12 h after re-stimulation and the cells were harvested after 24 h. DNA synthesis by adventitial fibroblasts was determined similarly, except that the cells were made quiescent in serum-free DMEM for 24 h.

Abbreviations used: FCS, foetal-calf serum; DMEM, Dulbecco's modification of Eagle's medium; TGF-β, transforming growth factor type β; VSMC, vascular smooth-muscle cell.

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Cells were prepared for counting by haemocytometer from triplicate culture dishes as previously described [19]. 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 the cells could not migrate into or out of the area being counted during the experiment. Cells in each of four squares in two separate wells were counted at each time point. All cell-counting experiments were repeated on at least three separate cultures as indicated, and cell numbers are plotted as means ± S.E.M.

A stock solution of tamoxifen (5 mM) was made up in 10% (v/v) ethanol and diluted in DMEM + 10% FCS to give the final concentration required. Where data are presented as 'percentage of control', the effects of each tamoxifen concentration are compared with the effects observed in control wells containing the same final concentration of ethanol vehicle. Recombinant TGF-β1 (Amersham International) was dissolved in 25 mM Tris/HCl to give a 5 μg/ml stock solution and sterile-filtered through a Spinnex tube. Neutralizing antiserum to TGF-β (BDA19; R & D Systems) was reconstituted in sterile MilliQ water. At 10 μg/ml this antibody completely abolished the activity of 10 ng/ml recombinant TGF-β both on subcultured (passage 8) VSMCs, assayed as previously described [21], and on DNA synthesis of mink lung epithelial cells as described below.

Assays for TGF-β

The TGF-β activity present in medium conditioned on various cells was determined by DNA-synthesis assay on mink lung epithelial (MvLu; American Type Culture Collection, Rockville, MD, U.S.A.) cells, by a modification of the assay described previously [22]. MvLu cells were subcultured at 1:5 dilution in DMEM + 10% FCS. After 24 h the medium was replaced with the conditioned medium to be tested in the absence or presence of the neutralizing antiserum to TGF-β at 10 μg/ml. DNA synthesis during a 1 h pulse of [^{3}H]thymidine (5 μCi/ml) was determined 23 h after addition of test medium. TGF-β activity was calculated as the proportion of the inhibition of DNA synthesis which was reversed in the presence of neutralizing antibody, using a standard curve to convert the inhibition values into quantities of TGF-β. The TGF-β1 standards and conditioned media both contained 10% FCS in DMEM.

The total latent and active TGF-β present was determined by a sandwich e.l.i.s.a. Maxisorp 96-well e.l.i.s.a. plates (Gibco) were coated with neutralizing antiserum against TGF-β (BDA19) at 2 μg/cm^2 in PBS overnight at room temperature. The plates were washed between each step with Tris-buffered saline containing 0.1% Triton X-100. The plates were incubated with samples for 2 h, with a second antibody to TGF-β (BDA5; R & D Systems) at 0.1 μg/ml for 2 h, with anti-rabbit IgG peroxidase-conjugated antibody (Sigma) for 1 h, and with the chromogenic substrate *o*-phenylenediamine (Sigma), made up according to the manufacturer's instructions, for 15 min. Under the conditions used, BDA19 and BDA5 specifically bind both latent and active TGF-β. A_{492} values were converted into quantities of TGF-β protein by using a standard curve. Both conditioned media and standards were assayed in the presence of 10% FCS in DMEM. This assay was linear for TGF-β concentrations in the range 0.1–20 ng/ml in the presence of 10% FCS in DMEM.

RNA preparation and Northern-blot analysis

Total cytoplasmic RNA was isolated from cultured VSMCs as previously described [23]. Northern-blot analysis was performed by electrophoresis of total cytoplasmic RNA in 1.5% agarose gels in a buffer containing 2.2 M formaldehyde, 20 mM Mops, 1 mM EDTA, 5 mM sodium acetate and 0.5 μg/ml ethidium bromide. The integrity of the RNA was checked by observing the gel under u.v. illumination before transfer on to Hybond N (Amersham International) as specified by the manufacturer. Filters were hybridized as previously described [23], by using a ^{32}P-labelled mouse TGF-β1 probe (Dr. R. Akhurst, University of Glasgow) corresponding to amino acids 68–228 in the precursor region of the TGF-β1 polypeptide [24].

RESULTS AND DISCUSSION

We have previously shown that subcultured VSMCs from the aortae of adult rats proliferate with a cell cycle time of approx. 35 h in DMEM + 10% FCS [19,23]. Addition of tamoxifen decreased the rate of proliferation, with maximal inhibition at concentrations above 33 μM (Figure 1a); with 50 μM tamoxifen the increase in cell number 96 h after addition of serum was decreased by 66 ± 5.2% (n = 3). The slower rate of proliferation might be due to a complete block on 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 proportion of the cells passing through M phase and the time course of entry into cell division were determined.

Quiescent VSMCs were stimulated with DMEM + 10% FCS in the absence or presence of 33 μM tamoxifen, and the cell number was determined at 8 h intervals by time-lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the VSMCs had divided by 40 h, whereas there was no significant increase in cell number in the presence of tamoxifen (33 μM) until after 48 h. However, by 64 h more than 90% of the cells had divided in the presence of tamoxifen (Figure 1b). The time taken for 50% of the cells to divide after stimulation by serum was increased from 35 ± 3 h (n = 7) to 54 ± 2 h (n = 3) by 33 μM tamoxifen. Since tamoxifen does not decrease significantly the proportion of cells completing the cell cycle and dividing, the data imply that the inhibition was due to an increase in the cell cycle time of nearly all (> 90%) of the proliferating cells.

To determine whether tamoxifen increased the duration of the cell cycle of VSMCs by increasing the duration of the G_0-to-S phase, the effect of tamoxifen on entry into DNA synthesis was analysed. Tamoxifen at concentrations up to 50 μM did not affect significantly the time course or the proportion of cells entering DNA synthesis after serum stimulation of quiescent VSMCs (DNA synthesis between 12 h and 24 h after stimulation was measured by [^{3}H]thymidine incorporation: control 17614 ± 1714 c.p.m.; 10 μM tamoxifen 16898 ± 3417 c.p.m.; 50 μM tamoxifen 18002 ± 4167 c.p.m.). Since the duration of S phase is approx. 12 h [21], we conclude that tamoxifen has not significantly affected the time course of entry into DNA synthesis. These results imply that tamoxifen decreases the rate of proliferation of serum-stimulated VSMCs by increasing the time taken to traverse the G_2-to-M phase of the cell cycle.

These observations suggested that tamoxifen had similar effects to TGF-β on subcultured VSMCs in the presence of serum [5–8]. Since tamoxifen is known to induce TGF-β activity in cultures of breast carcinoma cell lines [17,18], we determined whether tamoxifen decreased the rate of proliferation of VSMCs by inducing TGF-β activity. When VSMCs were stimulated with 10% FCS in the presence of 50 μM tamoxifen and 10 μg/ml neutralizing antiserum against TGF-β, the cells proliferated at the same rate as control cells in the presence of ethanol vehicle alone (Figure 1c).

To confirm that the VSMCs produced TGF-β in response to tamoxifen, VSMCs were treated with tamoxifen for 96 h in the

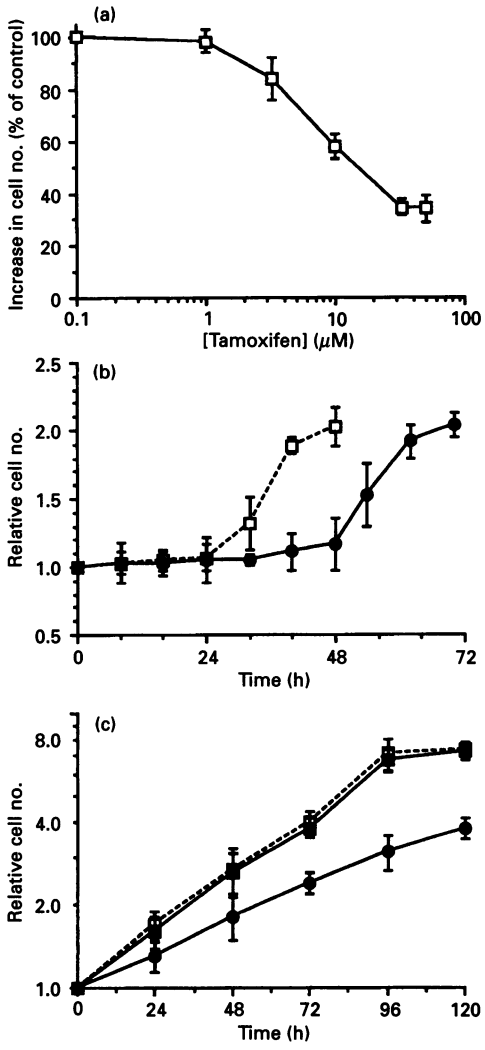

Figure 1 Effects of tamoxifen on proliferation of VSMCs

(**a**) Dose–response curve for the effects of tamoxifen on VSMC proliferation. Confluent cells (passages 7–9) were released with trypsin/EDTA, diluted 1:3 in DMEM + 10% FCS and grown for 24 h. They were made quiescent by incubation in serum-free DMEM for 48 h and re-stimulated with DMEM + 10% FCS at 0 h in the presence of various concentrations of tamoxifen or ethanol vehicle alone. Cell number was determined by counting released cells with a haemocytometer (see the Materials and methods section). Values are means of triplicate determinations from three separate experiments, expressed as percentage of control value; error bars are S.E.M. values. (**b**) Effect of tamoxifen on time course of cell division. Confluent cells (passages 6–10) were released with trypsin/EDTA, diluted 1:3 in DMEM + 10% FCS and grown for 24 h. The cells were made quiescent by incubation in serum-free DMEM for 48 h and re-stimulated with DMEM + 10% FCS at 0 h in the presence of 33 μM tamoxifen (●) or ethanol vehicle alone (□). Cell number was determined by time-lapse photomicroscopy of cells within deeply grooved grids (see the Materials and methods section). Values are means of counts in four separate fields in each of two wells from three separate culture experients; error bars are S.E.M. values. (**c**) Modulation of tamoxifen effects on proliferation by anti-TGF-β antibody. Confluent VSMCs (passages 9–11) were released with trypsin/EDTA, diluted 1:3 in DMEM + 10% FCS and grown for 24 h. At this time (termed 0 h) 50 μM tamoxifen (●), ethanol vehicle alone (□) or 50 μM tamoxifen and 10 μg/ml neutralizing antiserum to TGF-β (■) were added. Cell number was determined at various times by counting released cells with a haemocytometer (see the Materials and methods section). Values are means of triplicate determinations on three separate culture experiments; error bars are S.E.M. values.

presence of 10% FCS. The conditioned medium was then collected and TGF-β activity was determined by a modified mink lung epithelial (MvLu) cell assay (see the Materials and methods section). Tamoxifen increased the TGF-β activity in the medium by >50-fold (Table 1). Addition of tamoxifen (50 μM) in fresh DMEM + 10% FCS to the MvLu cells had no effect on DNA synthesis, demonstrating that tamoxifen did not induce production of active TGF-β by the MvLu cells.

Table 1 Effect of tamoxifen on production of TGF-β by VSMCs and activation of the propeptide

Adult rat aortic VSMCs (passage 8–11) were subcultured into DMEM + 10% FCS, made quiescent in serum-free DMEM for 48 h, and then re-stimulated in DMEM + 10% FCS (control) or DMEM + 10% FCS supplemented with tamoxifen or ethanol vehicle as indicated. After 96 h the conditioned medium was collected and TGF-β activity was determined by a modified mink lung epithelial cell assay (see the Materials and methods section). Total latent TGF-β plus active TGF-β present in the conditioned media was determined by sandwich e.l.i.s.a. (see the Materials and methods section). Values are means ± S.E.M. ($n = 3$) of triplicate determinations on conditioned media from three separate experiments. As a control, DMEM + 10% FCS was treated with 33 μM tamoxifen at 37 °C for 96 h in the absence of VSMCs.

Treatment of VSMCs	TGF-β (ng/ml)	
	Active	Latent plus active
DMEM + 10% FCS	< 0.1	2.1 ± 1.8
Ethanol vehicle	< 0.1	4.1 ± 2.8
10 μM tamoxifen	4.3 ± 0.7	12.7 ± 2.5
33 μM tamoxifen	5.6 ± 1.1	16.2 ± 2.4
DMEM + 10% FCS plus 33 μM tamoxifen in the absence of cells	< 0.1	1.3 ± 0.7

TGF-β is produced as a latent propeptide which can be activated outside the cell by proteases such as plasmin [11–13,16,25,26]. To determine whether tamoxifen increased TGF-β activity by promoting the activation of latent TGF-β or by stimulating the production of the latent propeptide which was subsequently activated, the total latent plus active TGF-β present in the conditioned medium was determined by a sandwich e.l.i.s.a. (see the Materials and methods section). After 96 h in the presence of tamoxifen (50 μM), the total TGF-β protein present was increased by approx. 4-fold (Table 1). Furthermore, the proportion of the TGF-β present which was active was increased from <5% in the medium conditioned on VSMCs in the presence of ethanol vehicle alone to approx. 35% in the medium conditioned on cells treated with 50 μM tamoxifen for 96 h. These results suggest that tamoxifen increases the TGF-β activity in cultures of rat VSMCs by stimulating the production of latent TGF-β and increasing the proportion of the total TGF-β which has been activated.

We have previously shown that heparin increases the TGF-β activity in medium conditioned on VSMCs [21]. The mechanism involves release of TGF-β from inactive complexes present in serum [4,21,27], since pretreatment of serum with heparin immobilized on agarose beads is as effective as direct addition of free heparin to the cells. To determine whether tamoxifen acts to release TGF-β from sequestered complexes in serum which are not immunoreactive in our e.l.i.s.a. assay, we treated 10% FCS in DMEM with 50 μM tamoxifen for 96 h at 37 °C in the absence of cells. Medium treated in this way contained similar levels of TGF-β protein and activity to untreated medium (Table 1). We conclude that tamoxifen, unlike heparin, does not act by releasing TGF-β from inactive complexes present in serum.

We also analysed the content of TGF-β1 mRNA by Northern analysis after addition of tamoxifen. Subcultured rat VSMCs (passage 6 in exponential growth) in the absence (Figure 2; lane C) or presence of ethanol vehicle alone (Figure 2; lane V)

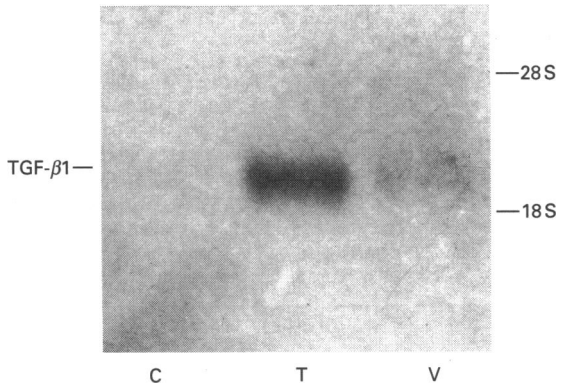

Figure 2 Effect of tamoxifen on TGF-β mRNA content of VSMCs

Confluent VSMCs (passage 5) were released with trypsin/EDTA, diluted 1:2 in DMEM + 10% FCS and grown for 48 h. The cells were then treated with 10 μM tamoxifen or ethanol vehicle alone. Cells were harvested with trypsin/EDTA 24 h later, and RNA was prepared from the pelleted cells (see the Materials and methods section). Northern-blot analysis was performed by using a probe against mouse TGF-β1, against 10 μg of total cytoplasmic RNA per lane. Lanes: C, control cells in the absence of tamoxifen and ethanol vehicle; T, cells in the presence of tamoxifen (10 μM) in ethanol vehicle; V, cells in the absence of tamoxifen and in the presence of ethanol vehicle alone. Positions of 18S and 28S rRNAs are indicated.

contain very little mRNA for TGF-β1. However, by 24 h after addition of tamoxifen (10 μM), TGF-β1 mRNA was strongly induced (Figure 2; lane T).

Although TGF-β decreases the rate of proliferation of VSMCs [5–8], it does not affect the rate of proliferation of fibroblasts [5]. Tamoxifen may therefore be expected to decrease the rate of proliferation of VSMCs selectively compared with adventitial fibroblasts. Consistent with this prediction, tamoxifen at concentrations up to 50 μM did not decrease the rate of proliferation of subcultured adventitial fibroblasts, assayed by cell counting. Tamoxifen is therefore a selective inhibitor of VSMC proliferation, with an ED_{50} at least 10-fold lower for VSMCs than for adventitial fibroblasts.

Tamoxifen has been widely used as a drug for treating breast-cancer patients. Although the mechanism is thought to involve anti-oestrogen effects, recent studies have suggested that tamoxifen stimulated TGF-β production independent of effects on the oestrogen receptor [18]. It remains a possibility that the up-regulation of TGF-β activity is at least partially responsible for the beneficial effects of tamoxifen on the prognosis of breast-cancer sufferers. Although tamoxifen and TGF-β increase the duration of the cell cycle in VSMCs stimulated by FCS, several studies have shown that TGF-β inhibits DNA synthesis in VSMCs stimulated by epidermal growth factor or platelet-derived growth factor in the absence of serum [5,28]. Nevertheless, any protective effect of elevated TGF-β levels during inappropriate proliferation of VSMCs remains controversial (reviewed in [9]). It should be possible to investigate the role of TGF-β in these cardiovascular disease processes in various animal models, such as the balloon injury of the rat carotid artery [29], using tamoxifen as a tool to elevate TGF-β activity. If these studies show a beneficial effect, then tamoxifen could be used relatively quickly to elevate TGF-β in patients undergoing therapeutic angioplasty, since it is known to have few adverse side-effects.

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