Transforming Growth Factor-β–induced Growth Inhibition and Cellular Hypertrophy in Cultured Vascular Smooth Muscle Cells

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Abstract. We have explored the hypothesis that hypertrophy of vascular smooth muscle cells may be regulated, in part, by growth inhibitory factors that alter the pattern of the growth response to serum mitogens by characterizing the effects of the potent growth inhibitor, transforming growth factor-β (TGF-β), on both hyperplastic and hypertrophic growth of cultured rat aortic smooth muscle cells. TGF-β inhibited serum-induced proliferation of rat aortic smooth muscle cells (ED_{50} = 2 pM); this is consistent with previously reported observations in bovine aortic smooth muscle cells (Assoian et al. 1982. *J. Biol. Chem.* 258:7155–7160). Growth inhibition was due in part to a greater than twofold increase in the cell cycle transit time in cells that continued to proliferate in the presence of TGF-β. TGF-β concurrently induced cellular hypertrophy as assessed by flow cytometric analysis of cellular protein content (47% increase) and forward angle light scatter (32–50% increase), an index of cell size. In addition to being time and concentration dependent, this hypertrophy was reversible. Simultaneous flow cytometric evaluation of forward angle light scatter and cellular DNA content demonstrated that TGF-β–induced hypertrophy was not dependent on withdrawal of cells from the cell cycle nor was it dependent on growth arrest of cells at a particular point in the cell cycle in that both cycling cells in the G_2 phase of the cell cycle and those in G_1 were hypertrophied with respect to the corresponding cells in vehicle-treated controls. Chronic treatment with TGF-β (100 pM, 9 d) was associated with accumulation of cells in the G_2 phase of the cell cycle in the virtual absence of cells in S phase, whereas subsequent removal of TGF-β from these cultures was associated with the appearance of a significant fraction of cycling cells with >4c DNA content, consistent with development of tetraploidy. Results of these studies support a role for TGF-β in the control of smooth muscle cell growth and suggest that at least one mechanism whereby hypertrophy and hyperploidy may occur in this, as well as other cell types, is by alterations in the response to serum mitogens by potent growth inhibitors such as TGF-β.

CELLULAR enlargement or hypertrophy plays a prominent role in the postnatal growth of many tissues, as well as in physiological and pathological hypertrophy of a variety of tissues (4). However, relatively little is known regarding the mechanisms that control cell size. Even less is known concerning the control mechanisms for the DNA endoreduplication and polyploidy that often accompanies cellular hypertrophy, although its occurrence is widespread in eukaryotic cells in vivo, occurring in terminally differentiated cardiac myocytes (14, 15, 29), and neurons (22), as well as in nonterminally differentiated hepatocytes (8), smooth muscle cells (10, 23, 30), and other cell types (see review by Brodsky and Uryvaeva [8]).

Our laboratory has been particularly interested in the medial hypertrophy of smooth muscle that develops in large arteries of hypertensive animals and humans (17, 23, 30) and has been implicated in the pathogenesis of this disease. Aortic hypertrophy is characterized by enlargement of existing smooth muscle cells with little or no change in cell number and is accompanied by development of polyploidy in a large fraction of smooth muscle cells (10, 23, 24, 30). Development of polyploidy in these cells does not appear to be due to an inherent loss in the capacity of cells to divide since tetraploid cells can be induced to proliferate in vitro in serum containing medium after isolation from intact vessels and separation on the basis of DNA content using a fluorescence activated cell sorter (19) or in vivo by subjecting vessels that contain polyploid cells to balloon embolectomy-induced injury (Owens, G. K., manuscript in preparation). This suggests that the growth response of smooth muscle cells, i.e., hypertrophy vs. hyperplasia, is a function of the nature of the growth stimulus. However, the precise factors that control hypertrophic vs. hyperplastic growth of smooth muscle cells

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are not known and virtually nothing is known regarding the mechanisms responsible for development of polyploidy in this cell.

There are several possible mechanisms to explain development of cellular hypertrophy. One hypothesis is that hypertrophy results from incomplete growth stimulation whereby the cell receives signals for the increased cell mass and DNA replication associated with cell cycle progression but not for cell division (5, 18). An alternative hypothesis, and the principal focus of the present study, is that cellular hypertrophy may be regulated, in part, by growth inhibitory factors that alter the pattern of the growth response to mitogens resulting in incomplete growth stimulation. An attractive candidate for a substance that might elicit the latter response in smooth muscle cells is transforming growth factor-β (TGF-β).[1] This 25-kD polypeptide homodimer (2) has been shown to be a potent growth inhibitor for vascular smooth muscle cells (3) as well as a variety of other anchorage-dependent cells (32), and it has been suggested that it may be a mediator of smooth muscle cell growth in vivo during wound repair and atherogenesis (3, 12). Of particular interest, treatment of primary cultures of renal proximal tubule cells with BSC-1 growth inhibitor, which has similar biological properties as TGF-β, is associated with development of cellular hypertrophy (16).

The principal aim of the present study was to explore the possible mechanisms for development of hypertrophy and hyperploidy in vascular smooth muscle cells by characterizing the role of TGF-β in control of hyperplastic growth of cultured rat aortic smooth muscle cells, and determining whether TGF-β–induced growth inhibition was associated with development of cellular hypertrophy and/or accumulation of cells with tetraploid DNA content.

Materials and Methods

Cell Culture

Rat thoracic aortic smooth muscle cells were isolated and cultured as previously described (25) with the following modifications: (*a*) 150–175-g rats were used instead of 200–225-g rats; and (*b*) the following enzymes in Hanks' balanced salt solution were used for cell isolations: 1 mg/ml collagenase (type II, 158 U/mg), 0.25 mg/ml elastase (type I, 3 U/mg), 1 mg/ml soybean trypsin inhibitor (all from Cooper Biomedical Inc., Malvern, PA).

Cells were harvested for passaging at confluency (~5-d intervals) with a trypsin-EDTA (0.05% trypsin, 0.02% EDTA, Gibco Laboratories, Grand Island, NY) solution and plated at 2–5 × 10^3 cells/cm^2. Cells were grown in either: (*a*) a 1:1 mixture of Dulbecco's modified Eagle's medium (DME, Gibco Laboratories) and Ham's F12 medium (Gibco Laboratories), containing 10% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT) (designated DF10); or (*b*) Medium 199 plus either 5% or 10% FCS; or (*c*) DME and Ham's F-12 (1:1) containing insulin (5 × 10^{-7} M), transferrin (5 μg/ml), and ascorbate (0.2 mM) all from Sigma Chemical Co. (St. Louis, MO), designated serum-free medium (SFM). All media were supplemented with L-glutamine (0.68 mM, Sigma Chemical Co.), penicillin (100 U/ml), and streptomycin (100 μg/ml). The SFM has been shown to maintain smooth muscle cells in a quiescent, noncatabolic state for extended periods of time and to promote expression of smooth muscle cell-specific contractile proteins (25). Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air with media changes every 2–3 d.

1. *Abbreviations used in this paper*: FALS, forward angle light scatter; SFM, serum-free medium; T_c, cell cycle transit time; TGF-β, transforming growth factor-β.

Growth Curves

Cultures were washed with a calcium- and magnesium-free phosphate-buffered saline (PBS: NaCl 137 mM, Na_2HPO_4 8.1 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, pH 7.4) harvested with trypsin-EDTA, diluted with 0.85% NaCl (Columbia Diagnostics Inc., Camden, SC), and counted on an Electrozone Celloscope (Particle Data Inc., Elmhurst, IL) with orifice size of 95 μm and sample volume of 500 μl. Triplicate wells were counted for each group at every time point.

[^{3}H]Thymidine Autoradiography

For determination of the fraction of cells in the S phase of the cell cycle, cultures were pulse labeled with [^{3}H]thymidine (1 μCi/ml, 6.7 Ci/mmol, New England Nuclear, Boston, MA) for 1 h in regular culture medium. Cultures were then washed twice with PBS, fixed in 2% glutaraldehyde for 5 min, dehydrated, and coated with Kodak NTB2 emulsion (diluted 1:1 with distilled water). Dishes were exposed for 5–7 d at 4°C and then developed in D-19 (Eastman Kodak Co., Rochester, NY), fixed with Rapid-Fix (Eastman Kodak Co.) and stained with hematoxylin. The percentage of cells synthesizing DNA was determined by counting the number of labeled cells (nuclei) in a random sample of at least 1,000 cells from each dish. Triplicate dishes were analyzed for each group.

Determination of labeling indices over a 72-h labeling period (e.g., see Table I) was done as above with the exception that cells were labeled continuously with 0.01 μCi/ml of [^{3}H]thymidine (renewed daily).

[^{3}H]Thymidine Incorporation

Relative rates of DNA synthesis were assessed by determination of [^{3}H]thymidine incorporation into trichloroacetic acid (TCA)-precipitable material. Cells were pulsed for 2 h with [^{3}H]thymidine (2 μCi/ml), washed with PBS followed by one wash with 10% (wt/vol) cold TCA at 4°C (10 min), and one wash with 10% TCA at 22°C. Cells were then dissolved in 0.5 N NaOH, placed in RediSolve EP (Beckman Instruments, Inc., Palo Alto, CA), and counted. Quadruplicate dishes were analyzed per sample point.

Flow Microfluorimetric Analysis of Cellular DNA, Forward Angle Light Scatter (FALS), and Protein Content

Cultures were washed with PBS and harvested using the trypsin-EDTA solution described above. Samples were divided for protein and DNA analysis and then centrifuged (113 *g*, 6 min). For the protein staining, cells were fixed in 75% (vol/vol) methanol in PBS on ice for 30 min, centrifuged (113 *g*, 6 min), stained with fluorescein isothiocyanate (FITC, 75 ng/ml, Sigma Chemical Co.; 0.5 M $NaHCO_3$, pH 8) on ice for 30 min, then centrifuged (113 *g*, 6 min) and resuspended in 0.85% (wt/vol) NaCl (11). For the measurement of cellular DNA and FALS, cells were fixed in methanol as above, centrifuged (113 *g*, 6 min) and stained with Hoechst 33258 (Sigma Chemical Co.) (0.58 μg/ml; NaCl 145 mM; Tris 100 mM, pH 7.4). All samples were kept on ice until analyzed on a Coulter Epics V fluorescence-activated cell sorter (Coulter Electronics Inc., Hialeah, FL). The Epics V was calibrated in terms of fluorescent signals and FALS using fluorescent polystyrene beads (9.7 μm diam, Coulter Electronics Inc.) in order to normalize machine settings between different sample runs. Cell clumping was <1% based on analysis of peak versus integrated fluorescence. Statistical evaluation of FALS and protein data was done using the standard statistical software package provided by Coulter Electronics Inc. This involved selecting minimal and maximal values that encompassed the entire cell population and calculation of the mean and standard deviation for each individual sample. Cell cycle fitting of DNA histograms was done by modification of the method of Dean and Jett (13) as previously described (23).

Preparation of Transforming Growth Factor-β

TGF-β was prepared from outdated human platelets as previously described (2). Fractions containing TGF-β from Bio-Gel P-60 chromatography with 1 M acetic acid containing 6 M urea as an eluent were pooled and TGF-β was further purified by high-performance liquid chromatography (HPLC) using a reverse-phase C18 column (SynChropak RP-P, 4.6 × 250 mm, SynChrom, Inc., Linden, IN) and water/acetonitrile solvent system containing 0.1% (vol/vol) TCA. Rechromatography of the purified TGF-β on HPLC indicated the protein to be homogeneous and the protein concentration was

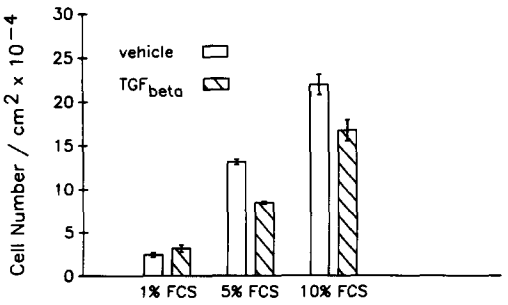

Figure 1. Serum dependence of TGF-β–induced growth inhibition. Cells were plated at 3 × 10^3/cm² in Medium 199 containing 10% FCS for 24 h to facilitate cell attachment and growth, and then switched to Medium 199 plus the FCS concentrations shown for 3 d before addition of TGF-β (100 pM) or vehicle (1 mg/ml BSA). Cell counts were determined in triplicate wells of each group after growth in each of the respective media for 6 d. Media were changed at 2-d intervals and fresh TGF-β or vehicle added. Values shown are mean ± SD.

determined by amino acid analysis using the Pico-Tag method (Waters Associates, Milford, MA) (7). The biological activity of purified TGF-β was assessed using a soft agar assay with normal rat kidney target cells (2). The ED_{50} was 10 pM.

Statistics

All results were analyzed by either Student's *t* test or an analysis of variance combined with Newman-Keuls multiple range test for intergroup comparisons. Probabilities of 0.05 or less were considered statistically significant. All experiments were repeated at least in duplicate. Values reported in the text are means plus or minus standard deviation (SD).

Results

TGF-β–Induced Inhibition of Cell Growth

Initial studies demonstrated that the effects of TGF-β on proliferative growth of rat aortic smooth muscle cells were dependent on serum concentration. After growth for 6 d, cell number was reduced by 36% in TGF-β–treated cultures vs. controls in cells grown in 5% FCS, whereas only a 20% reduction was observed in cells grown in 10% FCS (Fig. 1). TGF-β had no significant effect on cell number in quiescent cultures maintained in 1% FCS. Since the percent reduction in cell number was greater in cells grown in 5% FCS than in 10% FCS, all subsequent experiments, unless otherwise noted, were carried out using 5% FCS.

TGF-β was extremely potent in inhibiting cell growth showing maximal inhibition at a concentration of 20 pM, and an ED_{50} of 2 pM (Fig. 2). Mean cell-doubling times for TGF-β (20 pM) and vehicle-treated control cultures were 33 and 22 h, respectively, over the 3-d treatment period. To ensure that TGF-β did not reduce cell number simply by inducing increased cell death or detachment, growth inhibition was also assessed by determination of [^{3}H]thymidine incorporation into TCA-precipitable material at 22–24 (1 d), 46–48 (2 d), and 70–72 (3 d) h after addition of TGF-β or vehicle, and by determination of the fraction of cells in the S phase of the cell cycle by flow cytometric analysis of cellular DNA content. Consistent with cell number data, the rate of cell proliferation as judged by [^{3}H]thymidine incorporation was decreased by 40% after 1 d of TGF-β treatment, and by 70–75% after 2 and 3 d of treatment. Maximal inhibition of [^{3}H]thymidine incorporation was seen at TGF-β concentrations of 20 pM or greater. Although significant inhibition was observed with as short as 24 h of TGF-β treatment, maximal inhibition required 2 d or more of TGF-β treatment. Results of flow cytometric assays (not shown) showed a reduction in the fraction of cells in S phase in TGF-β–treated cultures as compared to controls, which was evident as early as 1 d after initiation of treatment.

Effects of TGF-β on Cell Cycle Transit Time

TGF-β–induced growth inhibition was further characterized by addressing the following two questions: (*a*) is there a subfraction of cells that either fail to replicate their DNA or replicate it at extremely slow rates in the presence of TGF-β? and (*b*) is the cell cycle transit time (T_c) altered in those cells that continue to replicate in the presence of TGF-β? In this experiment cells were plated in Medium 199 containing 10% FCS for 24 h and then switched to Medium 199 plus 5% FCS with or without 100 pM TGF-β for a pretreatment period of either 1 or 2 d. Cells were then grown in the continuous presence of 0.01 μCi/ml [^{3}H]thymidine (renewed daily) for a period of 72 h (this exceeds the doubling times of both control and TGF-β–treated cultures), either between 1 and 4 d or 2 and 5 d after initiation of TGF-β treatment. Culture media were changed and fresh TGF-β added at 2-d intervals. Cell counts were determined in replicate dishes at the beginning and end of the labeling period and autoradiography was performed at the end of the experiment to determine the fraction of [^{3}H]thymidine-labeled and unlabeled cells. The fraction of cells present at the initial time point (i.e., the time of addition of [^{3}H]thymidine) that replicated their DNA over the 72-h labeling period was calculated as: one minus the ratio of unlabeled final cell number/initial cell number. Results (Table I) demonstrated that a significantly smaller fraction of TGF-β–treated cells as compared to vehicle-treated cells replicated their DNA over the 72-h

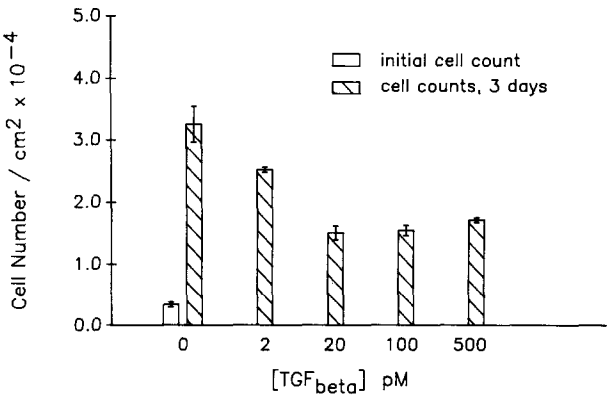

Figure 2. Concentration dependence of TGF-β–induced growth inhibition as assessed by analysis of cell number. Cells were plated at 3 × 10^3/cm² in Medium 199 containing 10% FCS for 24 h to facilitate cell attachment and growth, and then switched to Medium 199 containing 5% FCS with or without TGF-β at the concentrations indicated for 3 d. Control cultures were treated with TGF-β vehicle. Cell number was assessed at the time of addition of TGF-β or vehicle and at 3 d. Values shown are mean ± SD, n = 4.

Table I. Growth Fraction and Cell Cycle Time of TGF-β-(100 pM) and Vehicle-treated Smooth Muscle Cell Cultures

Group	* Duration of pretreatment	‡ Cell number		Percent Labeled cells (final)	Percent cycling cells (initial)§	Percent increase in cycling cells (1→2 d)‖	Doubling time of cycling cells (i.e., T_c)
		Initial	Final				
	d	*per cm²*					*h*
TGF-β	1	5,384 ± 200	8,024 ± 50	80.3 ± 1.9	71	45	45
	2	7,024 ± 284	8,896 ± 580	71.3 ± 0.6	64		
Vehicle	1	5,936 ± 364	12,840 ± 206	93.4 ± 0.3	86	126	20
	2	12,536 ± 328	19,136 ± 364	94.2 ± 1.0	91		

Values shown are means ± SE.
* Duration of treatment with TGF-β or vehicle before initiation of [³H]thymidine labeling. [³H]thymidine labeling was then done for 72 h with continuous treatment with TGF-β or vehicle.
‡ Cell number was determined at the time of initiation of [³H]thymidine labeling and 72 h thereafter. Thus, for the 1-d pretreatment groups the initial and final cell counts were done on days 1 and 4, respectively, whereas for the 2-d pretreatment groups initial and final cell counts were done on days 2 and 5, respectively. Cell count samples were treated identically as samples used for autoradiography including addition of [³H]thymidine.
§ Percentage of cells present at time 0 (i.e., initial time) which are labeled after growth in the continuous presence of [³H]thymidine (0.01 μCi/ml) for 72 h.
‖ This was calculated as the change in cell number between days 1 and 2 divided by the number of cycling cells present (i.e., mean cycling fraction at 1-2 d × initial cell count). This calculation assumes that all new cells must be derived from the cycling fraction and that cell death was negligible.

labeling period, indicating that there is a substantial fraction of cells that either do not cycle or cycle at extremely slow rates in the presence of TGF-β. Note that we cannot definitively conclude that these cells have actually withdrawn from the cell cycle since we have not demonstrated that these cells might not eventually replicate their DNA with further extension of the labeling period.

To address whether TGF-β also influenced cell cycle transit times in cells that were observed to proliferate during TGF-β treatment, data were further analyzed by calculating both the fractional increase and doubling time of the cycling fraction of cells between 1 and 2 d of treatment with either vehicle or TGF-β. Note that this estimate of doubling time for the cycling fraction of cells provides an estimate of the mean interdivision time of cells (i.e., T_c) for each of the growth conditions. Results demonstrated (Table I) that the mean interdivision time was more than doubled in cultures treated with TGF-β (T_c = 44.7 h) as compared to control (T_c = 20.3 h).

TGF-β-induced Cellular Hypertrophy

Marked changes in cellular morphology were evident in TGF-β-treated cultures in the growth inhibition studies described above (Fig. 3). The most notable change was an apparent increase in cell size. To assess this on a more quantitative basis, and to ensure that it did not simply represent cell spreading, cell size was assessed by flow cytometric analysis of both FALS, a measure of relative cell size, and cellular protein content. In these experiments, smooth muscle cells were plated in 10% FCS for 24 h and then switched to 5% FCS with or without 100 pM TGF-β for 3 d. Results (Fig. 4) showed a significant rightward shift in both FALS, as well as protein content histograms in TGF-β-treated cultures as compared to vehicle-treated controls. TGF-β was extremely

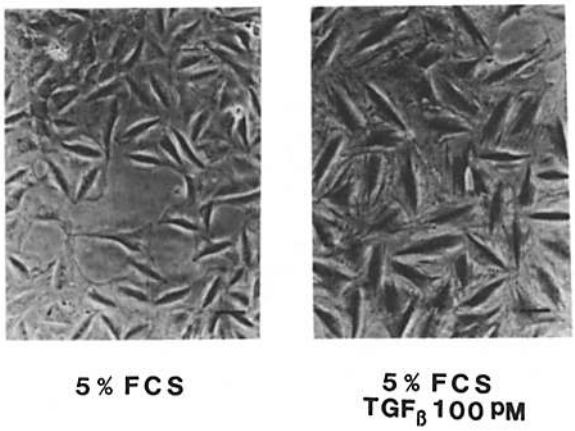

Figure 3. Phase micrograph of rat aortic smooth muscle cells after treatment with TGF-β (*right*) or vehicle (*left*) for 3 d in the presence of 5% FCS. Initial experimental conditions were as described in Fig. 2.

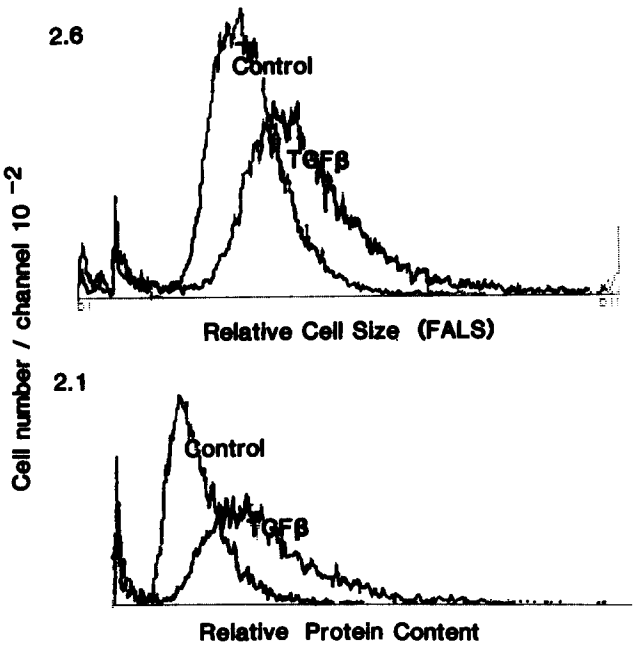

Figure 4. TGF-β-induced increases in relative cell size (FALS) and relative protein content as determined by flow microfluorimetry. Cells were plated in Medium 199 containing 10% FCS for 24 h and then switched to Medium 199 containing 5% FCS and either 100 pM TGF-β or vehicle (1 mg/ml BSA). Cells were treated for 3 d and then harvested for flow cytometric analysis of FALS and relative protein content as described in Materials and Methods. Between 5,000 and 10,000 cells were analyzed per sample.

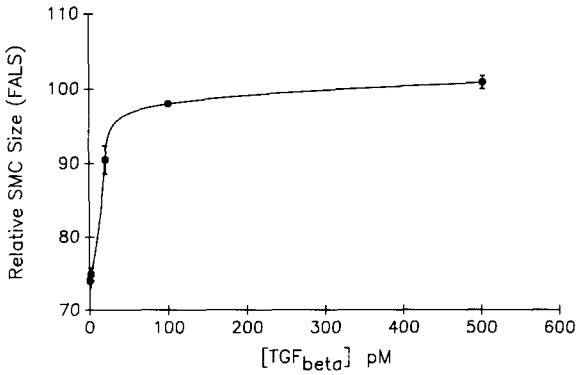

Figure 5. Concentration dependence of TGF-β-induced smooth muscle cell hypertrophy. Cells were plated at 3 × 10^3/cm² in Medium 199 containing 10% FCS for 24 h and then switched to Medium 199 containing 5% FCS plus either vehicle or TGF-β at the concentrations indicated. Cells were treated for 3 d and cellular hypertrophy assessed by flow cytometric analysis of FALS. Mean FALS values were determined for each individual sample from histograms such as those displayed in Fig. 5, and these values used for calculation of the group means (±SD, n = 3) shown.

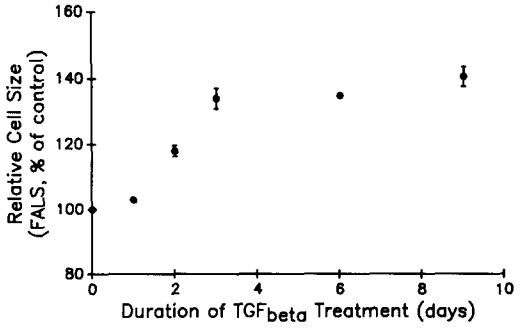

Figure 6. Time course of TGF-β-induced cellular hypertrophy. Cells were plated as described in Fig. 5 and then treated with 100 pM TGF-β or vehicle for the times indicated. Culture media, as well as TGF-β and vehicle, was replaced at 2-d intervals. Although flow cytometer settings (i.e., laser power, gain settings, etc.) were normalized as much as possible between runs on separate days, FALS data are expressed as a percentage of the corresponding vehicle-treated control to normalize for day-to-day fluctuations in machine performance. Triplicate samples were analyzed per time point per group. In some cases, the SD bars are less than the symbol size.

potent in inducing cellular hypertrophy causing a 47% increase mean cellular protein content and a 32–50% increase (range of means from four independent experiments) in FALS compared to controls after treatment with 100 pM TGF-β for 3 d. This cellular hypertrophy showed a steep concentration dependence with near-maximal effects after 3 d of treatment at a concentration of 100 pM and an ED_{50} = 20 pM (Figs. 5 and 6). Small but significant increases in relative cell size occurred after 1 d of TGF-β treatment (Fig. 6), although the most rapid increase in relative cell size occurred between 1 and 3 d of treatment. Hypertrophy was nearly maximal at 3 d, but there was an additional small increase in cell size between 3 and 9 d.

Reversal of TGF-β-induced Smooth Muscle Hypertrophy

To assess the reversibility of TGF-β-induced hypertrophy cells were first treated for 3 d with 100 pM TGF-β to induce near maximal hypertrophy, and then washed and switched to Medium 199 + 5 %FCS alone for 6 d. Relative cell size was then measured by flow cytometric evaluation of FALS. Results demonstrated that TGF-β-induced increases in FALS were reduced by >90% following removal of TGF-β and growth in 5% FCS alone for 6 d.

Cell Cycle Dependence of TGF-β-induced Smooth Muscle Cell Hypertrophy

Since TGF-β-induced growth inhibition was only partial, it was important to determine whether TGF-β-induced cellular hypertrophy was restricted to cells which were blocked at a particular point in the cell cycle. This was assessed by simultaneous analysis of cellular DNA content and FALS. A summary of comparisons of mean FALS of control and TGF-β-treated cells in either the G_0–G_1 or G_2 phase of the cell cycle is presented in Fig. 7. Results demonstrated that for each of the concentrations examined, the increase in cell size induced by TGF-β was the same for cycling cells in G_2 as for cells in G_0–G_1. Note that, although some of the cells in G_2 may not be cycling, a considerable fraction must be cycling, since a substantial fraction of cells continue to proliferate at this time in the presence of TGF-β (see Fig. 2). Thus, TGF-β-induced cellular hypertrophy was not limited to cells that were arrested at a particular point in the cell cycle nor to cells that have withdrawn from the cell cycle. These data indicate that if TGF-β-induced hypertrophy was

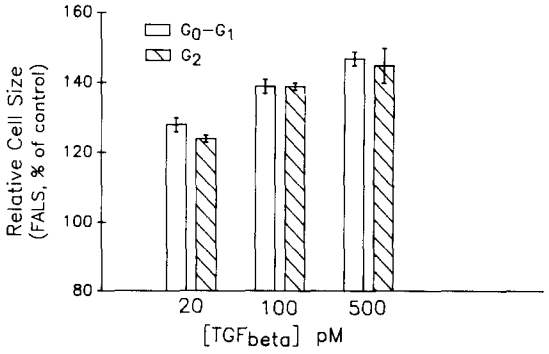

Figure 7. The cell cycle dependence of TGF-β-induced cellular hypertrophy was assessed by simultaneous analysis of FALS and cellular DNA content by flow microfluorimetry. Data analyses involved standard curve fitting to identify G_1 and G_2 peaks using software provided on the Epics V, calculation of peak means (±SD), setting of gating windows on DNA histograms based on these calculations (peak means ± 2 SD were used), followed by calculation of the mean (±SE) FALS values for that population. Cells were treated with either vehicle or TGF-β at the concentrations indicated for 3 d. Additional experimental details were identical to those described for Fig. 5. Comparisons are between control and TGF-β-treated cells identified as being either in the G_0–G_1 phase or the G_2 phase of the cell cycle based on examination of DNA histograms. Relative cell size of TGF-β is expressed as a percentage of the corresponding cells (in the same phase of the cell cycle) from vehicle-treated controls. Means ± SD (n = 3) are shown.

secondary to growth inhibition, it must occur by some mechanism that does not involve growth arrest and subsequent hypertrophy of cells at a particular point in the cell cycle. However, an alternative possibility that must be considered is that TGF-β-induced hypertrophy may not be a direct consequence of growth inhibition but simply occurs concurrently due to some additional effect of TGF-β distinct from growth inhibition.

TGF-β-induced Hypertrophy of Quiescent Smooth Muscle Cells in a Defined SFM

To determine whether TGF-β could induce hypertrophy in the absence of significant growth inhibition, cells were plated in medium containing 10% FCS for 24 h, switched to SFM for 5 d to induce quiescence, and treated with either 100 pM TGF-β or vehicle control for 3 d, and relative cell size was assessed by flow cytometric determination of FALS. Results showed a significant increase in FALS in TGF-β-treated cultures as compared to vehicle-treated controls (Fig. 8), whereas no detectable change in the fraction of cells in the S phase of the cell cycle was apparent by either flow microfluorimetric analysis or by [^{3}H]thymidine autoradiography. [^{3}H]thymidine-labeling indices (1-h pulse) from a representative experiment, were 0.9 ± 0.3% at the time of initiation of treatment and 0.6 ± 0.4% and 1.0 ± 0.5% ($P > 0.05$) in TGF-β and vehicle-treated control groups, respectively, at 48 h after initiation of treatment, and were typically <2% for all groups in replicate experiments. Consistent with these data, no significant changes in cell number were observed over the 3-d duration of these experiments. Significant increases in FALS were observed in TGF-β-treated cells in each of four independent experiments. However, for reasons that are not clear, the percent increase in mean FALS was quite variable between experiments ranging from 8 to 23% ($P < 0.01$ in all cases, paired t test). Results demonstrate that TGF-β can induce hypertrophy of quiescent smooth muscle cells in a defined SFM, although the magnitude of the cellular hypertrophy is considerably less than that observed in the presence of serum. However, TGF-β was extremely potent in inducing hypertrophy under these conditions showing maximal effects with concentrations as low as 2 pM (data not shown).

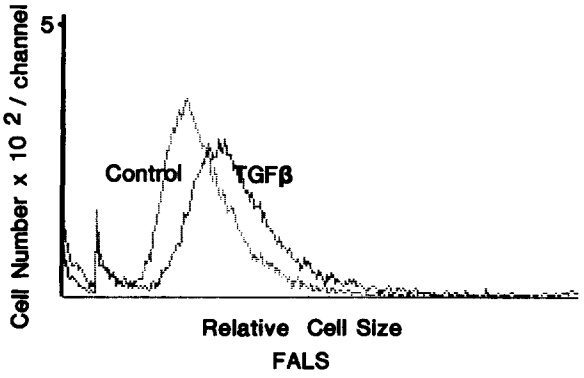

Figure 8. TGF-β-induced hypertrophy of smooth muscle cells in a defined SFM. Cells were plated at 3 × 10^3 cells/cm^2 in SFM (see Materials and Methods) containing 10% FCS for 24 h to promote cell attachment and spreading and then switched to SFM alone for 5 d (with a media change at 2.5 d) to induce quiescence. Under these conditions, [^{3}H]thymidine labeling indices (1-h pulse) were typically <2%. Cells were then treated with 100 pM TGF-β or vehicle for 3 d and relative cell size (i.e., FALS) and cellular DNA content (not shown) determined by flow microfluorimetry.

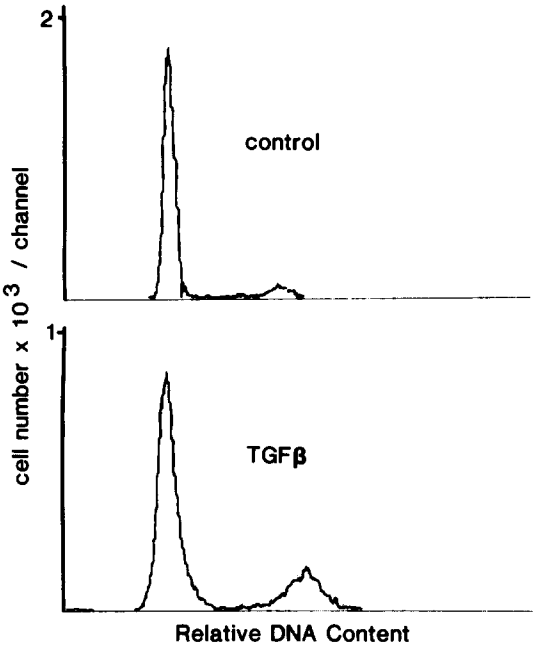

Figure 9. Cell cycle distribution of rat aortic smooth muscle cell cultures after treatment with 100 pM TGF-β (*lower panel*) or vehicle for 9 d. Cells were plated at 3 × 10^3 cells/cm^2 in Medium 199 containing 10% FCS for 24 h, switched to Media 199 containing 5% FCS, and treated with either 100 pM TGF-β or vehicle for 9 d with media changes and readdition of TGF-β or vehicle at 2-d intervals. Cells were then harvested, and FALS (not shown) and cellular DNA content were analyzed by flow microfluorimetry as described in Materials and Methods. A minimum of 10,000 cells were analyzed per sample.

Effects of Long-term Treatment with TGF-β on Cellular DNA Content

An interesting observation in earlier growth inhibition studies was what appeared to be a disproportionate fraction of cells in the G_2 phase of the cell cycle relative to the fraction of cells in S phase in TGF-β-treated cultures as compared to controls. This was of interest, since it is consistent with development of polyploidy, which is known to occur in association with smooth muscle cell hypertrophy in vivo (10, 23, 24, 30). However, a major difficulty in these short-term experiments in sparse cultures was the high frequency of cycling cells and the inability to distinguish cycling cells in G_2 from tetraploid cells. To at least partially circumvent this complication, subsequent experiments were done using a treatment period of 9 d, which had two advantages. First, in 9-d experiments both control and TGF-β-treated cultures grew to saturation density at which cell replication was considerably reduced in both groups. Second, long-term experiments permitted examination of the cumulative effects of TGF-β that might not be detectable in short-term experiments (e.g., the gradual accumulation of cells at a particular point in the cell cycle).

In these experiments, cells in Medium 199 containing 5%

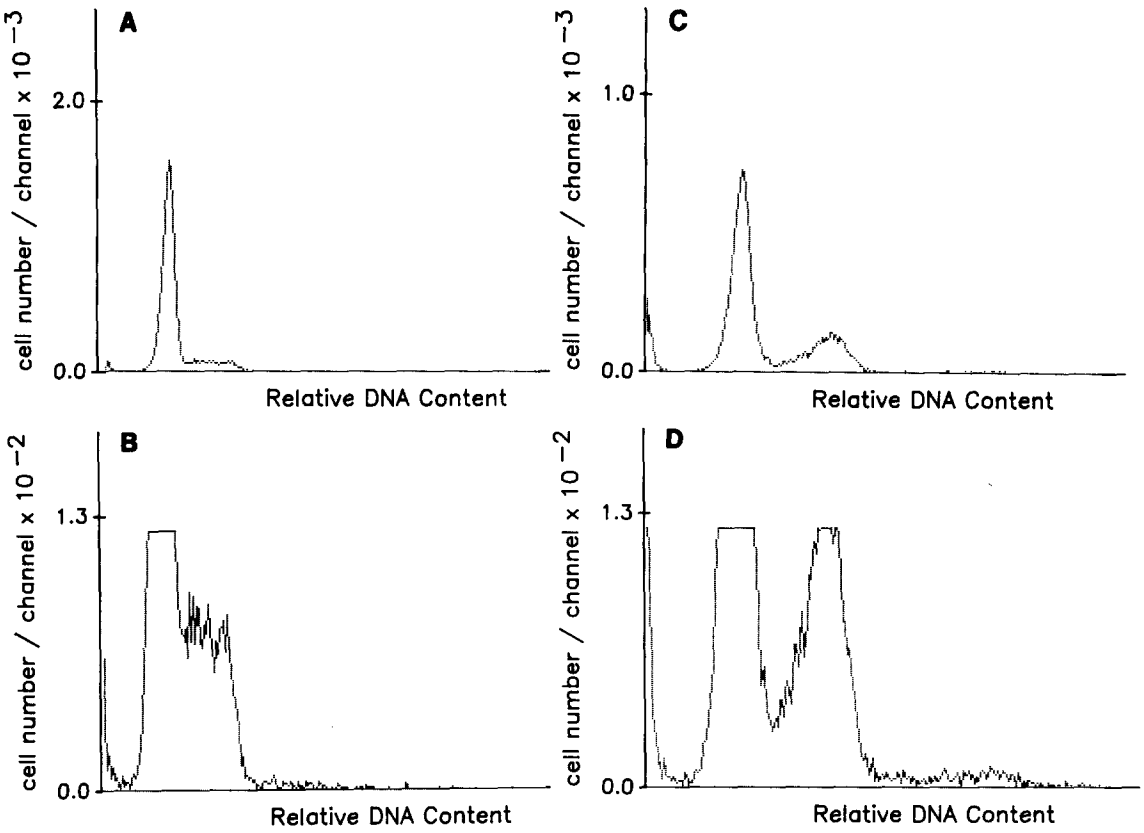

Figure 10. Representative cell cycle histograms from experiments exploring the growth response of putative tetraploid cells after removal of TGF-β and addition of media containing 10% FCS. Cells were treated with TGF-β (100 pmol) or vehicle in media containing 5% FCS for 9 d as described in the legend to Fig. 9, washed with fresh media, and then grown in Medium 199 containing 10% FCS. Samples were harvested for flow cytometric analysis of cellular DNA content (as described in Materials and Methods) 24 h later. (*A* and *B*) DNA histogram (at two different scales) from cells treated with vehicle during the initial 9-d treatment period; (*C* and *D*) histogram (again at two scales) from cells treated with TGF-β. An expanded scale is used in *B* and *D* to better visualize cells with >4c DNA content, which are present in relatively low abundance. A total of 15,000 cells were analyzed in each sample. A summary of the results of these experiments is presented in Fig. 11.

FCS were treated with 100 pM TGF-β or vehicle for 9 d and cellular DNA content assayed by flow microfluorimetry (Fig. 9). Results demonstrated a large fraction of cells in the G_2 phase of the cell cycle in TGF-β ($G_2\% = 26.5 \pm 1.6$; mean ± standard error of four experiments) as compared to vehicle-treated control cultures ($G_2\% = 7.6 \pm 0.5$), despite an extremely low fraction of cells in the S phase of the cell cycle based on examination of flow cytograms (see Fig. 9), or as measured by [^{3}H]thymidine autoradiography. These latter analyses showed 2.3 ± 0.4% and 16.4 ± 2.1% cells in S phase for TGF-β and vehicle-treated control groups, respectively. Observations are consistent with either arrest of cells in the G_2 phase of the cell cycle (4c DNA, but 2n chromosome number) or development of true tetraploidy (i.e., 4c DNA, 4n chromosome number in the resting, G_0–G_1, phase of the cell cycle). The fact that there is a significant increase in the fraction of cells with greater than tetraploid DNA in TGF-β–treated cultures as compared to controls (see Fig. 11, 0 time point), and that this fraction increases after removal of TGF-β and restimulation of cells with medium containing 10% FCS (Figs. 10 and 11) provide strong evidence that TGF-β has induced formation of tetraploid SMC.

Note that in the experiment presented in Figs. 10 and 11 the overall proliferative response of cells was quite small since cells were postconfluent and exhibited considerable contact inhibition of growth. To increase the magnitude of

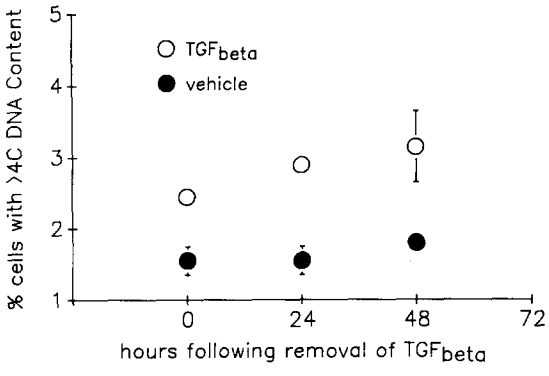

Figure 11. Growth response of tetraploid cells after removal of TGF-β or vehicle and growth in media containing 10% FCS. Additional experimental details are presented in Fig. 10. The fraction of cells with >4c DNA content was calculated by determining the fraction of cells with DNA contents between the G_2 mean plus 2 SD and the point at which the 8c peak returned to baseline. Each point represents the mean ± SD of at least three experimental samples.

the growth response after restimulation of growth in TGF-β-treated cells, experiments were also done in which cells were treated for 9 d with TGF-β or vehicle, and then washed and passaged into standard media containing 10% FCS. Consistent with the results above, flow cytometric analysis of cellular DNA content 48 h after passage demonstrated a 45% increase in cells with greater than 4c DNA in TGF-β-treated cells as compared to controls, supporting the idea that TGF-β treatment induces formation of tetraploid cells which are capable of further DNA replication.

Discussion

The initial objective in these studies was to characterize the nature of TGF-β-induced growth inhibition in cultured rat aortic smooth muscle cells. Consistent with previous reports on bovine aortic smooth muscle cells (3), TGF-β was a potent inhibitor of serum-induced growth of rat aortic smooth muscle cells showing an ED_{50} and maximal effects at concentrations of 2 and 20 pM, respectively. TGF-β-induced growth inhibition was associated with a marked increase in the cell cycle transit time in cells that continue to replicate in the presence of TGF-β, as well as the appearance of a subfraction of cells which did not replicate their DNA during a 72-h [^{3}H]thymidine-labeling period. Whereas it is not clear whether these latter cells have actually withdrawn from the cell cycle, it is clear that they must replicate at an extremely low rate. Roberts et al. (27) have reported a similar increase in cell cycle time for human lung carcinoma A-549 cells treated with TGF-β, although these data were based on determinations of doubling times and did not definitively assess the possible contributions of TGF-β-induced cell cycle withdrawal or increased cell turnover. Our flow cytometric analyses showed an increased fraction of cells in the G_1 phase of the cell cycle and a reduced fraction in S phase consistent with arrest in G_1, as reported previously by Heimark et al. (19a) for TGF-β-induced growth arrest in bovine endothelial cells. However, in the present study, TGF-β treatment was also associated with the accumulation of cells in the G_2 phase of the cell cycle suggesting that at least some cells may be growth arrested in G_2, although it appears that the rate at which cells accumulate in G_2 is quite low. In any event, it appeared that the major effect of TGF-β in inhibiting smooth muscle cell growth was due to an increase in cell cycle time rather than complete growth arrest at a particular point in the cell cycle.

TGF-β-induced growth inhibition was associated with development of marked cellular hypertrophy as well as the gradual accumulation of cells in the G_2 phase of the cell cycle in the virtual absence of cells in S phase. Development of cellular hypertrophy did not require withdrawal of cells from the cell cycle in that all cells appeared to be hypertrophied (see histograms, Fig. 4), and by far the majority of cells, if not all, continue to cycle in the presence of TGF-β although at a reduced rate (Table I). Furthermore, development of hypertrophy did not require arrest of cells at a particular point in the cell cycle in that both cells in G_1 as well as cycling cells in G_2 were hypertrophied as compared to the corresponding cells in vehicle treated controls (Fig. 7). Thus, hypertrophy was present irrespective of the position in the cell cycle. Since it is well established that serum evokes a large increase in cellular protein synthesis as part of the hypertrophic component of the proliferative response (5), one interpretation of these data is that TGF-β-induced cellular hypertrophy is the result of an increase in cell cycle transit time such that the hypertrophic component of cell cycle progression is prolonged and cells attain a larger mass. That is, TGF-β may selectively inhibit serum-stimulated increases in DNA replication, while having little or no effect on serum-induced increases in protein synthesis. Consistent with this idea, Like and Massague (20) demonstrated that TGF-β completely blocked serum or epidermal growth factor-induced increases in DNA replication in MvlLu mink lung epithelial cells, but did not block increases in S6 kinase activity, which has been implicated in the control of increased protein synthesis during cell cycle progression (9). Whether TGF-β-induced hypertrophy is indeed the result of selective inhibition of a subcomponent of those proliferative signals associated with DNA replication and cell division, and whether this is a generalized property of growth inhibitors that increase T_c, remains to be determined.

An alternative possibility is that TGF-β-induced hypertrophy represents a direct response to TGF-β that is independent of growth inhibition. Observations that TGF-β could induce hypertrophy of quiescent smooth muscle cells under serum free conditions support this idea. However, although TGF-β was extremely potent in causing hypertrophy under serum-free conditions (maximal effects were observed with concentrations as low as 2 pM), its efficacy was quite low eliciting a maximal increase in FALS of only 8–23% over control as compared with the 35–50% increase observed in serum containing experiments. The hypertrophic response elicited by TGF-β in SFM, may be similar to that evoked by angiotensin II in these cells. This contractile agonist induces hypertrophy, as well as apparent polyploidy of cultured rat aortic smooth muscle cells grown under serum-free conditions (18), consistent with the hypothesis that contractile agonists can directly stimulate smooth muscle cell hypertrophy. The high potency and low efficacy of TGF-β as a contractile agonist (6) would be consistent with its modest ability to directly stimulate cell hypertrophy. Thus, it is possible that part of the hypertrophic response observed in the presence of serum is due to direct hypertrophic effects of TGF-β. However, it is also possible that the cellular responses elicited under these two conditions are unrelated and occur by independent mechanisms.

An interesting observation in the present study was that prolonged TGF-β treatment was associated with a marked increase in the fraction of cells in the G_2 phase of the cell cycle in the absence of a significant fraction of cells undergoing DNA replication, consistent with either development of tetraploidy or arrest of diploid cells in the G_2 phase of the cell cycle. Observations of a significant fraction of cells with octaploid DNA content in TGF-β-treated cultures as well as the appearance of a significant fraction of cycling cells with greater than tetraploid DNA upon removal of TGF-β and restimulation with 10% FCS containing media strongly suggest that chronic treatment with TGF-β has induced formation of tetraploid cells. However, our data do not exclude the possibility that at least some of the cells in the 4n peak following TGF-β treatment represent cells arrested in the G_2 phase of the cell cycle. It should be noted that these two phenomenon may not be clearly separable experimentally and may be causally related. For example, TGF-β may in-

hibit passage through G_2 (i.e., G_2 arrest) which when prolonged may result in withdrawal from the cell cycle from G_2 and tetraploidy. Consistent with this idea, Zaitsu and Kimura (34) found that 3Y1 tsF121 cells, a temperature-sensitive mutant which arrests in either G_1 or G_2 at the non-permissive temperature, lose the ability to complete mitosis after prolonged G_2 arrest. Although many examples of G_2 arrest have been reported in the literature, most of these involve irradiation or chemical treatment of cells (26, 28, 34), and this is the first demonstration, to our knowledge, of induction of G_2 arrest and polyploidy by a naturally occurring, chemically defined substance. The fact that the G_2 cells were mononucleate (based on visual observation and flow cytometric analysis) indicates that neither karyokinesis or cytokinesis occurred. These data also strongly suggest that the observed polyploidy is not the result of cell fusion, since in this case one would expect to see multinucleate cells. The mechanisms whereby TGF-β might inhibit progression through G_2 are not known. Obvious possibilities are that TGF-β may disrupt cytoskeletal components or inhibit synthesis of G_2 specific proteins (26) necessary for mitosis, although direct evidence for either of these is currently lacking.

Although no direct evidence is available implicating a role for TGF-β in the hypertrophic response of smooth muscle cells in chronic hyptertension, the similarities in the growth responses observed in the present in vitro studies and that observed in vivo (10, 23, 24, 30) make it is tempting to speculate that TGF-β might play some role. This might involve release of TGF-β from platelets (2) adherent to a vessel wall damaged by increased hemodynamic stresses associated with hypertension (31). Alternatively, TGF-β might be derived from endothelial cells, smooth muscle cells, or activated macrophages which contain TGF-β mRNA (1, 21).

In summary, results of the present studies provide additional support for the hypothesis that TGF-β may be an important regulator of smooth muscle cell growth and suggest that one mechanism whereby cellular hypertrophy and polyploidy can occur in cells is by alterations in the response to serum mitogens by potent growth inhibitors such as TGF-β. Further studies will be required to determine: (*a*) whether development of cellular hypertrophy is a common response of many cell types to certain growth inhibitors; (*b*) the mechanisms of action of TGF-β in inhibiting cell proliferation, and inducing cellular hypertrophy and polyploidy; (*c*) the relationship between G_2 arrest and development of polyploidy; and (*d*) whether TGF-β is involved in mediation of vascular smooth muscle hypertrophy in vivo.

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