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Inhibition of carcinoma and melanoma cell growth by type 1 transforming growth factor β is dependent on the presence of polyunsaturated fatty acids

(serum-free culture medium/A549 lung carcinoma/B16 melanoma)

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ABSTRACT **Improved serum-free media were developed for the anchorage-dependent growth of A549 human lung carcinoma and B16 mouse melanoma cell lines *in vitro*. Type 1 transforming growth factor β (TGF-β1) inhibited the growth of A549 or B16 cells under serum-free conditions or in the presence of 10% serum by 15–33%. In contrast, in the presence of μg/ml concentrations of polyunsaturated fatty acids (PUFAs), picomolar concentrations of TGF-β1 irreversibly inhibited the serum-free growth of A549 or B16 cells by 90–100%. The PUFAs alone had little effect on cell growth. Cell growth inhibition by TGF-β1 was not potentiated by saturated fatty acids, monounsaturated fatty acids, or prostaglandins. Inhibition of A549 or B16 cell growth by TGF-β1 in the presence of PUFAs was almost completely reversed by the antioxidant vitamin E, suggesting a role for lipid peroxidation in this process. Inhibition of A549 or B16 cell growth by TGF-β1 in the presence of 5% fetal calf serum was also potentiated by PUFAs and partially reversed by antioxidants. The presence of retinoic acid was required for maximal PUFA-dependent growth inhibition of A549 or B16 cells by TGF-β1 under some, but not all, conditions. These results suggest that inhibition of carcinoma and melanoma cell growth by TGF-β1 is mediated, in large part, by PUFAs.**

Type 1 transforming growth factor β (TGF-β1) is a multifunctional protein found in all mammalian tissues. It has been implicated in the control of development, growth, immune system function, and carcinogenesis. Although TGF-β1 stimulates wound healing *in vivo*, the growth of many cell types *in vitro* is inhibited by this growth factor. In addition, the cellular growth response to TGF-β1 *in vitro* has been found to be dependent on both cell type and culture conditions (see refs. 1–5 for reviews; see ref. 6 for recent example). The ability of TGF-β1 to inhibit the growth of carcinoma and melanoma cells is of particular interest because it suggests its possible use as a chemotherapeutic agent (7, 8). One recent study has demonstrated a 60% inhibition of A549 human lung carcinoma tumor growth in athymic mice by TGF-β1 (9).

Inhibition of the anchorage-dependent growth of normal rat kidney fibroblasts by TGF-β1 is dependent on induction of type I collagen secretion (10). Inhibition of the growth of nontransformed and transformed epithelial cells by TGF-β1 is associated with inhibition of c-*myc* expression (see refs. 11 and 12 for examples and reviews). In addition, loss of growth inhibition of CCL 64 mink lung epithelial cells by TGF-β1 is associated with a loss of TGF-β receptor expression and (or) constitutive fibronectin expression (13). However, the precise mechanisms of cell growth inhibition by TGF-β1 are not known.

Because cellular response to TGF-β1 is often altered by the presence of other factors, such as growth factors and retinoids that are present in serum (see refs. 1–5 for reviews), it is advantageous to carry out studies of TGF-β1 action under serum-free conditions. Therefore, I have developed improved serum-free media for the anchorage-dependent growth of A549 lung carcinoma and B16 mouse melanoma cells. These studies have led to the observation that A549 and B16 cell growth inhibition by TGF-β1 is dependent on the presence of polyunsaturated fatty acids (PUFAs).

MATERIALS AND METHODS

Materials and Cells. Serum-free medium components were obtained and stock solutions were prepared as follows: Crystallized, fatty acid-free bovine serum albumin (BSA) catalog no. A 0281 (50 mg/ml in calcium- and magnesium-free phosphate-buffered saline, PBS); soybean trypsin inhibitor; insulin (20 mg/ml in 6 mM HCl); transferrin catalog no. T 5391 (5 mg/ml in PBS); hydrocortisone (500 μM in ethanol, stored under argon at −20°C); sodium selenite (50 μM); all-*trans*-retinoic acid (30 μg/ml in ethanol, stored in the dark under argon at −20°C); and triiodothyronine (20 nM in 10 mM NaOH) were from Sigma. Porcine TGF-β1 (in 4 mM HCl, containing BSA at 1 mg/ml) and basic fibroblast growth factor (bFGF) (in BSA at 1 mg/ml) were from R & D Systems (Minneapolis, MN). Receptor-grade epidermal growth factor (EGF) (in BSA at 1 mg/ml), vitamin E, and liquid RPMI 1640 medium were from GIBCO. Bovine plasma fibronectin was from GIBCO or Sigma. Bovine skin type I collagen was from Collagen Corp. Fatty acids were from either Sigma or Nu Check Prep (Elysian, MN) and were stored in ethanol, under argon, at either −20°C or −80°C. Similar results were obtained regardless of source or storage temperature. Custom fatty acid-free Dulbecco's modified Eagle's medium/Ham's F12 medium (DME/F12) was from Specialty Media (Lavallette, NJ). Milli-Q (Millipore) purified water was used in all experiments. Serum-free media component stock solutions were made fresh every 2–3 months.

A549 human lung carcinoma cells were from Lawrence Levine (Brandeis University), and B16-F1 mouse melanoma cells were from the American Type Culture Collection. Cells were maintained at 37°C (5% CO_2) in RPMI 1640 medium with 10% fetal calf serum (HyClone) and 15 mM Hepes. B16 melanoma cells were also maintained in the presence of penicillin and streptomycin at 100 units/ml each. Fresh cultures were initiated from frozen stocks every 2–3 months and were found to be free of mycoplasma infection.

Serum-Free Growth of A549 Lung Carcinoma Cells. A549 cells were grown by a modification of the method of Brower

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Abbreviations: TGF-β1, type 1 transforming growth factor β; PUFA, polyunsaturated fatty acid; BSA, bovine serum albumin; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

et al. (14). Tissue culture wells (2 cm^2) were treated with fibronectin at 10 μg/ml in 0.3 ml of 4°C RPMI 1640 medium overnight at 37°C, followed by aspiration and similar treatment with type I collagen. Wells were rinsed once with PBS before cell plating. A549 cells (100 mm dish) were washed once and then dissociated with 3 ml of 0.05% trypsin/0.53 mM EDTA in PBS, which was removed while the cells were still attached. Trypsin inhibitor (5 ml of 1 mg/ml in RPMI 1640) was added and the cells were dispersed and washed two times with RPMI 1640. Cells (5×10^3 per well) were plated in 0.5 ml of RPMI 1640 containing BSA at 1 mg/ml, insulin at 20 μg/ml, transferrin at 10 μg/ml, 0.5 mM sodium pyruvate, bFGF at 5 ng/ml, EGF at 5 ng/ml, 2 mM glutamine, 100 nM hydrocortisone, 50 nM sodium selenite, and retinoic acid at 3 ng/ml. TGF-β1, fatty acids, prostaglandins, and vitamin E were added 18–24 hr after plating, and then cell numbers were determined with a cell counter after an additional 3-, 4-, or 5-day incubation. Fatty acids and prostaglandins were added after dilution of concentrated stocks (20 mg/ml) into tissue culture medium containing BSA at 1 mg/ml (made fresh for each experiment). Treated cells were compared with control cells that received vehicle in all experiments. All experiments were carried out in triplicate and results are expressed as mean cell number ± SD.

Serum-Free Growth of B16 Melanoma Cells. Tissue culture wells were coated with 3 μg of fibronectin in fatty acid-free DME/F12/RPMI 1640 medium (1:1:2) as described above for A549 cells. Cells were incubated with nonenzymatic PBS-based cell dissociation solution (Sigma), then dispersed in DME/F12/RPMI 1640 and washed once with the same medium. The growth medium was a modification of that described by Fernandez-Pol *et al.* (15). Cells were plated in 0.5 ml of fatty acid-free DME/F12/RPMI 1640 containing BSA at 1 mg/ml, insulin at 5 μg/ml, transferrin at 5 μg/ml, 2 mM glutamine, 5 pM triiodothyronine, 10 nM sodium selenite, and 50 nM hydrocortisone. All other additions and determinations were carried out as described above for A549 cells.

RESULTS

Effects of TGF-β1 on Growth of A549 Cells in Serum-Containing and Serum-Free Media. An improved serum-free medium was developed for the growth of A549 lung carcinoma cells (Fig. 1). The medium was based on the ACL-3 medium developed by Brower *et al.* (14). Alterations included addition of bFGF and retinoic acid. No lag period was observed when A549 cells were grown in modified ACL-3 medium, and the doubling time was 27.1 hr, compared to 36 hr reported for growth of these cells in ACL-3 medium. Elimination of any of the components of the modified medium resulted in a significantly reduced rate of growth (data not shown). The doubling time in serum-containing medium (18.7 hr) was comparable to that reported by Brower *et al.* (14).

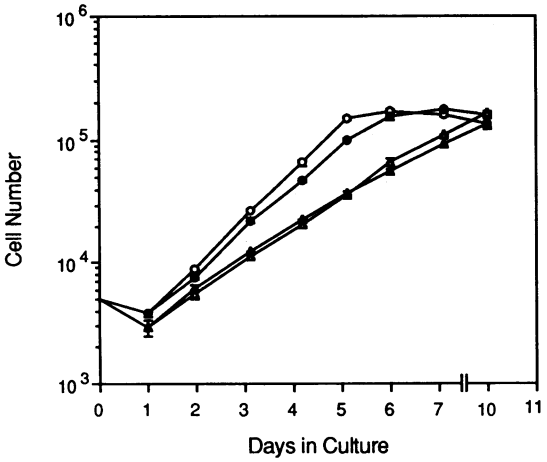

FIG. 1. Anchorage-dependent growth of A549 cells in 10% fetal calf serum and serum-free medium in the presence and absence of TGF-β1. Cells were grown in serum-free medium without (△) or with (▲) 200 pM TGF-β1, or in RPMI 1640 medium containing 10% fetal calf serum without (○) or with (●) 200 pM TGF-β1. Error bars indicate SD of triplicate determinations.

TGF-β1 has been found to reversibly inhibit the anchorage-dependent growth of A549 cells in medium containing 2% fetal calf serum by approximately 70% (7). In the present study, addition of 200 pM TGF-β1 inhibited subconfluent A549 cell growth in the presence of 10% fetal calf serum by 33%, whereas TGF-β1 was able to inhibit the serum-free growth of these cells by only 18–20% (Fig. 1).

Inhibition of A549 Cell Growth by TGF-β1 in the Presence of Exogenous PUFAs. PUFAs were tested for stimulatory effects on the serum-free growth of A549 cells. Addition of PUFAs at concentrations below 5 μg/ml had little effect on A549 growth. However, the presence of linoleic or α-linolenic acid significantly increased the cellular sensitivity to growth inhibition by TGF-β1. TGF-β1 inhibited the growth of A549 cells by almost 100%, relative to the cell density at the time of TGF-β1 addition, when the assay was carried out in the presence of linoleic or α-linolenic acid at 1–3 μg/ml (Fig. 2). Similar results were obtained with arachidonic, eicosapentaenoic, and docosahexaenoic acids. The presence of BSA in the growth medium was not required for, but did increase, PUFA-dependent growth inhibition by TGF-β1. In contrast, the presence of retinoic acid in the growth medium was required for significant PUFA-dependent growth inhibition by TGF-β1 (data not shown). Unsaturated and monounsaturated fatty acids were unable to increase A549 cellular sensitivity to growth inhibition by TGF-β1 (Fig. 2). Similar results were obtained with caprylic, lauric, palmitic, elaidic, and oleic acids. All fatty acids were nonspecifically toxic at concentrations above 10 μg/ml (data not shown).

Titrations of TGF-β1 revealed a biphasic response to this growth factor by A549 cells (Fig. 3). Subpicomolar concentrations of TGF-β1 reproducibly produced a small stimulation of cell growth in the absence or presence of linoleic acid. Picomolar concentrations of TGF-β1 inhibited A549 cell growth by a maximum of 20% in the absence of linoleic acid, as described above. Titration of TGF-β1 between 0.5 and 10 pM resulted in a dose-dependent 95–100% inhibition of cell growth in the presence of linoleic acid at 2 μg/ml. Final cell numbers in the presence of TGF-β1 were lower than the initial plating density or the cell density at the time of TGF-β1 addition.

Irreversible Inhibition of A549 Cell Growth by TGF-β1 in the Presence of PUFAs. A549 growth curves from experiments carried out in the presence of PUFAs and TGF-β1 demonstrated that little TGF-β1-mediated growth inhibition occurred during the first 1–2 days. However, treatment with PUFAs and TGF-β1 caused a complete cessation of growth by day 3 and resulted in destruction and loss of attached cells during subsequent incubation. Fig. 4 demonstrates that treatment of cells with linoleic acid and TGF-β1 resulted in both cell growth inhibition and subsequent cell destruction. This observation was confirmed by analysis of trypan blue exclusion by untreated and TGF-β1-treated cells. After growth in the presence of linoleic acid and TGF-β1, approximately 80% of the remaining cells were nonviable on the basis of trypan blue uptake. These results were also confirmed by direct replating of cells. Cells were grown in the presence of linoleic acid at 1 μg/ml with or without TGF-β1 for 4 days. Untreated wells contained an average of 10.24×10^4 cells and TGF-β1-treated wells contained 0.27×10^4 cells. Trypsinization

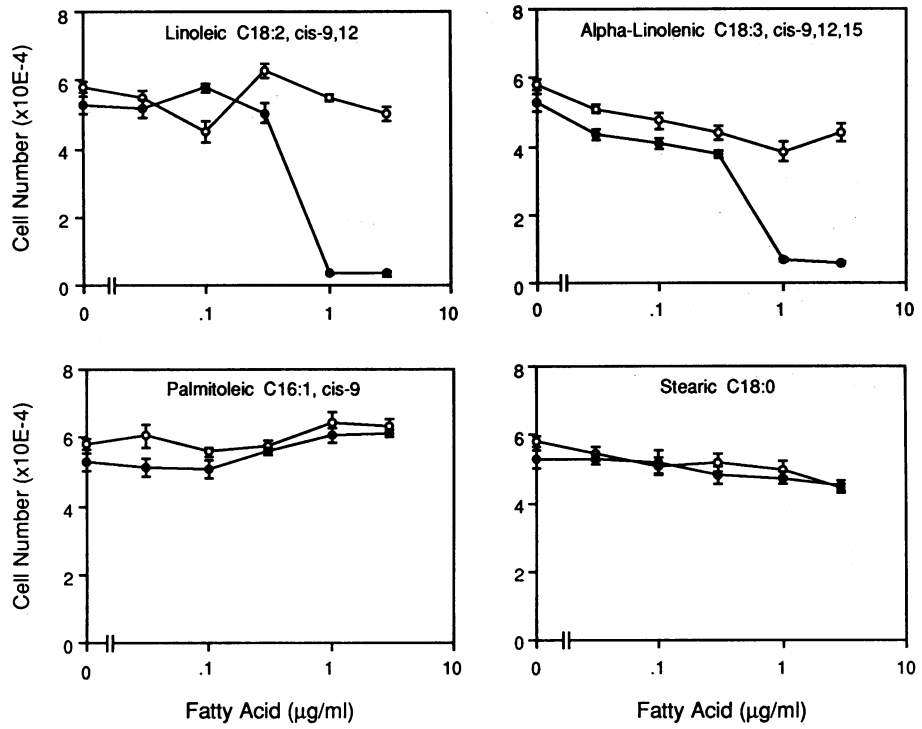

FIG. 2. Effects of fatty acids on A549 cell growth inhibition by TGF-β1. Cells were grown under serum-free conditions with the indicated fatty acids, in the absence (○) or presence (●) of 100 pM TGF-β1. Cell numbers were determined 5 days after TGF-β1 addition. ×10E-4 indicates × 10^{-4}. PUFA-dependent inhibition of A549 cell growth by TGF-β1 was observed in 12 independent experiments.

and replating of the TGF-β1-treated cells in medium containing 10% fetal calf serum resulted in the isolation of no, or only a few, colonies. Untreated cells were easily replated at high efficiency. The limited growth inhibition mediated by TFG-β1 in the absence of PUFAs was reversible.

Role of PUFAs in A549 Cell Growth Inhibition by TGF-β1. The possibility that a PUFA metabolite may be the mediator of A549 cell growth inhibition by TGF-β1 was tested. Fig. 5 demonstrates that, although linoleic, α-linolenic, or docosahexaenoic acid at 1 μg/ml was able to mediate growth inhibition by TGF-β1, prostaglandins E_1, E_2, D_2, and $F_{2\alpha}$ at a similar concentration were unable to act synergistically with TGF-β1 to inhibit the growth of A549 cells.

Linoleic acid (an $n = 6$ fatty acid), α-linolenic acid, and docosahexaenoic acid ($n = 3$ fatty acids) serve as precursors for the biosynthesis of different cyclooxygenase or lipoxygenase products. Thus, the results described above suggest that PUFAs themselves, or some product common to all PUFAs, must be responsible for mediating growth inhibition by TGF-β1. PUFAs are susceptible to peroxidation, resulting in the generation of toxic degradation products (see ref. 16 for review). Therefore, cell growth inhibition by linoleic acid and

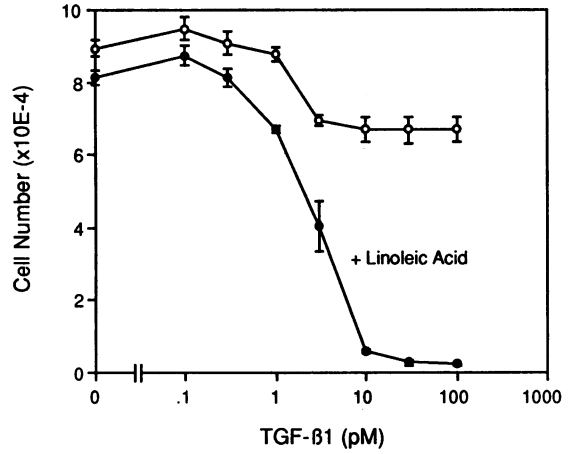

FIG. 3. Dose–response curve for inhibition of A549 cell growth by TGF-β1 in the presence and absence of linoleic acid. Cells were grown under serum-free conditions with the indicated concentrations of TGF-β1 in the absence (○) or presence (●) of linoleic acid at 2 μg/ml. Cell numbers were determined 5 days after TGF-β1 addition.

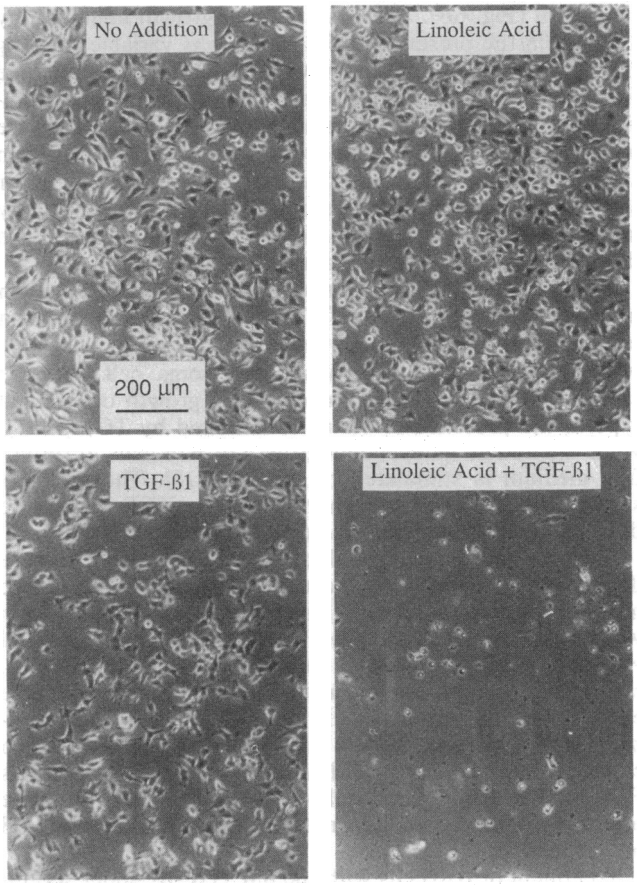

FIG. 4. Photographs of A549 cells grown in the absence and presence of linoleic acid at 1 μg/ml and 25 pM TGF-β1. Cells were grown under serum-free conditions with the indicated additions. Photographs were taken with a Nikon N2000 camera 4 days after addition of fatty acid and TGF-β1.

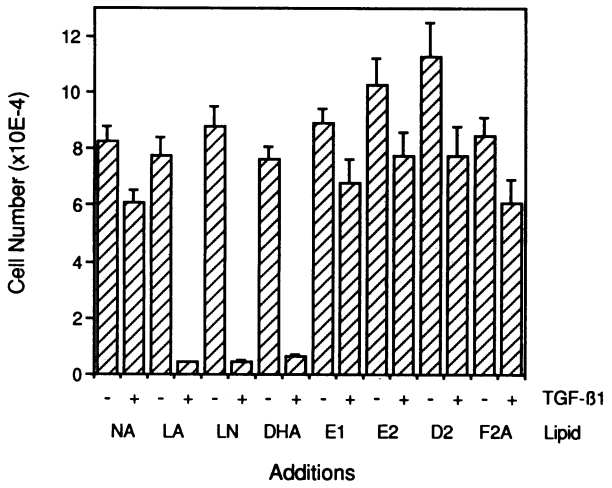

FIG. 5. Effects of PUFAs and prostaglandins on A549 cell growth inhibition by TGF-β1. Cells were grown under serum-free conditions with the indicated lipid at 1 μg/ml, in the absence (−) or presence (+) of 40 pM TGF-β1. Cell numbers were determined 4 days after TGF-β1 addition. NA, no addition; LA, linoleic acid; LN, α-linolenic acid; DHA, docosahexaenoic acid; E1, E2, D2, and F2A, prostaglandins E_1, E_2, D_2, and $F_{2\alpha}$, respectively.

TGF-β1 was examined in the presence and absence of the antioxidant vitamin E. Fig. 6*A* demonstrates that vitamin E was able to prevent linoleic acid-dependent A549 cell growth inhibition by TGF-β1.

When A549 cells were grown in the presence of 5% fetal calf serum, addition of 200 pM TGF-β1 resulted in a 70% inhibition of growth (Fig. 6*B*). The extent of cell growth inhibition by TGF-β1 in the presence of 5% serum was variable, ranging from 23% to 70%. Addition of vitamin E and the glutathione peroxidase cofactor sodium selenite prevented growth inhibition by TGF-β1 (Fig. 6*B*). Vitamin E and sodium selenite were unable to completely reverse growth inhibition by TGF-β1 in serum-containing or serum-free media. As observed under serum-free conditions, exogenous linoleic acid significantly increased A549 sensitivity to growth inhibition by TGF-β1 in the presence of 5% serum (data not shown).

PUFA-Dependent Inhibition of B16 Melanoma Cell Growth by TGF-β1. To determine if PUFAs play a general role in tumor cell growth inhibition by TGF-β1, a second cell type was examined. The B16 mouse melanoma cell line was chosen because anchorage-independent growth of this cell type in the presence of serum is inhibited by TGF-β1 (7), while anchorage-independent growth of B16 cells in serum-free medium is stimulated by TGF-β1 (15). These results suggest that B16 cell growth inhibition by TGF-β1 may be dependent on unidentified factors contained in serum.

As described above for A549 cells, the present studies were dependent on the development of an improved serum-free medium for the growth of B16 melanoma cells. The medium was based on DME/F12+H+F developed by Fernandez-Pol *et al.* (15). The modifications included the use of fibronectin, rather than serum, for promotion of cell attachment, the elimination of prostaglandin E_1, and the addition of crystalized fatty acid-free BSA. The basal medium was also changed, and was composed of a mixture of DME, Ham's F12, and RPMI 1640. B16 cell doubling times in the presence of serum-containing and serum-free media were 13.4 and 15.8 hr, respectively (Fig. 7*A*).

Treatment of B16 cells with 100 pM TGF-β1 under serum-free anchorage-dependent conditions resulted in a 10–15% inhibition of growth, as described above for A549 cells (Fig. 7*B*). Addition of α-linolenic acid alone at 10 μg/ml had little effect on the growth of the cells, but the presence of this PUFA allowed TGF-β1 to inhibit cell growth by 85%. Similar

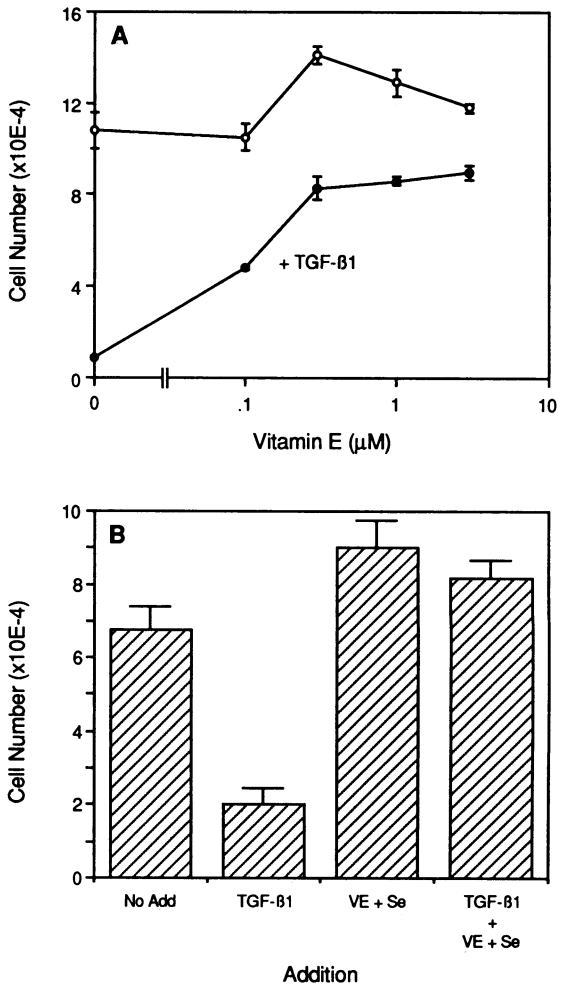

FIG. 6. Reversal of TGF-β1-mediated A549 cell growth inhibition by vitamin E. (*A*) Cells were grown under serum-free conditions with linoleic acid at 2 μg/ml and the indicated concentrations of vitamin E, in the absence (○) or presence (●) of 25 pM TGF-β1. Cell numbers were determined 5 days after addition of TGF-β1 and vitamin E. Similar results were obtained in three independent experiments. (*B*) Cells were plated at 2×10^3 per well in RPMI 1640 medium containing 5% fetal calf serum. TGF-β1 (200 pM) and (or) vitamin E (3 μM) and sodium selenite (30 nM) were added the following day and then cell numbers were determined after an additional 4-day incubation.

results were obtained with linoleic, γ-linolenic, arachidonic, and docosahexaenoic acids. As described for A549 cells, subpicomolar concentrations of TGF-β1 slightly stimulated the growth of B16 cells, and long-term treatment with picomolar concentrations of TGF-β1 and PUFAs caused an irreversible inhibition of B16 cell growth (data not shown). Inhibition of the growth of B16 cells by TGF-β1 in the presence, but not in the absence, of α-linolenic acid was reversed by vitamin E (Fig. 7*B*). α-Linolenic acid also increased B16 sensitivity to growth inhibition by TGF-β1 in the presence of 5% serum. The synergistic inhibitory effect of TGF-β1 and PUFAs in the presence of serum was dependent on the addition of retinoic acid, and was largely reversed by addition of vitamin E and sodium selenite (data not shown).

DISCUSSION

The development and use of improved serum-free media for the growth of lung carcinoma and melanoma cells has led to the finding that cell growth inhibition by TGF-β1 is highly dependent on the presence of exogenous PUFAs. The reversal of TGF-β1-mediated growth inhibition by vitamin E and sodium selenite in serum-containing or serum-free media

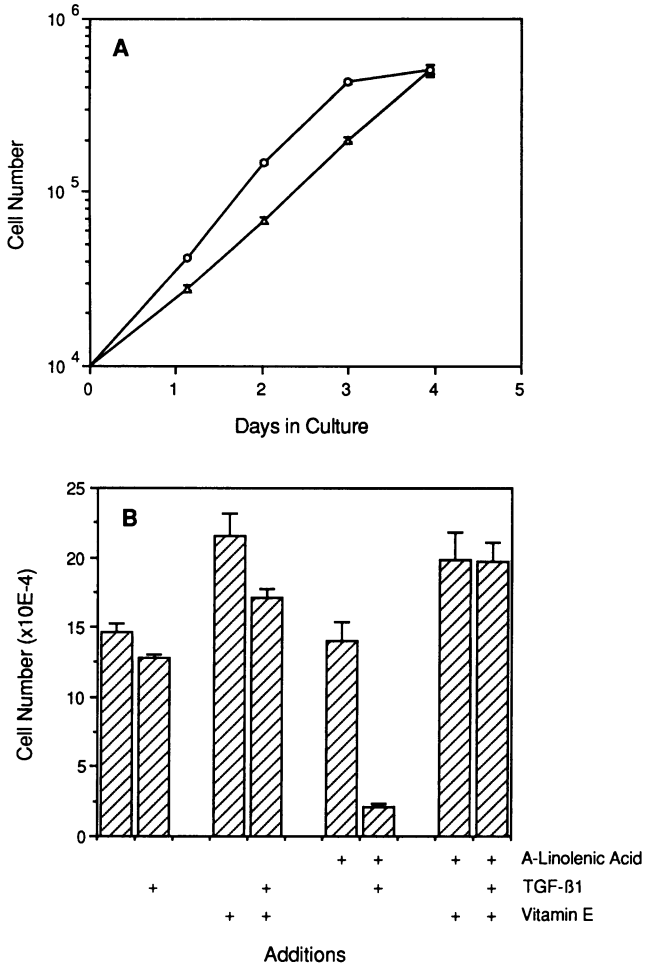

FIG. 7. (*A*) Anchorage-dependent growth of B16 melanoma cells in serum-containing and serum-free media. Cells (1×10^4 per well) were grown in serum-free medium (△) or RPMI 1640 medium containing 10% fetal calf serum (○). (*B*) Effects of α-linolenic acid and vitamin E on B16 cell growth inhibition by TGF-β1. Cells (5×10^3 per well) were grown under serum-free conditions in the absence or presence of α-linolenic acid, at 10 μg/ml, 100 pM TGF-β1, and (or) 1 μM vitamin E (added 1 day after cell plating). Cell numbers were determined 3 days after these additions. Similar results were obtained in four independent experiments.

suggests that inhibition by TGF-β1 is mediated, in part, by PUFAs under both conditions. The remarkably similar PUFA dependence observed with two different tumor cell lines from different species suggests that this may represent a general mechanism for tumor cell growth inhibition by TGF-β1. Indeed, preliminary studies have demonstrated PUFA-dependent MCF-7 human breast carcinoma cell growth inhibition by TGF-β1 (M.J.N. and A. M. Iannotti, unpublished data). Since fetal calf serum contains PUFAs (approximately 70 μg/ml), antioxidants such as vitamin E (3 μM), and retinoids in concentrations which are likely to vary between types and batches of serum (HyClone product information), my results suggest one explanation for the well-established variability of cellular response to TGF-β1 in the presence of serum.

Previous studies have demonstrated inhibition of the growth of at least 16 different tumor cell lines by exogenous PUFAs in the presence of serum. Inhibition was dependent on serum source and concentration, and it was reversed by antioxidants or sodium selenite, suggesting a role for lipid peroxidation (see refs. 17 and 18 for examples and reviews). My demonstration of a synergistic effect of TGF-β1 and PUFAs on inhibition of carcinoma and melanoma cell growth, and reversal of this inhibition by antioxidants under serum-free conditions, suggests that TGF-β1 present in serum may have contributed to the growth inhibition by PUFAs observed previously. The putative products of lipid oxidation directly responsible for transformed cell growth inhibition have not been identified. The serum-free model systems described in this communication will be useful for the identification of these compounds.

Bégin *et al.* (17, 18) have found that nontransformed cells are relatively resistant to growth inhibition by exogenous PUFAs. We have obtained preliminary evidence that, although the serum-free growth of nontransformed fibroblasts and nontransformed lung, skin, and breast epithelial cells is sensitive to inhibition by TGF-β1, exogenous fatty acids are not required for and do not potentiate this inhibition (M.J.N., D. J. Sarubbi, and A. M. Iannotti, unpublished data). These results support the conclusion that growth inhibition by TGF-β1 can be mediated by several different mechanisms.

At present, the nature of the synergistic interaction between PUFAs and TGF-β1 is unclear. TGF-β1 may stimulate PUFA uptake by cells, or it may stimulate PUFA peroxidation and (or) breakdown by a direct or indirect mechanism. Alternatively, TGF-β1 and PUFAs may function independently, with both pathways being required for significant growth inhibition.

The ability of TGF-β1 to inhibit tumor cell growth irreversibly in the presence of PUFAs and retinoic acid suggests that this combination may be useful in the development of TGF-β-based cancer chemotherapy. To test this possibility, the next step is to determine if combinations of TGF-β1, PUFAs, and retinoids act synergistically to inhibit or possibly prevent tumor growth *in vivo*.

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