Inhibition of Nb2 T-lymphoma cell growth by transforming growth factor- β

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Transforming growth factor- β (TGF- β) inhibits proliferation of Nb2 cells, a rat T lymphoma, in response to lactogens and interleukin-2. Prostaglandins may play an important role in the pathway through which TGF- β exerts its inhibitory actions, because prostaglandin E_2 also inhibits proliferation of Nb2 cells, and indomethacin, an inhibitor of prostaglandin synthesis, reverses the inhibitory effects of TGF- β on Nb2 cell proliferation.

INTRODUCTION

Transforming growth factor- β (TGF- β) is a multifunctional regulator of cell growth that exerts either stimulatory or inhibitory effects [1]. On cells of the immune system, TGF- β primarily exerts inhibitory actions. It blocks growth-factor-dependent proliferation and antibody secretion in B lymphocytes [2], suppresses natural killer-cell activity [3] and inhibits thymocyte proliferation [4]. In T lymphocytes, TGF- β inhibits induction of interleukin-2 (IL-2) receptors and proliferation in response to IL-2 [5]. These immunosuppressive effects of TGF- β may represent a physiologically important autocrine or paracrine regulatory mechanism, because activation of T cells [5] and macrophages [6] results in the synthesis and secretion of TGF- β .

Little is known about the mechanism of action of TGF- β in immune cells or in the numerous other cell types responsive to the growth factor. However, one report [7] suggests that prostaglandins of the E series may mediate the bone-resorbing actions of TGF- β . The immunosuppressive actions of TGF- β may involve a similar mechanism, because prostaglandins of the E series inhibit T-cell proliferation [8], and recent data [9] suggest that inhibition of prostaglandin synthesis is an important step in activation of T lymphocytes. Prostaglandins of the E series also may mediate inhibition of T-cell proliferation by monocytes [10] and inhibition of lymphocyte proliferation by some tumours [11]. We have used Nb2 cells, a rat T lymphoma [12] that proliferates in response to prolactin (PRL) [13] and IL-2 [14], to examine further the immunosuppressive actions of TGF- β .

EXPERIMENTAL

Materials

Human growth hormone (hGH; 79-7-23H) was a gift from Dr. H. G. Friesen, and purified TGF- β was obtained from Dr. M. B. Sporn and R & D Systems Inc. (Minneapolis, MN, U.S.A.). Rat IL-2 was obtained from

Collaborative Research Inc. (Lexington, MA, U.S.A.). Ovine prolactin (oPRL; NIH-P-I-2) was obtained from the Hormone Distribution Program, N.I.H. (Bethesda, MD, U.S.A.). Indomethacin and prostaglandins E_2 (PGE $_2$), E_1 (PGE $_1$) and $F_{2\alpha}$ (PGF $_{2\alpha}$) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture and quantification

The Nb2 cells used in this study, derived from a clone produced in the laboratory of Dr. H. G. Friesen, were grown in Fischer's medium supplemented with 10% (v/v) fetal bovine serum, 10% (v/v) horse serum, 1 mM-2-mercaptoethanol, 100 units of penicillin/ml and 100 μ g of streptomycin/ml, and were maintained in a humidified atmosphere of CO $_2$ /air (1:19) at 37 °C. In one series of experiments, Nb2 cells were cultured in a serum-free medium developed for this cell line [15]. Cell proliferation was quantified by the bioassay of Tanaka *et al.* [13]. Approx. 24 h before addition of test substances, the Nb2 cells were growth-arrested by removing fetal bovine serum from the medium (i.e. resuspended in 'assay medium'). Cell number and cell diameter were determined with a Coulter model ZM counter with channelyzer (Coulter Electronics, Hialeah, FL, U.S.A.). Cell number was converted into number of replications by the formula: $\log(\text{cell number}/\text{initial cell number})/\log 2$.

Thymidine incorporation

Cell samples were placed in sterile tubes containing [3 H]thymidine (final concn. 10 μ Ci/ml; ICN Radiochemicals, Costa Mesa, CA, U.S.A.) and incubated for 15 min at 37 °C in a shaking water bath. Labelling was stopped by placing the tubes in an ice/water bath for 30 s, followed by collection of the cells by centrifugation. The uptake of [3 H]thymidine into DNA was assessed by precipitation with 5% (v/v) trichloroacetic acid and collection of the precipitate on glass-fibre filters by vacuum filtration followed by liquid-scintillation counting in a Beckman LS5800 instrument. Portions of assay medium containing no cells were processed as

Abbreviations used: TGF, transforming growth factor; IL-2, interleukin-2; PRL, prolactin; PG, prostaglandin; hGH, human growth hormone (somatotropin).

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Fig. 1. Inhibitory effects of TGF- β on mitogen-stimulated proliferation of Nb2 cells

Cells were plated in assay medium at a concentration of 1×10^5 cells/ml in the wells of 24-well culture dishes. The cells were exposed to hGH (1 ng/ml) or rat IL-2 (20 half-maximal units/ml), with or without TGF- β (■, 0; □, 0.005 ng/ml; ▨, 0.05 ng/ml; ▤, 0.5 ng/ml; ▥, 5 ng/ml; ▦, 50 ng/ml) for 3 days. The error bars indicate $\pm$ S.E.M. ($n = 3$).

described above to determine non-specific [3 H]thymidine precipitation.

RESULTS AND DISCUSSION

Effects of TGF- β on Nb2 cell proliferation in response to lactogens and rat IL-2 were examined in initial studies. hGH, as well as oPRL, was used in these studies because it is a potent lactogen and it only acts through PRL receptors in Nb2 cells [13,16]. Moreover, in our studies, results obtained with hGH and oPRL were indistinguishable. A representative experiment in which hGH and IL-2 were used is shown in Fig. 1. Maximal stimulation of Nb2 cell proliferation is obtained with hGH or oPRL at 5–10 ng/ml and IL-2 at 40–80 half-maximal units/ml [14]; thus sub-maximal concentrations of hGH and IL-2 were used in this experiment. After 3 days in culture in a medium containing 10% horse serum, Nb2 cells exposed to mitogen plus TGF- β underwent approximately one less replication than did cells exposed to mitogen alone. TGF- β consistently inhibited mitogen-induced proliferation in five separate dose-response studies, and the degree of inhibition did not appear to be affected by the concentration of mitogen (1–10 ng of lactogen/ml, 20–40 half-maximal units of IL-2/ml) or the number of cells [$(0.5\text{--}5) \times 10^5$ /ml] (results not shown). Another consistent finding in these studies was that TGF- β never completely inhibited cell proliferation. Significant inhibition of proliferation in response to IL-2 was achieved with 0.05 ng of TGF- β /ml ($P < 0.001$), whereas significant inhibition of lactogen-stimulated proliferation required 5 ng of TGF- β /ml ($P < 0.05$). Thus IL-2-stimulated proliferation appears to be more sensitive to the inhibitory actions of TGF- β . The greater sensitivity of IL-2-stimulated proliferation does not appear to result from up-regulation of the receptor for TGF- β by IL-2, because binding of 125 I-TGF- β to IL-2-treated and oPRL-treated cells did not differ from that by quiescent cells (results not shown). In Fig. 1, TGF- β inhibited hGH-stimulated growth to a slightly greater degree; however, this was not a consistent finding. Serum factors were not required for TGF- β to exert its inhibitory

Fig. 2. Time course of the effects of TGF- β on lactogen-induced Nb2 cell growth

A representative experiment is shown in which hGH served as the lactogen. Nb2 cells growth-arrested in assay medium were resuspended in fresh assay medium (1×10^5 cells/ml) and maintained in spinner flasks. At zero time, the cells were exposed to hGH (1 ng/ml; ○) or hGH + TGF- β (5 ng/ml; △). Samples (1 ml) were withdrawn at the times indicated for determination of cell replication (a) and cell diameter (b). Samples (2 ml) were withdrawn at the indicated times for [3 H]thymidine labelling to assess entry of cells into S phase (c). In the Figure, [3 H]thymidine incorporation has been corrected for non-specific precipitation. Each point represents the mean of two determinations.

actions because, in a serum-free medium [15], the number of replications (see the Experimental section) of Nb2 cells exposed to 5 ng of oPRL/ml alone (1.85 ± 0.02 ; S.E.M., $n = 3$) was significantly greater ($P < 0.01$) than that of cells exposed to oPRL + 5 ng of TGF- β /ml (1.26 ± 0.03 , $n = 3$). Although serum factors do not appear to be required for TGF- β -induced inhibition, it cannot be discounted, however, that factors secreted by Nb2 cells play a role.

Fig. 3. Effect of prostaglandins on Nb2 cell proliferation

The effect of prostaglandins on oPRL- and IL-2-induced Nb2 cell proliferation is shown with PGF_{2α} (▨), PGE₁ (■) and PGE₂ (□) at concentrations of 1 and 10 μM. Growth-arrested Nb2 cells were resuspended in fresh assay medium (1 × 10⁵ cells/ml) and dispensed into the wells of 24-well culture plates. The cells were exposed to oPRL (5 ng/ml) or IL-2 (40 half-maximal units/ml) with or without prostaglandins for 24 h. The error bars indicate ± S.E.M. (*n* = 3).

The effects of TGF- β on lactogen-induced changes in cell proliferation, cell size and entry into S phase were examined in time-course studies. TGF- β consistently inhibited lactogen-induced cell proliferation in ten separate time-course studies, but it never inhibited any growth responses during the first cell cycle. In the representative experiment shown in Fig. 2, the inhibitory effects of TGF- β , which included delayed cell replication and decreased cell size, were first observed during the second cell cycle. [³H]Thymidine incorporation was monitored only during the first cell cycle, because some synchrony was lost after this time and it was difficult to predict the timing of TGF- β inhibition. The timing of TGF- β -induced inhibition was variable, because in some experiments these inhibitory effects were not observed before the fourth cell cycle. The factors regulating the timing of TGF- β -induced inhibition are unknown at present; however, delayed expression of the inhibitory effects of TGF- β accounts for the incomplete inhibition observed in the 3-day proliferation studies (see above).

Inhibitory effects similar to those obtained with TGF- β could be produced with prostaglandins. Nb2 cell proliferation in response to oPRL and IL-2 was inhibited by PGE₁ and PGE₂ (Fig. 3), but no inhibition was observed with PGE_{2α} at the same concentration. These data are in agreement with earlier studies [8,10,11] which showed that prostaglandins of the E series at a similar concentration inhibit T-lymphocyte proliferation. PGE₂ was chosen for use in subsequent studies because it was active in inhibiting proliferation in response to oPRL and IL-2, and PGE₂ has been shown to be produced by T lymphocytes [9]. As observed with TGF- β , PGE₂ inhibited the mitogen-induced increase in cell size and in cell number (Fig. 4). A decrease in cell size in response to PGE₂ (Fig. 4b) was observed within 4 h, and cells exposed to PGE₂ never attained the maximal size reached by cells exposed to mitogen alone. At least a

Fig. 4. Time course of the effects of PGE₂ on oPRL-stimulated Nb2 cell growth

Growth-arrested Nb2 cells were resuspended in fresh assay medium (1 × 10⁵ cells/ml) and maintained in spinner flasks. At zero time, the cells were exposed to oPRL (10 ng/ml; ○) or oPRL + PGE₂ (20 μM; △). Cell replication (a), cell diameter (b) and [³H]thymidine incorporation (c) were determined as described in the Experimental section. At 28 h (arrow), half of the cells exposed to oPRL + PGE₂ were washed and resuspended in fresh assay medium containing only oPRL (▲). Each point represents the mean of two determinations.

portion of the cells treated with oPRL plus PGE₂ entered S phase, as determined by [³H]thymidine incorporation (Fig. 4c); however, entry occurred later and the rate of [³H]thymidine incorporation was lower. It is possible that the decreased [³H]thymidine incorporation in response to PGE₂ reflects effects on thymidine uptake or differential labelling of intracellular pools; however, the decrease in [³H]thymidine incorporation is consistent with the minimal cell division observed over a period of 60 h. PGE₂ did not decrease the viability of the cells, because cell division resumed when PGE₂ was removed from the medium (Fig. 4a).

Fig. 5. Indomethacin reverses the inhibitory effects of TGF- β on oPRL-stimulated Nb2 cell growth

Growth-arrested Nb2 cells were resuspended in fresh assay medium (1×10^5 cells/ml) and maintained in spinner flasks. At zero time, the cells were exposed to oPRL (10 ng/ml; ○) or oPRL + TGF- β (5 ng/ml; △). At 52 h (arrow), indomethacin (2 μ M) was added to half of the cells exposed to oPRL + TGF- β (▲). Each point represents the mean of two determinations.

The similar effects of TGF- β and PGE₂ on Nb2 cell proliferation and size are consistent with the possibility that prostaglandins are involved in the actions of TGF- β . Thus we examined the ability of indomethacin, an inhibitor of prostaglandin synthesis, to mitigate the inhibitory actions of TGF- β . The concentration of indomethacin used in these studies was similar to those effective in decreasing prostaglandin synthesis in a T-cell line [9] and to those used to inhibit bone resorption in response to TGF- β [7]. Indomethacin completely reversed the inhibitory effects of TGF- β on oPRL-stimulated Nb2 cell proliferation (Fig. 5). Within 2–4 h after addition of indomethacin, TGF- β -treated cells proliferated at a rate indistinguishable from that of cells exposed to oPRL alone (Fig. 5). In contrast, the rate of proliferation of cells exposed to oPRL plus TGF- β in the absence of indomethacin was virtually zero. The addition of indomethacin to cells exposed to oPRL had a slight inhibitory effect on the rate of cell proliferation (results not shown), which is in agreement with results obtained in a previous report [17] in which indomethacin was used at a similar concentration. Inhibition of prostaglandin synthesis by indomethacin may shunt more substrate to the lipoxygenase pathway, but augmentation of this pathway in TGF- β -treated cells is likely of little importance, inasmuch as inhibitors of the lipoxygenase pathway have little effect on Nb2 cell proliferation [17].

Our data show that TGF- β inhibits Nb2 cell proliferation in response to both lactogens and IL-2, and that IL-2-induced proliferation is more sensitive to the actions of TGF- β . TGF- β inhibited proliferation in the absence and in the presence of serum; thus the actions of TGF- β are not dependent on serum factors. TGF- β did not inhibit the mitogenic actions of lactogens or IL-2 during the first round of cell division, but rather the 'growth factor' inhibited the second or subsequent cell cycles.

Furthermore, the data indicate that the immunosuppressive actions of TGF- β require ongoing prostaglandin synthesis, perhaps synthesis of PGE₁ or PGE₂. This possibility is consistent with previous studies showing that PGE₂ may mediate the bone-resorbing actions of TGF- β [7] and that inhibition of prostaglandin synthesis is an important step in lymphocyte activation [9]. In view of the delay in expression of the actions of TGF- β , it would seem that the anti-proliferative actions of TGF- β must entail more than a direct activation of prostaglandin synthesis. The mechanism(s) of action of TGF- β require investigation.

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