Transforming Growth Factors-β1 and β2 Are Mitogens for Rat Schwann Cells

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Abstract. Transforming growth factor-β1 (TGF-β1) and TGF-β2 were found to be potent mitogens for purified rat Schwann cells, each stimulating DNA synthesis in quiescent cells and also increasing their proliferation rate. Half-maximal stimulation of DNA synthesis occurred at ~0.1 ng/ml TGF-β1 or TGF-β2. Mitogenic stimulation by TGF-β1 and TGF-β2 was enhanced by forskolin, which activates adenylyl cyclase, at concentrations up to 0.5 μM forskolin. However, at 5 μM forskolin, the synergistic interaction between forskolin and TGF-β1 was abolished. These results are in contrast to the observed synergy between forskolin and another Schwann cell mitogen, glial growth factor (GGF). Both 0.5 and 5 μM forskolin were found to enhance the stimulation of DNA synthesis by partially purified GGF (GGF-CM). As well as being functionally distinct, TGF-β1 and GGF-CM activities were also physically separable by chromatography on a Superose 12 gel permeation column. Thus, TGF-β1 and β2 are rat Schwann cell mitogens, and Schwann cells are one of the few normal cell populations to respond mitogenically to TGF-β.

Schwann cells are the glial, or “support” cells of the peripheral nervous system. During embryogenesis, they migrate along growing nerve axons, proliferate, and synthesize a basal lamina consisting predominantly of laminin, collagen, and proteoglycans (Timpl and Martin, 1981). Eventually they ensheathe the axons, and myellinate larger axons (Webster and Favilla, 1984). In adult animals, Schwann cells play an important role in nerve regeneration after wounding, by reentering the cell division cycle, replacing damaged Schwann cells, and synthesizing a new basal lamina and myelin (Salzer and Bunge, 1880). In addition, the original basal lamina provides a directional “tube” along which new axons can grow (Aguayo and Roberts, 1984).

The factors controlling Schwann cell proliferation during embryogenesis and wound healing in vivo are unknown, although several mitogens have been identified in vitro. The best characterized Schwann cell mitogen is glial growth factor (GGF),1 which has been purified to homogeneity from bovine pituitary glands (Lemke and Brockes, 1984). A GGF-like factor has also been identified in human tumors of Schwann cell origin (Brockes et al., 1986), suggesting that it may play a role as a growth factor in tumorigenesis. Glia maturation factor is also a Schwann cell mitogen (Bosch et al., 1984), and basic fibroblast growth factor has been reported to be weakly mitogenic (Ratner et al., 1988); whereas many other soluble polypeptide growth factors, including platelet-derived growth factor, epidermal growth factor, and nerve growth factor, have been tested and found to be non-stimulatory for rat Schwann cells (Raff et al., 1978a; Lemke and Brockes, 1984; for review, see Ratner et al., 1986). Rat Schwann cells are stimulated to proliferate by neurites in serum-free medium in the absence of added factors (Salzer and Bunge, 1980). This mitogenic activity is believed to reside on the neurite cell surface, since axolemmal and PC12 membrane fractions stimulate Schwann cell proliferation (Ratner et al., 1986, and references therein); it can be dissociated from membranes, and binds heparin (Ratner et al., 1988; DeCoster and DeVries, 1989), but the mitogen has not been identified or purified. A mitogenic activity for Schwann cells is also secreted by macrophages that have phagocytosed myelin-enriched membrane fractions, and it has been suggested that this could contribute to the stimulation of Schwann cell proliferation during nerve regeneration (Baichwal et al., 1988). The extracellular matrix components laminin and fibronectin also induce DNA synthesis in Schwann cells in vitro (Baron-Van Evercooren et al., 1982; McGarvey et al., 1984), but it is not clear whether they are directly mitogenic, or whether they act by binding and presenting growth factors (Porter et al., 1987). Finally, agents that increase intracellular cAMP levels, such as cholera toxin and forskolin, are mitogenic for Schwann cells, and synergize with GGF-CM (partially purified GGF) to stimulate proliferation (Raff et al., 1978b; Porter et al., 1986). However, neither GGF nor mitogenic membrane fractions have been shown to act by elevating intracellular cAMP levels (Raff et al., 1978a; Meador-Woodruff et al., 1984).

1. Abbreviations used in this paper: GGF, glial growth factor; TGF-β, transforming growth factor-β; [3H]UdR, [125I]iododeoxyuridine.
lieved to be involved in development, wound healing and tumorigenesis in vivo (for review, see Roberts et al., 1988). Most cell types express TGF-β receptors (Wakefield et al., 1987), but the action of TGF-β on cell proliferation in culture varies from inhibition to stimulation, depending on cell type, growth conditions, and the growth factors present (for review, see Moses et al., 1985). Although osteoblasts are mitogenically stimulated by TGF-β (Centralla et al., 1987; Robey et al., 1987), it inhibits the proliferation of most normal cell populations (Sporn et al., 1987).

To investigate the role of TGF-β in Schwann cell development and tumorigenesis, it is important to establish what action, if any, TGF-β has on the growth of Schwann cells. We have shown that both TGF-β1 and TGF-β2 are potent mitogens for rat Schwann cells in vitro, and that the activity of TGF-β can be clearly distinguished from GGF-CM activity.

Materials and Methods

Materials

The following sources and materials were used: tissue culture plastics (Costar, Cambridge, MA); batch-tested FCS (Imperial Laboratories Ltd., Salisbury, England); poly-0·1%)-lysine hydrobromide (relative molecular mass of 30-70 kD) and rabbit complement (Sigma Chemical Co., St. Louis, MO); DME, 2·5% trypsin in PBS, and 0·05% trypsin/0·02% EDTA in modified Puck’s saline (Gibco Laboratories, Grand Island, NY); collagenase (type 1) (Worthington Biochemical Corp., Freehold, NJ); forskolin (Calbiochem-Behring Corp., San Diego, CA); anti-Thy-1.1 IgM (Serotec, Oxford, England); [125I]2-iododeoxyuridine (Amersham Corp., Arlington Heights, IL); highly purified porcine platelet-derived TGF-β1, TGF-β2, and purified rabbit anti-TGF-β polyclonal IgG (R&D Systems, Minneapolis, MN).

A crude preparation of GGF was prepared according to the methods of Lemke and Brockes (1984). Briefly, frozen bovine pituitaries (Northeast Biomedical Laboratories, Uxbridge, England) were homogenized and a protein precipitate collected at a concentration of between 40-70% ammonium sulphate. The ammonium sulphate precipitate was diluted and solubilized, filtered and concentrated on a 10-kD tangential flow filter, dialedyzed into 0·1 M sodium phosphate (pH 6·0), loaded onto a CM-cellulose column, washed with 0·05 M sodium chloride/0·1 M sodium phosphate (pH 6·0), and eluted with 0·2 M sodium chloride/0·1 M sodium phosphate (pH 6·0). The active fraction (known as GGF-CM), 0·2-0·3 mg protein/ml, was pooled and stored at -20°C.

Schwann Cell Culture

Schwann cells from rat sciatic nerves were isolated using the method of Brockes et al. (1979). Briefly, sciatic nerves were dissected from rat pups, dissociated with 0·1% collagenase and 2·5% trypsin in DME and plated onto poly-lysine-coated tissue culture flasks in DME containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (PS). After 2-4 d the medium was replaced with fresh medium containing 10 μM 1-β-D-arabinofuranosylcytosine. The cells were washed 3-4 d later, and fed with DME containing 10% FCS, 5 μg/ml forskolin, and 0·5 μg/ml GGF-CM. Once confluent, 2-3 d later, the cells were detached with 0·05% trypsin/0·02% EDTA in modified Puck’s saline, washed, and treated with mouse monoclonal anti-Thy-1.1 antibody and rabbit complement for 30 min to remove contaminating fibroblasts. Schwann cells prepared by this method have been shown to be 99·5% pure as determined by immunofluorescent staining for N-CAM, lamin B and collagen type IV (see also Ridley et al., 1988). Schwann cells were grown on poly-lysine-coated tissue culture plastic (poly-lysine was necessary to maintain long-term proliferation of Schwann cells in culture; see Porter et al., 1986) in DME containing 10% FCS, 5 μg/ml forskolin, 0·5 μg/ml GGF-CM, and passed every 4-7 d as described above.

Measurement of Schwann Cell Proliferation

First passage Schwann cells were seeded onto poly-lysine-coated 24-well dishes (Falcon Labware, Oxnard, CA) at 104 cells/well, in DME containing 3% FCS, 1 μg/ml forskolin, and 0·5 μg/ml GGF-CM. After 48 h, cells were washed with DME and 3% FCS/DME containing the appropriate concentrations of TGF-β1, TGF-β2, or forskolin were added. Cells were fed every 2 d. To count cells, triplicate wells were trypsinized and counted with a Coulter counter.

Assay of Schwann Cell DNA Synthesis

The method for measuring DNA synthesis was based on that described by Raff et al. (1978a). Cells were cultured in the absence of GGF-CM or forskolin for 2-3 d, then seeded in 96-well plates (Costar) at 10,000 cells/well in 100 μl/well DME, 10% FCS. 16-24 h later, growth factors, aliquots from column fractions, and forskolin were added to the wells. GGF-CM, TGF-β and forskolin were serially diluted in DME and added to give a constant volume of 120 μl. A 1-2 μg/ml TGF-β stock was prepared in 4 mM HCl, 1 mg/ml BSA (fatty acid free), and a 5-mM forskolin stock was prepared in DMSO. 5 μl of each column fraction was added per well, using column running buffer as a control. The cells were then incubated for 48 h, 0·2 μCi/well [125I]2-iododeoxyuridine being added for the last 24 h. Medium was aspirated, cells detached using 0·05% trypsin/0·02% EDTA in modified Puck’s saline, harvested onto glass fiber filter mats, washed with H2O followed by ethanol, and incorporated radioactivity was counted.

Column Chromatography

GGF-CM and TGF-β were diluted into 50 mM sodium phosphate (pH 6·0), loaded onto a Mono S HR 5/5 cation exchange column (Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with a 40 ml/40 min linear gradient up to 2 M sodium chloride/50 mM sodium phosphate (pH 6·0) buffer. Fractions were collected into tubes containing 100 μl BSA in PBS, as carrier protein, giving a final concentration of 0·1 mg/ml BSA.

Gel permeation was carried out on a Superose 12 HR 10/30 column (17·038-01; Pharmacia Fine Chemicals) equilibrated and run in 50 mM sodium phosphate, 750 mM sodium chloride (pH 6·0) (running buffer). The column was calibrated using bovine thyroglobulin, bovine gamma globulin, chicken ovalbumin, horse myoglobin, and vitamin B-12 standards (Bio-Rad Laboratories, Cambridge, MA). 100 ng of TGF-β1 and 100 μg GGF-CM samples were run through the column separately, or together, with 35 μl of running buffer, and collected in the presence of 0·1 mg/ml carrier BSA. 5 μl samples of each fraction were assayed for Schwann cell mitogenic activity, in the presence of 0·5 or 5 μg/ml forskolin.

Results

TGF-β1 and β2 Are Mitogenic for Schwann Cells

To test the mitogenic response of rat Schwann cells to TGF-β, serial dilutions of TGF-β1 and TGF-β2 were assayed for their ability to stimulate DNA synthesis. Highly purified porcine platelet TGF-β1 and TGF-β2 were both mitogenic for quiescent Schwann cells, as determined by incorporation of [125I]2-iododeoxyuridine ([125I]UdR) (Fig. 1 a). Half-maximal stimulation of DNA synthesis was obtained with TGF-β1 at 0·1 ng/ml, and with TGF-β2 at 0·07 ng/ml. Although the background DNA synthesis varied considerably between different experiments, TGF-β1 consistently induced a 5-10-fold stimulation of DNA synthesis in six independent experiments. The mitogenic action of both the TGF-β1 and TGF-β2 preparations was inhibited by purified rabbit anti-TGF-β polyclonal IgG (Fig. 1 b); 50 μg/ml IgG completely blocked the stimulation of DNA synthesis induced by 1 ng/ml TGF-β1, and 10 μg/ml neutralized the action of 1 ng/ml TGF-β2. Similar concentrations of this anti-TGF-β IgG neutralized the action of TGF-β both as a mitogen for AKR-2B cells (Keski-Oja et al., 1987), and as an inhibitor of DNA synthesis in mink lung epithelial cells (as reported by R & D Systems); thus mitogenic stimulation of Schwann cells was likely to be specifically induced by TGF-β molecules in the purified preparations, and not by contaminants. In addition, 50 μg/ml of anti-TGF-β IgG had no effect on the mitogenic activity of partially purified GGF (GGF-CM) obtained from
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bovine pituitary glands (data not shown); it is therefore unlikely that TGF-β and GGF share the common epitope that this antibody recognizes on TGF-β molecules.

As well as stimulating DNA synthesis, TGF-β1 and TGF-β2 also stimulated the proliferation rate of Schwann cells, as measured by increase in cell number over 9 d (Fig. 2). At 1 ng/ml TGF-β1 or TGF-β2, the doubling time in the exponential phase of growth was ~4 d, whereas the doubling time of cells without added TGF-β over the same period was longer than 8 d. This concentration of TGF-β is in the same range as that required to stimulate the growth of osteoblasts (Centralla et al., 1987), and of normal rat kidney fibroblasts in soft agar (Anzano et al., 1983).

TGF-β1 Synergizes with Forskolin in Stimulating Schwann Cell Proliferation

The Schwann cell mitogenic activity in GGF-CM acts synergistically with agents that increase intracellular cAMP levels to stimulate proliferation (Raff et al., 1978; Porter et al., 1986). To establish whether TGF-β action could also be enhanced by increasing cAMP levels, the mitogenic activity of TGF-β was tested in combination with forskolin, a diterpene drug that raises the intracellular cAMP concentration by stimulating adenylate cyclase (Seamon and Daly, 1981). While forskolin alone stimulated DNA synthesis only very slightly, in combination with TGF-β it synergistically increased the induction of DNA synthesis (Fig. 3 a). However, this activity was highly dependent on the concentration of forskolin present. Synergy between TGF-β1 and forskolin was detectable at concentrations as low as 0.005 μM forskolin, and maximal stimulation of the TGF-β1 response occurred at 0.5 μM forskolin. In contrast, 5 μM forskolin did not stimulate or slightly suppress the TGF-β1-induced DNA synthesis response in three independent experiments. For example, 0.25 ng/ml TGF-β1 induced an approximately fivefold stimulation of DNA synthesis in the absence of forskolin, whereas in 5 μM forskolin DNA synthesis was increased only approximately threefold (Fig. 3 a). This pattern of response to different forskolin concentrations was seen at both suboptimal and optimal concentrations of TGF-β1 (Fig. 3 b).

The proliferation rate of Schwann cells in TGF-β1 and TGF-β2 was also enhanced by forskolin, as shown in Fig. 2. Forskolin at 0.5 μM synergized with both TGF-β1 and TGF-β2 in stimulating Schwann cell proliferation, decreasing the doubling time during exponential growth from 4 to 2 d.

The critical dependence of the TGF-β mitogenic response on forskolin concentration contrasts with the synergy observed between GGF-CM and forskolin. In the presence of both suboptimal and optimal mitogenic concentrations of GGF-CM, 5 μM forskolin was found to be more stimulatory than 0.5 μM forskolin (Fig. 3 c). The responses of rat Schwann cells to GGF-CM and TGF-β1 can thus be distinguished on the basis of their synergistic action with forskolin, suggesting that these two mitogenic activities act via different mechanisms. Since the action of a third Schwann cell mitogen, glia maturation factor, is not enhanced by increased intracellular cAMP levels (Bosch et al., 1984), the synergism of TGF-β1 with forskolin also distinguishes it from glia maturation factor.

Separation of TGF-β1 and GGF-CM Activities by Column Chromatography

The results above provide a biological assay for distinguishing TGF-β from the Schwann cell mitogenic activity in GGF-CM, through their differential mitogenicity in combination with forskolin on Schwann cells. However, to distinguish the two activities unambiguously on a biochemical level, and to
Figure 3. DNA synthesis in Schwann cells in the presence of forskolin and either TGF-β1 or GGF. (a) Schwann cells were exposed to serial dilutions of forskolin in the presence of 0 ng/ml TGF-β1 (○), 0.0025 ng/ml TGF-β1 (■), 0.025 ng/ml TGF-β1 (▲), or 0.25 ng/ml TGF-β1 (△). Cells were also incubated with serial dilutions of TGF-β1 (b), or GGF-CM (c), in the presence of 0 μM forskolin (○), 0.05 μM forskolin (△), 0.5 μM forskolin (▲), or 5 μM forskolin (■). Induction of DNA synthesis was measured by incorporation of [3H]TdR. Values represent the mean of duplicate determinations, which differed by <10% from the mean. Similar results were obtained in two independent experiments.

Discussion

TGF-β can act either as an inhibitor or stimulator of cell proliferation, depending on both cell type and growth conditions (for review, see Moses et al., 1985). Since the responsiveness of a cell type to growth stimulatory and inhibitory factors can alter upon expression of oncogenes and transformation (Armelin et al., 1984; Stern et al., 1986; Jetten et al., 1986), it is important to study the behavior of normal cells towards factors to understand their potential roles in development, regeneration, and disease. Our results demonstrate that both TGF-β1 and TGF-β2 are mitogens for normal rat Schwann cells in vitro. Of other normal cell populations that have been studied, osteoblasts are one of the few to be stimulated by TGF-β (Centralla et al., 1987; Robey et al., 1987). In contrast, TGF-β inhibits the proliferation of most normal cell types, including keratinocytes, B and T lymphocytes, bronchial epithelial cells and endothelial cells (Sporn et al., 1987, and references therein). It is thus important to have identified Schwann cells as another cell type for which TGF-β mitogenic activity was detected in the Schwann cell DNA synthesis assay with 0.5 μM forskolin, and gave greater activity with 5 μM forskolin, thus displaying the same properties as GGF-CM. The second activity was detected in the presence of 0.5 μM forskolin, but not with 5 μM forskolin, and was therefore identified as TGF-β1. This separation method should allow the detection and identification of TGF-β-like factors, in tumor samples and in conditioned medium from cell lines, which stimulate Schwann cell proliferation.

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is a mitogen to further our understanding of the molecular basis for its pleiotropic actions.

TGF-β1 acts synergistically with forskolin, which stimulates adenylate cyclase, to stimulate Schwann cell proliferation, and experiments are in progress to investigate its action in the presence of other Schwann cell mitogens. In addition, it is possible that serum factors, or autocrine factor(s) secreted by Schwann cells (Porter et al., 1987), may synergize with TGF-β. Preliminary evidence suggests that TGF-β induces DNA synthesis 8-9 h later than GGF-CM in quiescent Schwann cells (J. Davis, unpublished data). One explanation for this delayed action of TGF-β could be that it stimulates the Schwann cells to secrete an autocrine growth factor, for example GGF or glia maturation factor (Bosch et al., 1984; Lim et al., 1988). Such an action for TGF-β has been described in the mouse cell line AKR-2B, where TGF-β stimulates secretion of a PDGF-like factor, which acts as a direct mitogen (Leof et al., 1986).

TGF-βs are a group of closely related polypeptides, of which four members have been cloned (Derynck et al., 1985; Madisen et al., 1988; ten Dijke et al., 1988; Derynck et al., 1988; Jakowlew et al., 1988). The two factors TGF-β1 and TGF-β2 have been best characterized, and are 71% homologous in amino acid sequence. Little is known about possible differential functions of the two factors in vivo, but a few studies have directly compared the actions of TGF-β1 and TGF-β2 in vitro. In adipocytes, both inhibit expression of differentiation-specific genes (Cheifetz et al., 1987), whereas DNA synthesis in aortic endothelial cells is strongly inhibited by TGF-β1 but not TGF-β2 (Jennings et al., 1988). We have shown that both TGF-β1 and TGF-β2 stimulate rat Schwann cell DNA synthesis and proliferation with similar potency.

Since TGF-β is a mitogen for Schwann cells, it is possible that autocrine secretion of TGF-β could be involved in the development of Schwann cell tumors. Many cell types have been shown to secrete TGF-β (Assioian et al., 1985), most of which is in an inactive, latent form that can be activated by proteolysis or acid treatment (Lawrence et al., 1984; Lyons et al., 1988). As nearly all cell types express TGF-β receptors (Wakefield et al., 1987), it has been postulated that activation of latent TGF-β could be important in controlling TGF-β action in vivo (Roberts et al., 1988). Schwann cells which secreted TGF-β, and were able to activate it, would stimulate their own proliferation, thus escaping the requirement for exogenous mitogenic factors. Northern analysis has shown that rat Schwann cells contain TGF-β mRNA (A. Ridley, unpublished data), thus it is probable that normal Schwann cells in culture synthesize latent TGF-β. The ability to activate TGF-β may be linked to the transformation process, and could play a crucial role in the development of a tumorigenic phenotype. A Schwann cell population has been isolated which grows in the absence of added growth factors in vitro (Porter et al., 1987). These cells produce a Schwann cell mitogen, and are tumorigenic in nude mice (Langford, et al., 1988). Transformed Schwann cells expressing the oncogenes SV40 large T and v-Ha-ras can also proliferate in the absence of added mitogens (Ridley et al., 1988). It is possible that TGF-β could be acting as a autocrine factor in both these cases. In addition, a number of tumors of Schwann cell origin contain polypeptide factor(s) mitogenic for normal Schwann cells (Brookes et al., 1986), and it was suggested that these tumors might secrete GGF. It should now be possible to identify whether TGF-β is a major mitogen present in Schwann cell tumor extracts, both by testing for Schwann cell mitogenic activity in combination with forskolin, and also by using neutralizing antibodies to TGF-β. In addition, the nature of the Schwann cell mitogen(s) in neurile membranes is not known (Ratner et al., 1986; DeCoster and DeVries, 1989), and a possible relationship to either GGF or TGF-β could also be investigated by these methods.

Aside from its effects on cell proliferation, TGF-β can induce or enhance the formation of extracellular matrix in many cell types (Sporn et al., 1987), and there is some evidence that this action could be involved in wound healing in vivo (Roberts et al., 1988). In vitro, it stimulates the transcription of both the collagen and fibronectin genes in normal rat kidney cells (Igniotz et al., 1987). In addition, it inhibits the degradation of newly formed matrix proteins, both by increasing the synthesis of protease inhibitors, and by decreasing the secretion of certain proteases (Sporn et al., 1987). Schwann cells are highly specialized for the synthesis of extracellular matrix, and secrete collagen type IV, laminin, and heparan proteoglycans that form the basal lamina around nerve axons (Timpl and Martin, 1981). It will be important to determine whether TGF-β enhances extracellular matrix production by Schwann cells in vitro, and whether it can stimulate expression of myelin-specific proteins such as P0 and myelin basic proteins (Lemke, 1986). Schwann cells present a good model for studying TGF-β action, since the proliferation and differentiation of purified Schwann cells can be studied in vitro. TGF-βs could play a specific role in controlling Schwann cell function in vivo, both stimulating proliferation and concomitantly promoting synthesis of differentiation-specific extracellular matrix molecules. Such a role would be relevant to peripheral nerve regeneration after wounding, and also to the development of nerve tracts during embryogenesis.

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