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BSC-1 growth inhibitor transforms a mitogenic stimulus into a hypertrophic stimulus for renal proximal tubular cells: Relationship to Na^+/H^+ antiport activity

(transforming growth factor β/insulin/hydrocortisone/amiloride)

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ABSTRACT **Renal hypertrophy is characterized by an increase in cell size and protein content with minimal hyperplasia. The mechanisms of control of this pattern of cell growth have not been determined. The present studies examined whether the growth inhibitor elaborated by BSC-1 kidney epithelial cells (GI), which has nearly identical biological properties to transforming growth factor β (TGF-β), could transform a mitogenic stimulus into a hypertrophic stimulus for rabbit renal proximal tubular cells in primary culture. Insulin (10 μg/ml) plus hydrocortisone (50 nM) increased the amount of protein per cell, cell volume, and [^{3}H]thymidine incorporation at 24 and 48 hr in these cells. GI/TGF-β (10 units/ml) led to a minimal stimulation of [^{3}H]thymidine incorporation. When added together with insulin plus hydrocortisone, GI/TGF-β inhibited the stimulatory effect of these mitogens on [^{3}H]thymidine incorporation but did not block the increase in protein per cell and cell volume—i.e., the cells underwent hypertrophy. The fact that this pattern persisted for 48 hr indicated that GI/TGF-β exerted a prolonged inhibitory effect on mitogenic-stimulated DNA synthesis rather than delaying its onset. Amiloride-sensitive Na^+ uptake (indicative of Na^+/H^+ antiport activity) correlated with protein per cell and cell volume rather than with DNA synthesis. P60 gel chromatographic fractionation of conditioned medium harvested from proximal tubular cells yielded a fraction that inhibited [^{3}H]thymidine incorporation in BSC-1 cells and CCL 64 cells; the relative inhibitory activity on these cell lines and the chromatographic behavior were similar to those observed with GI/TGF-β. These studies indicate that the control of cell size may be regulated by autocrine mechanisms mediated by the elaboration of growth inhibitory factors that alter the pattern of the growth response to mitogens.**

Renal hypertrophy is characterized by an increase in cell size and protein content with only minimal hyperplasia (1). Why the kidney should demonstrate a hypertrophic rather than a mitogenic response to growth stimuli *in vivo* is unclear, but the possibility exists that a factor produced by the cell modifies its response to growth stimuli that otherwise would cause cell proliferation. When BSC-1 (African green monkey kidney) cells are grown to confluence, they produce a polypeptide growth inhibitor (GI), which causes G_1 arrest of these cells at low density (2, 3). This growth inhibitor has been shown to have nearly identical biological activity with type β transforming growth factor (TGF-β) derived from human platelets, in that it competes for binding to the same receptor and either stimulates or inhibits proliferation, depending on the experimental conditions, in a manner similar to TGF-β (4). Both TGF-β and GI inhibit thymidine incorporation into BSC-1 epithelial cells in a dose-dependent manner (4), and TGF-β has been shown to prolong cell-cycle time in NRK fibroblasts (5). The possibility that this transforming growth factor plays a role in determining the growth pattern of kidney cells with regard to cell size has not been investigated.

We have recently shown that hypertrophy of renal proximal tubular cells in primary culture can be achieved by exposure of the cells to single growth factors (e.g., insulin, prostaglandin E_1, hypertonic NaCl), whereas combinations of growth factors (e.g., insulin with hydrocortisone) are mitogenic in this system (6). Although a number of studies have suggested that the mitogenic response is associated with an increase in Na^+/H^+ antiport activity (7–13), recent evidence indicates that these events may be dissociated (6, 14–16). We have reported an early increase in Na^+/H^+ antiport activity in association with *in vitro* hypertrophy of rabbit renal proximal tubular cells (6), suggesting that stimulation of this transport process may be associated with growth in cell size rather than initiation of DNA synthesis.

In this paper, we examine the effects of GI/TGF-β on the response of proximal tubular cells to the mitogenic stimulus of insulin with hydrocortisone. This combination of growth factors increases protein content (protein/cell), DNA synthesis, and Na^+/H^+ antiport. We show that GI/TGF-β blocks the DNA stimulatory effect of insulin with hydrocortisone but allows protein/cell, cell volume, and Na^+/H^+ antiport to increase. Thus, cell hypertrophy rather than hyperplasia was associated with the increase Na^+/H^+ antiport. Furthermore, since the proximal tubular cells produced a growth inhibitor that inhibited the growth of BSC-1 cells, it is possible that they are able to regulate their size and total protein content in the presence of the appropriate growth stimuli by an autocrine mechanism.

MATERIALS AND METHODS

Materials were obtained from the following sources: bovine insulin and hydrocortisone were from Sigma, carrier-free $^{22}Na^+$ was from Amersham, and amiloride was from Merck Sharp & Dohme. GI/TGF-β was purified by high-performance liquid chromatography as described (3). One unit of activity was defined as the amount of inhibitor required to inhibit incorporation of [*methyl*-^{3}H]thymidine by 50% in 2- to 3-day cultures of BSC-1 cells in 5 ml of Dulbecco's modified Eagle's medium (DME medium) supplemented with 0.4 μg of biotin per ml and 0.1% calf serum. One unit is ≈4 ng of protein.

Primary cultures of rabbit proximal tubular cells (6, 17) were grown to confluence in DME/Ham's F-12 media (1:1)

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Abbreviations: GI, growth inhibitor produced by BSC-1 kidney epithelial cells; TGF-β, transforming growth factor β.

supplemented with human transferrin (1 μg/ml), bovine insulin (5 μg/ml), and hydrocortisone (50 nM) as described. The insulin and hydrocortisone were then removed for 48 hr to achieve growth arrest in G_0/G_1. Insulin (10 μg/ml) with hydrocortisone (50 nM) were then added for various times with or without GI/TGF-β. [^{3}H]Thymidine incorporation (2-hr pulse), protein/cell, and cell volume (by Coulter counter) were measured as described (6).

Na^+/H^+ antiport was measured as amiloride-sensitive Na^+ uptake (10 mM NaCl) into Na^+-depleted ouabain-treated cells using $^{22}Na^+$ as a tracer (6). Na^+ uptake was measured in the absence and presence of 0.1 mM amiloride.

To determine whether proximal tubular cells produce a growth inhibitor that inhibits DNA synthesis by BSC-1 cells, cells were grown to confluence, the medium was removed, and the cells were washed twice with DME medium/Ham's F-12 medium. Fresh medium (DME/Ham's F-12) containing transferrin (1 μg/ml) was then added for 48 hr and the medium was then harvested. One liter of conditioned medium was concentrated to 1 ml by ultrafiltration using a YM 10 membrane, and the concentrate was subjected to gel filtration on a 0.7 × 30 cm Bio-Gel P60 column (3). Fractions (1 ml) were collected and were assayed for protein and for growth inhibitory activity on BSC-1 cells and CCL64 cells as described (3, 18).

RESULTS

Effects of GI/TGF-β on [^{3}H]Thymidine Incorporation. The dose–response of GI/TGF-β on [^{3}H]thymidine incorporaion and cell count after 24 hr exposure in the absence and presence of insulin and hydrocortisone are shown in Fig. 1. GI/TGF-β alone resulted in minimal stimulation of DNA synthesis, but only at a concentration of 10 units/ml. In contrast, GI/TGF-β inhibited the stimulatory effect of insulin and hydrocortisone on [^{3}H]thymidine incorporation in a dose-dependent fashion with a maximal effect at 5–10 units/ml. There was no effect of these factors on cell count at 24 hr.

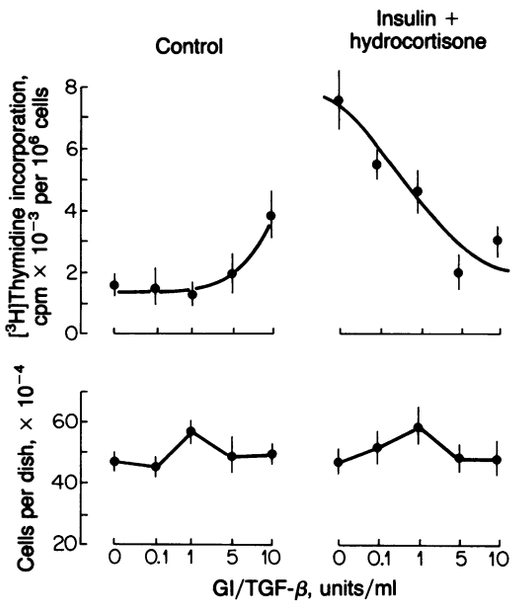

FIG. 1. Dose–response effect of GI/TGF-β on [^{3}H]thymidine incorporation and cell number in proximal tubular cells. GI/TGF-β was added for 24 hr in the absence (control) and presence of insulin (10 μg/ml) and hydrocortisone (50 nM). Confluent cells were maintained in DME medium/Ham's F-12 medium containing transferrin for 48 hr prior to the addition of these agents.

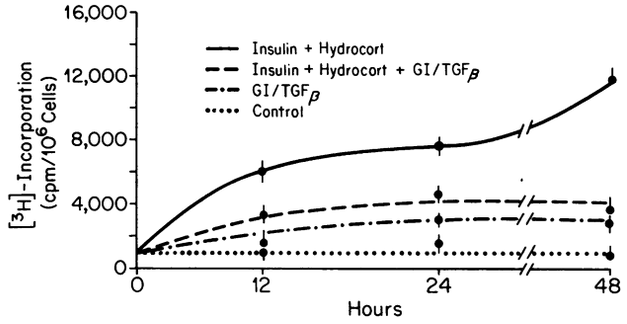

FIG. 2. Effect of GI/TGF-β (10 units/ml) with and without insulin and hydrocortisone on the time course of [^{3}H]thymidine incorporation.

Since TGF-β has been shown to increase the length of the cell cycle by prolonging G_1 (5), we examined the time course of GI/TGF-β on [^{3}H]thymidine incorporation at 0, 12, 24, and 48 hr in the presence and absence of insulin plus hydrocortisone. [Quiescent proximal tubular cells initiate DNA synthesis 10–12 hr after addition of this combination of growth factors (6).] In Fig. 2, it may be seen that the stimuatory effect of insulin plus hydrocortisone was inhibited at 12, 24, and 48 hr, indicating that GI/TGF-β exerted a prolonged inhibitory effect on DNA synthesis rather than delaying its onset.

Effects of GI/TGF-β on Protein/Cell and Cell Volume. The effects of GI/TGF-β on protein/cell were examined at 12, 24, and 48 hr in the absence and presence of insulin with hydrocortisone. Whereas blockade of stimulation of DNA synthesis by these growth factors was observed in the presence of GI/TGF-β at 24 and 48 hr, protein/cell increased significantly at both time points (Fig. 3). An increase in cell volume within 24 hr was also observed (Fig. 4).

Effects of GI/TGF-β on Na^+/H^+ Antiport. Uptake of 10 mM NaCl, using $^{22}Na^+$ as a tracer, was studied at 1 min or 3 min. As shown in Fig. 5, 2 hr exposure to GI/TGF-β alone had little or no effect on amiloride-sensitive Na^+ uptake. Amiloride-sensitive Na^+ uptake by quiescent cells was stimulated after 2 hr exposure to insulin and hydrocortisone; the addition of GI/TGF-β did not block this increased Na^+ uptake. The increased Na^+/H^+ antiport correlated with the increase in protein/cell and cell volume rather than with the rate of [^{3}H]thymidine incorporation.

Production of an Inhibitor of DNA Synthesis by Proximal Tubular Cells. Medium conditioned 48 hr by confluent proximal tubular cells was concentrated by ultrafiltration and the concentrate was fractionated by P60 gel chromatography. The ultraviolet absorption of the fractions at 280 nm indicated a peak of protein in fractions 3 and 4. Assays of aliquots of the fractions indicated growth inhibitory activity in fractions 5 and 6. Inhibition of [^{3}H]thymidine incorporation was 30% with BSC-1 cells and 97% with CCL 64 cells, using ≈40 ng of protein per ml. Thus, the chromatographic behavior and the relative activity of the proximal tubular cell-growth inhibitor on the two cell lines are similar to those observed with the BSC-1 cell-growth inhibitor, although the yield per liter of conditioned medium was much less (≈10%).

DISCUSSION

The recently described homology between the BSC-1 GI and TGF-β (4) has raised the possibility that this substance, which has been isolated from a variety of normal tissues including bovine kidney (19), human placenta (20), and human platelets (21), may act as an important regulator of cell growth. Both TGF-β (19–21) and the BSC-1 inhibitor (3) have been purified, and the amino acid composition and partial amino acid sequence of TGF-β have been determined (19–21). Release of

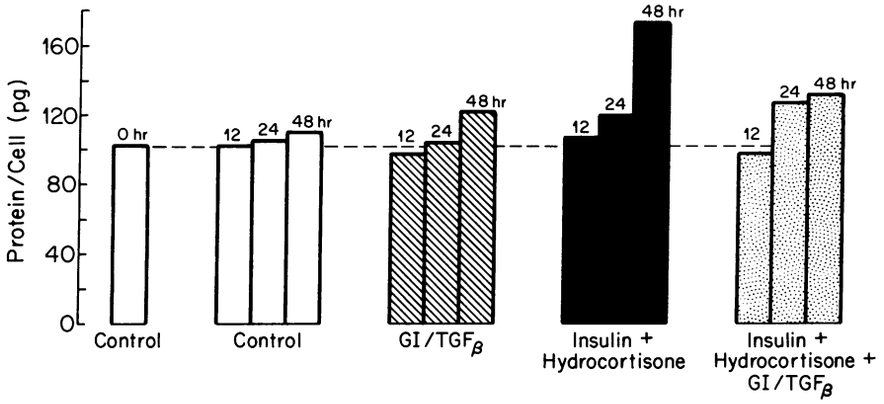

FIG. 3. Effect of GI/TGF-β (10 units/ml) on protein/cell in the absence and presence of insulin (10 μg/ml) and hydrocortisone (50 nM).

this substance into the medium could act by attaching to specific receptors on the same cells that produce it, leading to autocrine control of the pattern of cell growth.

The present study examined whether the combination of the BSC-1 growth inhibitor (GI/TGF-β) with a stimulus that is normally mitogenic to proximal tubular cells in primary culture (i.e., insulin plus hydrocortisone) could transform the pattern of growth stimulation from one of hyperplasia to one of hypertrophy. Confluent proximal tubular cells arrested in G_0/G_1 responded to high concentrations of GI/TGF-β alone with a small increase in thymidine incorporation but responded in the opposite manner when GI/TGF-β was added to the mitogenic combination of insulin plus hydrocortisone—i.e., stimulation of DNA synthesis was blocked. Of interest is the fact that protein/cell and cell volume increased at 24 and 48 hr. Thus, these cells conformed to the pattern of compensatory renal growth that occurs *in vivo*, in which situation protein/cell (or protein/DNA) increases with only a minor increase in cell number (1).

The role of increased Na^+/H^+ antiport in the process of cell proliferation has received a great deal of attention. Despite the associations that have been drawn between the mitogenic effects of growth factors, Na^+/H^+ antiport activity, and intracellular pH (7–13), evidence for a dissociation of these effects also exists (6, 14–16). Work with mutant fibroblasts lacking the Na^+/H^+ antiport demonstrates the importance of the antiport, but also shows that it is not needed if the cells are grown in a HCO_3^--containing medium (22). These experiments also show that increase in intracellular pH is necessary for mitogenesis (22). Selective inhibition of Na^+/H^+ antiport by a high-affinity analog of amiloride in BALB/c 3T3 cells fails to inhibit the mitogenic response to epidermal growth factor (EGF) (16). EGF stimulates cytoplasmic alkalinization in A431 cells via stimulation of Na^+/H^+ exchange without stimulation of mitogenesis (14) and Lys-bradykinin stimulates amiloride-sensitive Na^+ uptake in WI-38 cells without stimulation of DNA synthesis (15). Finally, we have recently reported that early stimulation of the Na^+/H^+ antiport occurs when cells hypertrophy *in vitro* in the absence of stimulation of DNA synthesis (6).

The finding that GI/TGF-β failed to block Na^+/H^+ antiport activity either alone or in combination with insulin and hydrocortisone is consistent with the observed lack of an effect of an impure preparation of the BSC-1 growth inhibitor on amiloride-sensitive Na^+ uptake by isolated rabbit renal brush border membrane vesicles (B. Sacktor and J. Kinsella, personal communication). Although GI/TGF-β has been shown to decrease the Na^+ content of BSC-1 cells (23), the effect observed here on basal-stimulated or insulin/hydro-

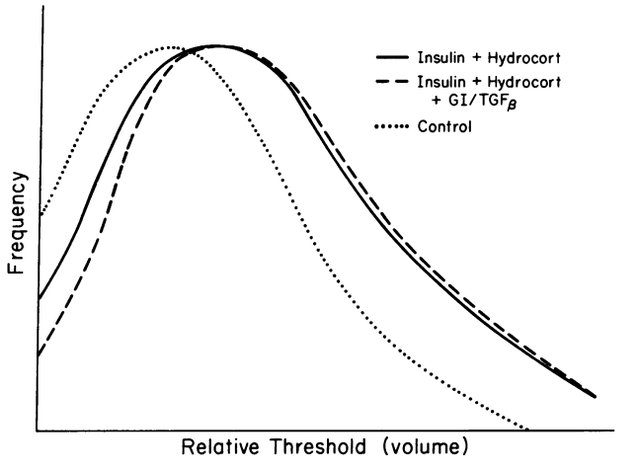

FIG. 4. Cell volume profiles of control cells and cells exposed to GI/TGF-β with or without insulin and hydrocortisone for 24 hr. Cell volume is plotted as relative threshold, with a right shift indicating an increase in cell volume.

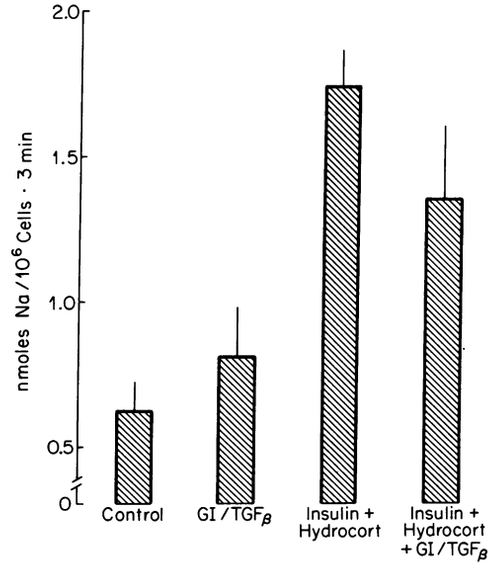

FIG. 5. Amiloride-sensitive Na^+ uptake of cells exposed for 2 hr to GI/TGF-β (10 units/ml) in the absence and presence of insulin (10 μg/ml) and hydrocortisone (50 nM). A 3-min uptake at 37°C was carried out (6) on Na^+-depleted oubain-treated cells by adding 5×10^5 cells in 20–80 μl of uptake buffer containing 10 mM NaCl, 110 mM Me_4NCl, 3.4 mM KCl, 17.7 μM Tris base, and 28.3 mM Hepes, and $^{22}Na^+$ (1 μCi per sample; 1 Ci = 37 GBq) with or without 0.1 mM amiloride.

cortisone-stimulated Na^+ influx (total or amiloride sensitive) was minimal.

Of interest was the dissociation between Na^+/H^+ antiport and thymidine incorporation in these studies. Insulin and hydrocortisone increased Na^+/H^+ antiport, protein/cell, and thymidine incorporation; addition of GI/TGF-β blocked stimulation of thymidine incorporation with little effect on the other two parameters. Thus, the previously described association of early stimulation of Na^+/H^+ antiport and a subsequent increase in cell size and protein content was also observed in the present situation.

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1. Johnson, H. A. & Vera Roman, J. M. (1966) *Am. J. Pathol.* **49,** 1–13.
2. Holley, R. W., Armour, R. & Baldwin, J. H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1864–1866.
3. Holley, R. W., Bohlen, P., Fava, R., Baldwin, J. H., Kleeman, G. & Armour, R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5989–5992.
4. Tucker, R. F., Shipley, G. D., Moses, H. L. & Holley, R. W. (1984) *Science* **226,** 705–707.
5. Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F. & Sporn, M. B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 119–123.
6. Fine, L. G., Badie-Dezfooly, B., Lowe, A., Hamzeh, A., Wells, J. & Salehmoghaddam, S. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1736–1740.
7. Moolenaar, W. H., Mummery, C. L., van der Saag, P. T. & de Laat, S. W. (1981) *Cell* **23,** 789–798.
8. Moolenaar, W. H., Yarden, Y., de Laat, S. W. & Schlessinger, J. (1982) *J. Biol. Chem.* **257,** 8502–8506.
9. Pouyssegur, J., Chambard, J. C., Franchi, A., Paris, S. & Van Obberrghen-Schilling, E. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3935–3939.
10. Schuldiner, S. & Rozengurt, E. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7778–7782.
11. Frelin, C., Vigne, P. & Lazdunski, M. (1983) *J. Biol. Chem.* **258,** 6272–6276.
12. Boonstra, J., Moolenaar, W. H., Harrison, P. H., Moed, P., van der Saag, P. T. & de Laat, S. W. (1983) *J. Cell Biol.* **97,** 92–98.
13. Moolenaar, W., Tsien, R., van der Saag, P. & de Laat, S. (1983) *Nature (London)* **304,** 645–648.
14. Rothenberg, P., Glaser, L., Schlesinger, R. & Cassel, D. (1983) *J. Biol. Chem.* **258,** 12644–12653.
15. Owen, N. E. & Villareal, M. L. (1983) *Cell* **32,** 979–985.
16. Besterman, J. M., Tyrey, S. J., Cragoe, E. J., Jr., & Cuatrecasas, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6762–6766.
17. Sakhrani, L. M., Badie-Dezfooly, B., Trizna, W., Mikhail, N., Lowe, A. G., Taub, M. & Fine, L. G. (1984) *Am. J. Physiol.* **246,** F757–F764.
18. Holley, R. W., Armour, R., Baldwin, J. H. & Greenfield, S. (1983) *Cell Biol. Intl. Reports* **7,** 141–147.
19. Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blacher, R., Pan, Y. C. E., Stein, S., Lehrman, S. R., Smith, J. M., Lamb, L. C. & Sporn, M. B. (1983) *Biochemistry* **22,** 5692–5698.
20. Frolick, C. A., Dart, L. L., Meyers, C. A., Smith, D. M. & Sporn, M. B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3676–3680.
21. Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M. & Sporn, M. B. (1983) *J. Biol. Chem.* **258,** 7155–7160.
22. Pouyssegur, J., Sardet, C., Franchi, A., L'Allemain, G. & Paris, S. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 4833–4837.
23. Walsh-Reitz, M. M., Toback, F. G. & Holley, R. W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 793–796.