Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 436-442

TRANSFORMING GROWTH FACTOR β INHIBITS DNA SYNTHESIS IN HEPATOCYTES ISOLATED FROM NORMAL AND REGENERATING RAT LIVER

A.J. Strain, A. Frazer, D.J. Hill, and R.D.G. Milner

Department of Pediatrics, University of Sheffield,
Clinical Sciences Centre, Northern General Hospital,
Sheffield S5 7AU, United Kingdom

Received April 13, 1987

The inhibitory action of transforming growth factor β (TGFβ) on DNA synthesis in hepatocytes isolated from the liver of normal rats or from the liver remnant of rats 18 h following partial hepatectomy was compared. Continuous exposure to TGFβ inhibited DNA synthesis of cultured hepatocytes to a similar degree in both groups when labelled with ^{3}H thymidine from 24-48 h or 48-72 h. At 20 pM TGFβ, ^{3}H-thymidine incorporation was reduced by 64-78% in hepatocytes from normal liver and by 60-73% in cells from 18 h regenerating liver. The nuclear labelling index was reduced by 70-80% in all cells. Exposure to TGFβ at concentrations up to 500 pM from 0-24 h had no effect on ^{3}H-thymidine incorporation, but exposure at 20 pM for 24 h periods thereafter was uniformally effective. These results indicate that there is no change in sensitivity of hepatocytes from 18 h regenerating liver to TGFβ, compared with normal cells, and that TGFβ may act at some point in the G_1 phase of the cell cycle to inhibit hepatocyte growth.

Transforming growth factor β is a polypeptide of MW 25 kd found in many normal tissues including platelets, kidney, placenta and whole extracts of rat and mouse embryos which is expressed by a wide range of cell-types in vitro (1,2). TGFβ is a potent bifunctional regulator of cell growth and differentiation; for example it inhibits the differentiation of myoblasts and pre-adipocytes (3,4), but enhances differentiation of bronchial and intestinal epithelial cells (5,6). TGFβ can either stimulate or inhibit growth of normal and transformed fibroblasts but is a potent growth inhibitor of many epithelial cell-types (1,2,7-9).

Recently we have shown that TGFβ reversibly inhibits DNA synthesis in isolated human fetal hepatocytes with half-maximal activity below 1 pM while other hepatic cell-types are unaffected by over 100 fold higher concentrations (10). TGFβ also reversibly inhibits the increase in rat hepatocyte DNA synthesis in response to insulin and EGF (11,12). Adult hepatocytes are

Abbreviations: TdR, thymidine; EGF, epidermal growth factor; TGFβ, transforming growth factor β; PHx, partial hepatectomy; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

0006-291X/87 $1.50

normally quiescent but following partial hepatectomy, regeneration of the tissue begins with initiation of DNA synthesis in hepatocytes after a lag period of 14-16 h (13). Liver regeneration is a carefully regulated process with growth arrest following restoration of the original tissue mass. The existence of negative regulatory factors has therefore been proposed (13). In this study we have examined the response of hepatocytes isolated from regenerating liver to determine whether a decrease in sensitivity to TGFβ might be responsible for their proliferative capacity.

MATERIALS AND METHODS

Collagenase was obtained from Boehringer-Mannheim Ltd, Lewes, Sussex; Falcon 35 mm tissue-culture petri-dishes were from Becton-Dickinson UK Ltd, Cowley, Oxford; Arginine-free William's E medium was prepared by Imperial Laboratories Ltd, Salisbury, Wiltshire; ^{3}H-thymidine (5 Ci/mmol) was purchased from NEN, Stevenage, Hertfordshire; EGF was from Gibco Ltd, Uxbridge, Middlesex; Insulin (Actrapid) from Novo Research, Farillon Ltd, Romford, Essex and Hydrocortisone from Sigma, Poole, Dorset; Ilford K5 photographic emulsion was purchased from Ilford Ltd, Mobberley, Cheshire; TGFβ was supplied by Drs A Roberts and M Sporn, NIH, Bethesda, MD, USA.

Hepatocytes were isolated from the livers of normal 200-250 g male Wistar rats or from the liver remnant following 2/3 partial hepatectomy (14), by two-step collagenase perfusion as previously described (15,16). Briefly, livers were perfused with Ca^{2+}/Mg^{2+}-free Krebs-Ringer buffer containing 10 mM Hepes (pH 7.4) for 10 min at 25 ml/min and then with the same buffer supplemented with 0.05% collagenase and 5 mM $CaCl_2$ for 12.5 min. The resultant cell suspension was filtered through 60 μm nylon mesh, gravity sedimented and resuspended in medium containing 10% newborn calf serum. Cells were counted, viability assessed by trypan-blue dye exclusion and plated (3 x 10^5 cells in 2 ml) into 35 mm tissue-culture petri dishes previously coated with rat-tail tendon collagen (16). After 20-30 min attachment, cells were washed with PBS, and were refed 'basal' medium with or without growth factor additions. Medium was renewed every 24 h as appropriate and ^{3}H-TdR (1 μCi/ml) was added for the final 24 h in culture. 'Basal' medium consisted of arginine-free William's E medium, supplemented with ornithine (0.4 mM), hydrocortisone (5 μM) and insulin (100 nM). Hepatocyte DNA synthesis was determined by autoradiography and by liquid scintillation counting of DNA extracts as previously described in detail (15,16).

RESULTS

Hepatocytes were isolated from the liver of normal rats or from the liver remnant of rats 18 h following partial hepatectomy (18 h PHx) and were maintained in primary monolayer culture for up to 72 h. During the first 24 h in culture ^{3}H-TdR incorporation in hepatocytes from normal rats remained low and cells failed to respond to EGF (Fig 1A). Beyond this time, at 24-48 h and 48-72 h basal ^{3}H-TdR incorporation increased and was further stimulated by EGF. Although TGFβ had no effect on the relatively low level of DNA synthesis in cells up to 24 h in culture, there was significant dose-dependent inhibition with continuous exposure to TGFβ for 48 h and 72 h (Fig 1A). At 20 pM TGFβ, DNA synthesis was reduced by 66% and 64% at 24-48 h and 48-72 h respectively in the absence of EGF and by 78% and 71% respectively in the

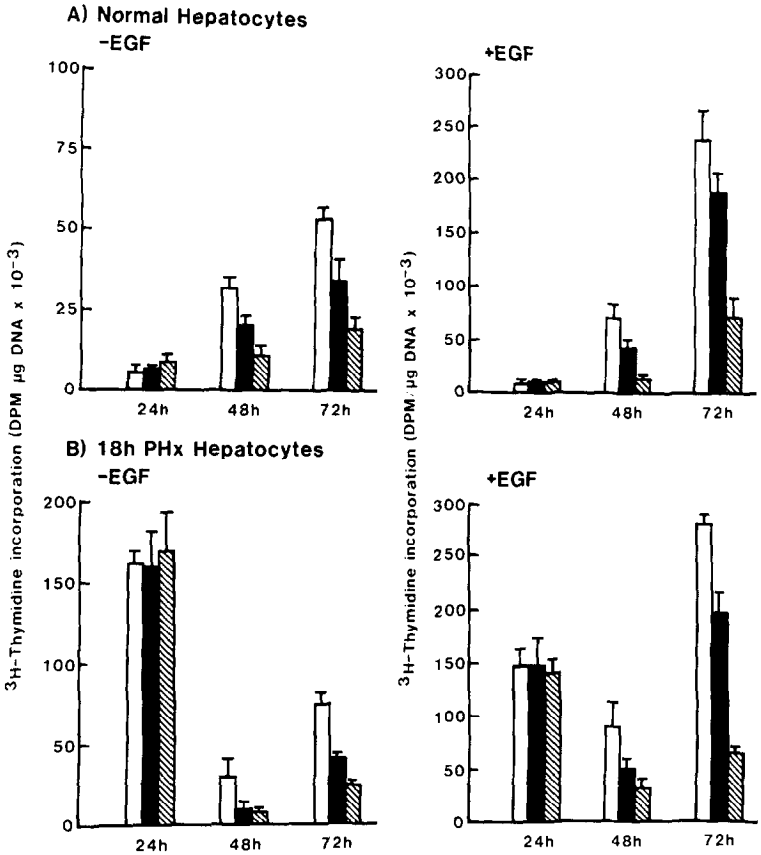

Figure 1. Effect of TGFβ on DNA synthesis in hepatocytes isolated from normal rats (A) or from rats 18 h following partial hepatectomy (B). Cells were maintained for the times indicated in 'basal' medium (see materials and methods), or in medium supplemented with 0.85 nM EGF, in the absence of TGFβ (open bars) or in the presence of 5 pM (solid bars) or 20 pM (cross-hatched bars) TGFβ. ^{3}H-thymidine was added for the final 24 h. Results represent the mean ± S.D. of triplicate cultures.

presence of EGF (Fig 1A). Hepatocytes from 18 h regenerating liver demonstrated over 30 fold higher ^{3}H-TdR incorporation during the 1st 24 h in culture although there was no further rise in response to EGF (Fig 1B). The basal level of incorporation was reduced at 24-48 h and at 48-72 h incubation, but cells did respond to EGF. Furthermore, TGFβ inhibited DNA synthesis by a similar degree compared with normal cells (67% at both 24-48 h and 48-72 h in the absence of EGF and 60% and 73% respectively in the presence of EGF). The higher rate of DNA synthesis in 18 h PHx hepatocytes compared with normal cells during the first 24 h was unaffected by TGFβ (Fig 1B). The growth response of cells was also examined by autoradiography (Fig 2). In confirmation, during the first 24 h in culture TGFβ failed to alter the labelling index of both normal and PHx cells, while a consistent 70-80% reduction was seen in response to TGFβ in both groups at the later times (Fig 2).

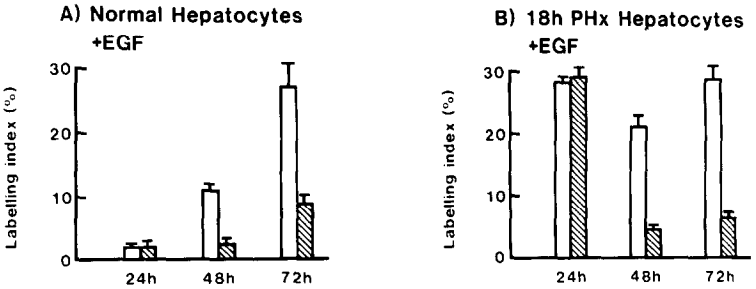

Figure 2. Effect of TGFβ on the hepatocyte nuclear labelling index of cells isolated from normal rats (A) or from animals 18 h following partial hepatectomy (B). Cells were maintained in medium containing 0.85 nM EGF without TGFβ (open bars) or with 20 pM TGFβ (cross-hatched bars). Results represent the mean ± S.D. of triplicate cultures.

To determine whether hepatocytes from 18 h PHx rats were resistant to TGFβ action or were simply less sensitive during the first 24h in culture, considerably higher doses were tested. As shown in Table 1 ^{3}H-TdR incorporation remained unaffected by up to 500 pM TGFβ. This concentration of TGFβ also failed to inhibit the low level of incorporation in normal hepatocytes (results not shown). Finally, to examine the possibility that exposure to TGFβ for more than 24 h was required for inhibition to occur, we measured DNA synthesis in hepatocytes after exposure to TGFβ for 24 h periods only (Table 2). While as expected, there was no inhibition in the first 24 h, DNA synthesis was substantially inhibited both in the absence and presence of EGF, when TGFβ was present from 24-48 h or 48-72 h only (Table 2).

DISCUSSION

The present data are consistent with our previous observations and those of others demonstrating the reversible growth inhibitory action of TGFβ on primary fetal and adult hepatocytes at low pM concentrations (10-12). In addition our findings indicate that hepatocytes isolated from rats 18 h

TABLE 1. ^{3}H-TdR incorporation in hepatocytes isolated from 18 h regenerating liver

	^{3}H-Thymidine Incorporation (DPM/μg DNA x 10^{-3})	
TGFβ (pM)	-	+ EGF (0.85 nM)
0	85.8 ± 9.2	80.7 ± 15.4
20	71.8 ± 19.5	82.9 ± 20.0
100	68.6 ± 12.2	77.3 ± 8.8
500	83.7 ± 15.1	86.3 ± 16.3

Hepatocytes were maintained in culture for 24 h with or without TGFβ in the presence of ^{3}H-TdR. Results represent the mean + S.D. of triplicate cultures.

TABLE 2. ^{3}H-TdR incorporation in hepatocytes from normal rat liver

	TGFβ (20 pM)	^{3}H-Thymidine Incorporation (DPM/μg DNA x 10^{-3}) -	+ EGF (0.85 nM)
A.	0	5.5 ± 0.6	30.8 ± 10.7
	0 - 24 h	4.9 ± 0.2	36.4 ± 10.8
	24 - 48 h	3.6 ± 0.1	15.7 ± 1.5
	0 - 48 h	3.0 ± 0.2	9.9 ± 4.6
B.	0	71.8 ± 13.1	253.3 ± 24.6
	24 - 72 h	17.0 ± 6.6	176.3 ± 11.0
	24 - 48 h	14.9 ± 0.3	101.7 ± 14.0
	48 - 72 h	21.5 ± 7.4	102.4 ± 9.4

Cells were maintained in culture for 48 h (A) or 72 h (B) and TGFβ was present for the times indicated. ^{3}H-TdR was added for the final 24 h. Results represent the mean ± S.D. of triplicate cultures.

following partial hepatectomy are equally sensitive to TGFβ beyond 24 h in culture when compared with cells from control animals.

Although several growth inhibitors have been isolated from rat liver (17-20) they have remained poorly characterised. An inhibitor functioning as a negative growth regulator in the adult liver, must be expressed continually to maintain hepatocytes in a state of quiescence. The observation that TGF-β-like peptides are not present in adult rat liver extracts (21) and TGFβ mRNA is undetectable in adult human liver (22) together with our present data suggest that TGFβ does not play such a role *in vivo*. However, since TGFβ is active at extremely low concentrations *in vitro*, it is possible that low levels of expression, difficult to detect experimentally, may be sufficient to maintain the necessary concentration within the microenvironment of the cells. In a recent preliminary report, a reduction in the expression of TGFβ receptors in freshly isolated attached hepatocytes 3 h following PHx has been described (23) with a gradual recovery during the next 18 h. Studies of the biological activity of TGFβ on hepatocyte DNA synthesis at earlier times following partial hepatectomy, therefore, may highlight differences not apparent at 18 h.

The high rate of ^{3}H-TdR incorporation and high labelling index during the first 24 h in culture of 18 h PHx cells indicate that they have already traversed the G1/S-phase boundary prior to isolation. This is expected since there is a lag-period of 14-16 h following partial hepatectomy *in vivo*, before initiation of DNA synthesis (13). In addition, this lag-period may explain the lack of EGF response during the first 24 h in culture, an observation which we and others have previously reported (15,24).

The reasons for the failure of TGFβ to inhibit DNA synthesis during this period are not clear. Although exposure to TGFβ for 24 h only is sufficient

to inhibit DNA synthesis (Table 2), it is possible that plasma membrane receptors are damaged following collagenase disaggregation of cells. However, receptor-mediated responses to hormones such as insulin and glucagon are readily demonstrable during the first 24 h in both monolayer and suspension cultures of hepatocytes (24-27) and TGFβ receptors are present on freshly isolated cells from 18 h regenerating liver (23). We suggest that TGFβ may inhibit hepatocyte proliferation at some point in the G_1-phase of the cell-cycle and that when cells have progressed into S-phase, they are no longer susceptible to inhibitory action. This possibility is supported by the observation that TGFβ does not simply lower the rate of ^{3}H-TdR incorporation in cells, but substantially reduces the recruitment of cells into S-phase.

ACKNOWLEDGMENTS: We are grateful to Drs A.B. Roberts and M.P. Sporn, NIH, Bethesda, Maryland, USA for supplying purified TGFβ. This work was supported by grants from Birthright, the Yorkshire Cancer Research Campaign and the SmithKline (1982) Foundation.

REFERENCES

1. Massague, J. (1985) Trends Biochem. Sci. 10, 237-240.
2. Sporn, M.B., Roberts, A.B., Wakefield, L.M. and Assoian, R.K. (1986) Science. 233, 532-534.
3. Florini, J.R., Roberts, A.B., Ewton, D.Z., Falen, S.L., Flanders, K.C. and Sporn, M.B. (1986) J. Biol. Chem. 261, 16509-16513.
4. Ignotz, R.A. and Massague, T. (1985) Proc. Natl. Acad. Sci. 82, 8530-8534.
5. Masui, T., Wakefield, L.M., Lechner, J.F., La Veck, M.A., Sporn, M.B. and Harris, C.C. (1986) Proc. Natl. Acad. Sci. 83, 2438-2442.
6. Kurokowa, M., Lynch, K. and Podolsky, D.K. (1987) Biochem. Biophys. Res. Commun. 142, 775-782.
7. Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F. and Sporn, M.B. (1985) Proc. Natl. Acad. Sci. 82, 119-123.
8. Hill, D.J., Strain, A.J., Elstow, S.F., Swenne, I. and Milner, R.D.G. (1986) J. Cell. Physiol. 128, 322-328.
9. Tucker, R.F., Shipley, G.D., Moses, H.L. and Holley, R.W. (1984) Science. 226, 705-707.
10. Strain, A.J., Hill, D.J. and Milner, R.D.G. (1986) Cell Biol. Int. Rep. 10, 855-860.
11. Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K. and Ichihara, A. (1985) Biochem. Biophys. Res. Commun. 133, 1042-1050.
12. Carr, B.I., Hayashi, I., Branum, E.L. and Moses, H.L. (1986) Cancer Res. 46, 2330-2334.
13. Bucher, N.L.R. and McGowan, J.A. (1984) (ed. R. Wright) In: Liver and Biliary Disease, pp 251-265, W.B. Saunders, London.
14. Higgins, G.M. and Anderson, R.M. (1931) Arch. Pathol. 12, 186-202.
15. McGowan, J.A., Strain, A.J. and Bucher, N.L.R. (1981) J. Cell. Physiol. 108, 353-363.
16. Strain, A.J., McGowan, J.A. and Bucher, N.L.R. (1982) In Vitro. 18, 108-116.
17. Verly, W.G. (1973) Nat. Cancer Inst. Monogr. 38, 175-184.
18. Kur, C.Y. and Yoo, T.J. (1977) J. Natl. Cancer Inst. 59, 1691-1695.
19. McMahon, J.B., Farrelly, J.G. and Iype, P.T. (1982) Proc. Natl. Acad. Sci. 79, 456-460.

20. Makowka, L., Falk, R.E., Teodorczyk-Injeyan, J., Venturi, D., Rotstein, L.E., Falk, W., Langer, B., Blendis, L.M. and Phillips, M.J. (1983) Cancer. 51, 2180-2190.
21. Hill, D.J., Strain, A.J. and Milner, R.D.G. (1986) Cell. Biol. Int. Rep. 10, 915-922.
22. Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B. and Goeddel, D.V. (1985) Nature. 316, 701-705.
23. Carr, B.I., Thall, A., Whitson, R.H. and Itakura, K. (1986) J. Cell. Biol. 103, 443a.
24. Friedman, D., Claus, T., Pilkis, S. and Pini, G. (1981) Exptl. Cell Res. 135, 282-290.
25. Fehlmann, M., Morin, O., Kitabgi, P. and Freychet, P. (1981) Endocrinol. 109, 253-261.
26. Morin, O., Fehlmann, M. and Freychet, P. (1982) Mol. Cell. Endocrinol. 25, 339-352.
27. Nakamura, T., Tomomura, A., Noda, C., Shimoji, M. and Ichihara, A. (1983) J. Biol. Chem. 258, 9283-9289.
28. Shimoyama, R., Savage, C.R. and Boden, G. (1985) J. Clin. Endocrinol. Metab. 60, 928-933.