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A growth factor for hepatocytes is inactivated by sub-lethal γ-irradiation in vivo or in vitro

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Serum from partially hepatectomised rats promoted DNA synthesis in primary adult rat hepatocyte cultures. If the rats had been exposed to sub-lethal γ-irradiation immediately following operation or if their serum, collected at 3 h, was exposed to irradiation in vitro, the growth-promoting activity was destroyed. Prostaglandin E_2 also stimulated DNA synthesis in the cultures; if PGE_2 was irradiated in serum from intact or partially hepatectomised rats its growth-promoting activity was markedly diminished.

Liver growth factor; Prostaglandin E_2; Liver regeneration; Ionizing irradiation

1. INTRODUCTION

Primary adult rat hepatocyte cultures [1] can be induced to grow by various factors including EGF, serum from partially hepatectomised rats and eicosanoids [1–5]. When liver growth is promoted in vivo by partial hepatectomy [6], regeneration is delayed if rats are exposed to ionizing irradiation immediately before or up to 3 h after operation [7,8]. The atypical sensitivity of liver in G_0 phase to ionizing irradiation [7,8] is unexplained; many of the biochemical events associated with hepatocyte transition from G_1 to S phase, such as the induction of thymidine kinase, are affected. Recent experiments [8] indicated that levels of cyclic AMP, which are markedly elevated 4–6 h after partial hepatectomy [9] are lower if the animals had received sub-lethal γ-irradiation 0–2 h after operation. Adenylate cyclase activity in plasma membranes isolated from the livers of the partially hepatectomised, irradiated rats, was low but responded normally to stimulation by β-agonists, suggesting ionizing irradiation might be affecting the signal for regeneration, rather than the response of the hepatocytes to the signal. The growth-promoting effects of serum collected from partially hepatectomised rats 3 h after operation were therefore compared with those of serum from rats which had been exposed to sub-lethal γ-irradiation.

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2. MATERIALS AND METHODS

2.1. *Hepatocyte cultures*

Hepatocytes from adult male Wistar rats (130–150 g) were prepared by the two-step collagenase method [10]. Viability of the cells was checked by trypan blue exclusion and was always greater than 85%. The hepatocytes were plated in triplicate at 5×10^5 cells/2 ml on 60 mm collagen-coated dishes [1] in Dulbecco's modified Eagle's medium (MEM), arginine-free, supplemented with 10% MEM essential amino acid solution, 10% MEM non-essential amino acid solution, 1.63 μM glutathione, 0.5 mM L-ornithine, 10 mM Na-pyruvate and 50 $\mu g \cdot ml^{-1}$ gentamycin. All culture media contained insulin (0.5 $\mu g \cdot ml^{-1}$). The cells were allowed to attach for 2 h at 37°C in humidified 5% CO_2/95% air, the medium was changed and the growth factors were added. Culture medium was changed at 24 h and fresh additions were made. Appropriate assays were made on each dish.

2.2. *Serum collection*

Serum was collected under ether anaesthesia from sham-operated or partially hepatectomised [6] rats 3 h after operation [5]. Where indicated the animals were exposed to 4 Gy ^{60}Co γ-

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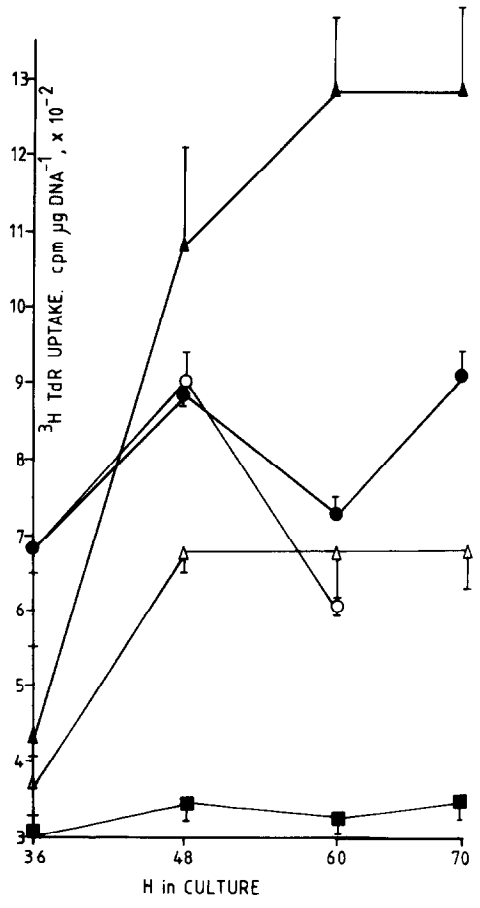

Fig.1. The effect of 4 Gy γ-irradiation in vivo on the growth-promoting properties of rat serum on adult liver hepatocyte cultures. Hepatocytes were plated at 5×10^5 cells/dish. Culture medium was changed at 24 h and fresh additions made. Serum (10% v/v) was obtained and incorporation into DNA measured over 2 h periods as in section 2. [^{3}H]TdR uptake into DNA is expressed as cpm μg DNA^{-1} ± SEM. (▲—▲) Serum collected 3 h after partial hepatectomy; (△—△) serum collected 3 h after partial hepatectomy, from rats given 4 Gy in vivo; (●—●) serum from sham-operated animals; (○—○) serum from sham-operated animals, from rats given 4 Gy in vivo; (■—■) control cultures (insulin only).

irradiation (dose rate, 4 Gy/5 min) immediately after operation. Serum was stored for up to 10 days at −15°C. Serum was irradiated in vitro in air at 0°C in closed, plastic tubes (dose 4 Gy in 5 min).

2.3. *[^{3}H]TdR incorporation into DNA*

Cultures were incubated with 4 μCi [^{3}H]methylthymidine (spec. act. 92 Ci·mmol^{-1}, Amersham International) for 2 h periods. At the end of the labelling period the cells were washed 3 times with ice-cold phosphate-buffered saline and frozen in liquid N_2 until radioactivity in the DNA was determined [1].

Protein was estimated by the method of Lowry et al. [11]; DNA was determined fluorimetrically [12]. Calf thymus DNA was used as a standard.

3. RESULTS AND DISCUSSION

Serum taken at 3 h from animals which were exposed to 4 Gy immediately after laparotomy was indistinguishable from that obtained from non-exposed animals in its growth promotion of the hepatocytes (fig.1). Serum obtained from rats 3 h

Table 1

The effects of serum from partially hepatectomised rats on growth-associated responses in hepatocyte cultures

	cAMP levels	Prostaglandin release into medium							
		PGE_2				$PGF_{2\alpha}$			
Time in culture (h):	6	0	1	6	24	0	1	6	24
Serum from:									
Sham-operated rats	27 ± 0.66	9 ± 0.5	90 ± 2.5	165 ± 5	140 ± 3.5	14 ± 1	230 ± 5	215 ± 2.5	187 ± 5
Collected 3 h after partial hepatectomy	44 ± 0.80	29 ± 0.5	104 ± 5	237 ± 7.5	208 ± 5	39 ± 1	310 ± 5	332 ± 5	195 ± 2.5
Collected 3 h after partial hepatectomy + 4 Gy	17 ± 0.27	30 ± 2	68 ± 5	102 ± 5	85 ± 5	36 ± 2	117 ± 1	135 ± 2.5	109 ± 2.5

Cultures were set up with 5×10^5 cells/dish. Serum was collected from sham-operated or partially-hepatectomised rats 3 h after operation. Where indicated, the animals were exposed to 4 Gy ^{60}Co γ-irradiation immediately after operation. The serum was present at 10% (v/v). Cyclic AMP levels [16] are expressed as pmol·mg protein^{-1} ± SEM. PGE_2 and $PGF_{2\alpha}$ in the culture medium were determined by radioimmune assay [17] and are given as pg·ml^{-1}

after partial hepatectomy enhanced DNA synthesis especially by 60 h (fig.1). If the partially hepatectomised rats had been exposed to 4 Gy immediately after operation, the enhanced growth-promoting activity of the serum was lost. Irradiating the hepatocyte cultures or using hepatocytes obtained from irradiated rats did not affect their response to serum or EGF (not shown).

Other growth-associated parameters were examined. Cyclic AMP levels in the cells supplemented with serum from irradiated, partially hepatectomised rats were diminished (table 1) and prostaglandin release into the culture medium [5] after 1, 6 and 24 h was also lowered (table 1).

Whilst the decreased cyclic AMP levels and lowered DNA synthesis found with the hepatocyte cultures resembled the results following irradiation of partially hepatectomised rats, effects of total body exposure are complex, so that the changes in growth-promoting activity of the serum are difficult to interpret. Serum from sham-operated animals or which had been collected from rats 3 h after partial hepatectomy was therefore irradiated with 4 Gy in vitro. No effect was observed in the growth-promoting activity of serum from the laparotomised animals (table 2B) but the expected stimulation produced by serum from the partially hepatectomised rats was diminished (table 2D).

The period of sensitivity of liver regeneration to inhibition by the cycloxygenase inhibitor indomethacin [13], coincides with that found for the sensitivity of partially hepatectomised rats to sub-lethal irradiation in vivo (−0.5 to +3 h after operation) [8]. Eicosanoids stimulate DNA synthesis in hepatocyte cultures [4,5]; the growth-promoting effects of PGE_2 were therefore tested in combinations with serum ± irradiation.

PGE_2 is available commercially in solutions sterilised by irradiation; predictably therfore its effects on hepatocyte growth were not diminished by exposure to 4 Gy (table 2A). In other experiments where PGE_2 or serum from partially hepatectomised rats was present in the culture medium for only 2–6 h after attachment, both PGE_2 and serum caused DNA contents of the hepatocytes to be doubled by 67 h [5]; lower uptake of [^{3}H]TdR into DNA was observed if the growth factors were continuously present. In the experiments presented here, where the growth factors were present continuously, the increments in [^{3}H]TdR incorporation into DNA when PGE_2 and serum from laparotomised rats were present together, over that caused by insulin alone (fig.1), were approximately the sums of those found when the growth factors were present separately (table 2A,B,C). A similar result was obtained when serum from partially hepatectomised rats was used, but even in the presence of added PGE_2 [^{3}H]TdR uptake into DNA was lower if serum from the partially hepatectomised rats had been exposed to 4 Gy in vitro (table 2E). When, however, PGE_2 was added to either serum and the mixture irradiated, growth-

Table 2

The effects of γ-irradiation in vitro on the growth-promoting activity of rat serum

Serum from	[^{3}H]TdR uptake into DNA		
	46–48 h	58–60 h	70–72 h
A. PGE ± 4 Gy			
−	1817 ± 86	2697 ± 212	2022 ± 692
+	1342	2434	4022
B. Sham-operated rats ± 4 Gy			
−	866 ± 13	730 ± 133	900 ± 29
+	798 ± 40	810 ± 15	–
C. Sham-operated rats ± 4 Gy + PGE_2 (unirradiated)			
−	2406 ± 319	3352 ± 461	2850
+	1720	3630	3100
D. Partially hepatectomised rats collected at 3 h ± 4 Gy			
−	1080 ± 137	1286 ± 100	1299 ± 169
+	697	902	907
E. Partially hepatectomised rats ± 4 Gy + PGE_2 (unirradiated)			
−	2200	3400	4002
+	2001	2430	3600
F. Sham-operated rats + PGE_2 exposed together to 4 Gy			
	1128 ± 104	1510 ± 92	–
G. Partially hepatectomised rats + PGE_2 exposed to 4 Gy			
	1220	1360	–

Cultures were set up at 5×10^5 cells/dish. Serum was collected from sham-operated or partially hepatectomised rats 3 h after operation and was present at 10% (v/v). Where indicated the serum was exposed to 4 Gy in vitro. PGE_2 was present at 6 μg/dish [4]. It was dissolved in 10% EtOH. Amounts of ethanol added (1–2 μl) to the medium did not affect the viability of the cells. The medium was changed and fresh growth factor added, after 24 h. 4 μCi of [^{3}H]TdR were added to the cultures 46, 58 or 70 h after the initial addition of the growth factors and the incorporation into DNA measured after 2 h. Thymidine uptake is expressed as cpm·μg DNA^{-1}. The results are the means of two or more experiments

promoting activities were greatly diminished (table 2F,G).

About 80% of PGE_2 in serum is bound to protein in an alkali-labile linkage which has to be dissociated before radioimmune assay [14,15]. PGE_2 levels in serum from irradiated partially hepatectomised rats were not different from those in the unirradiated groups (table 1 and [7]) indicating that the alkali-labile linkage was not irreversibly affected by exposure to γ-rays. However, as the stimulatory activity of (serum + PGE_2) is so markedly lowered when the two are exposed together, irradiation may alter a protein/PGE_2 complex thus diminishing its growth-promoting potential.

The relative concentrations and potencies of different growth-promoting factors in serum from partially hepatectomised rats are unknown. The similarity in behaviour of serum + PGE_2, when irradiated together, to that observed after irradiation in vivo or after exposing serum from partially hepatectomised animals suggests that prostaglandins are involved in the promotion of liver growth.

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REFERENCES

[1] Nakamura, T. and Ichihara, A. (1985) Cell Struct. Funct. 10, 1–16.
[2] Paul, D., Leffert, H., Sato, G. and Holley, R.W. (1972) Proc. Natl. Acad. Sci. USA 69, 374–377.
[3] Michalopoulos, G., Gauciulli, H.D., Norotry, A.R., Keigerman, A.D., Strous, S.C. and Jirtle, R.W. (1982) Cancer Res. 42, 4673–4682.
[4] Skouteris, G.G., Ord, M.G. and Stocken, L.A. (1988) J. Cell. Physiol. 135, 516–520.
[5] Little, P., Skouteris, G.G., Ord, M.G. and Stocken, L.A. (1988) J. Cell Sci. 91, 549–553.
[6] Higgins, G. and Anderson, R.M. (1933) Arch. Pathol. 12, 186–202.
[7] Holmes, B.E. (1956) in: CIBA Foundation Symposium on Ionizing Radiation and Cell Metabolism, pp.225–238, J. and A. Churchill Ltd, London.
[8] Skouteris, G.G., Ord, M.G. and Stocken, L.A. (1987) Biochem. Soc. Trans. 15, 706–707.
[9] MacManus, J.P., Franks, D.J., Youdale and Braceland, B.M. (1972) Biochem. Biophys. Res. Commun. 49, 1201–1207.
[10] Seglen, P.O. (1976) Methods Cell Biol. 13, 29–83.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Kapp, L.N., Brown, S.L. and Levecz, R. (1974) Biochim. Biophys. Acta 361, 140–143.
[13] Rixon, R.H. and Whitfield, J.F. (1982) J. Cell. Physiol. 113, 281–288.
[14] Maclouf, J., Kindahl, H., Granstrom, E. and Samuelsson, B. (1980) Eur. J. Biochem. 109, 561–566.
[15] Lecomte, M., Nunez, D. and Boeynaems, J.M. (1986) Prostaglandins 32, 150–154.
[16] Steiner, A.L., Pagliara, A.S., Chase, L.R. and Kipnis, D.M. (1972) J. Biol. Chem. 247, 1114–1120.
[17] Bothwell, W., Verburg, M., Wynalda, M., Daniels, E.G. and Fitzpatrick, F.A. (1982) J. Pharmacol. Exp. Ther. 220, 229–235.