EFFECT OF PROSTAGLANDINS AND HORMONES ON CYCLIC AMP FORMATION IN RAT HEPATOMAS AND LIVER TISSUE

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Summary.—The formation of cyclic AMP was studied in normal liver, subcutaneous hepatomas derived from MH₁C₁ cells, and premalignant liver and primary hepatomas induced by the carcinogens 2-acetylaminofluorene (AAF) and 4-dimethylamino-azobenzene (DAB). While only very slight effects of prostaglandins (PG) were seen in slices of normal liver, all the hepatomas responded strongly to PGE₁ and PGE₂. The hepatomas also had increased PGE₁-sensitive adenylate-cyclase activity. PGF_{1α} and PGF_{2α} did not increase the cAMP level significantly either in the liver or in the hepatomas. During AAF carcinogenesis the response to PGE₁ increased slightly during the carcinogen feeding, and was greatly elevated only in the fully developed hepatomas. This is in contrast to the increase in adrenalin response seen during carcinogenesis, which starts much earlier, and reaches a peak value within 8–10 weeks. It is concluded that various hepatomas have elevated responsiveness to PGE₁ and PGE₂ as well as to adrenalin, but the course of change in the tissues' ability to respond to these agents during carcinogenesis is very different.

CYCLIC AMP (cAMP) seems to be a regulator of cell growth, but its precise role is incompletely understood (Ryan & Heidrick, 1974; Pastan *et al.*, 1975; Friedman, 1976). The metabolism of cAMP is altered in many cancers and malignant cell lines. In rat hepatomas the cAMP system is regulated differently from that of normal liver tissue. In most hepatomas adenylate cyclase is less responsive to glucagon than the liver enzyme (Allen *et al.*, 1971; Emmelot & Bos, 1971; Christoffersen *et al.*, 1972; Tomasi *et al.*, 1973; Boyd *et al.*, 1974; Hickie *et al.*, 1975; Criss & Morris, 1976; Hickie *et al.*, 1977). However, increased effects of adrenalin (Brown *et al.*, 1970; Christoffersen *et al.*, 1972; Boyd *et al.*, 1974) and prostaglandin E₁ (PGE₁) (Chayoth *et al.*, 1973) have also been described. Furthermore, during treatment of rats with chemical carcinogens, before tumour development,

alterations in the response pattern of the adenylate cyclase have been observed, including increased responsiveness to adrenergic agents (Christoffersen *et al.*, 1972, 1974; Christoffersen, 1975; Boyd *et al.*, 1974, 1976) and to PGE₁ (Chayoth *et al.*, 1973).

The present study was undertaken in order to get more information on how control of cAMP formation differs in hepatomas and livers. Particular interest has been devoted to the effect of prostaglandins, which have also been proposed as taking part in growth regulation (Sykes, 1976; Jimenez de Asua *et al.*, 1975; Hial *et al.*, 1976). Some primary and transplanted hepatomas were studied, to see whether the increased responsiveness to PGE₁ that has been reported for ethionine-induced hepatomas (Chayoth *et al.*, 1973) is present also in other hepatomas, and also includes other prostaglandins. We also wanted to

examine the time course of the changes in responsiveness of the liver tissue towards PGE₁ and hormones during chemical carcinogenesis.

MATERIALS AND METHODS

Materials.—2-Acetylaminofluorene (AAF) and 4-dimethylaminoazobenzene (DAB) were obtained from Koch–Light Laboratories, UK, adrenalin bitartrate from Rhone Poulenc Paris, glucagon from, Novo, Copenhagen, cyclic AMP, theophylline and pyruvate kinase from Sigma Chemical Co., St Louis, Mo, U.S.A., methylisobutylxanthine from Aldrich Chemicals, Milwaukee, Wis., U.S.A., cyclic [³H]AMP (24.1 Ci/mmol) from New England Nuclear, Boston, U.S.A., [^α32P]ATP from The Radiochemical Centre, Amersham, and phosphoenol pyruvate from Boehringer, Mannheim Germany. The prostaglandins were kindly provided by Dr John Pike, The Upjohn Company, Kalamazoo, Mich., U.S.A.

Carcinogen treatment.—Male albino Wistar rats, 12 weeks old at the beginning of the carcinogen treatment, were used. The animals were fed a basal diet (Christoffersen, 1975). This diet was supplemented with 0.025% AAF for the treated group. Paired control animals received the basal diet. Liver tissue designated as pre-malignant was taken from rats killed at various times of treatment, before development of tumours. After 8 months the carcinogen treatment was terminated, and the animals were given standard laboratory chow, until they were killed 5 months later. At that time, the majority of treated rats had liver tumours, often multiple nodules of varying diameter. Tumours of diameter 0.5–1.5 cm were used in the experiments described here. The tumours were examined histologically (courtesy of Dr S. B. Refsum, Rikshospitalet, Oslo), and were found to be hepatocarcinomas. We also studied a single tumour from a rat treated with 0.05% DAB for 7 months, followed by 9 months on carcinogen-free diet.

Inoculation of hepatoma cells.—Buffalo rats of either sex were used in these experiments. The clonal rat hepatoma cell line MH₁C₁ derived from Morris hepatoma 7795 (Richardson *et al.*, 1969) was cultured as described by Gaudernack *et al.* (1973). When the rats were about 2 weeks old, suspensions of MH₁C₁ cells containing

~200 µg cell protein suspended in 0.5 ml growth medium (Dulbecco modified Eagle's medium) were inoculated into each flank at the lower back region. After 2–5 months, when tumours of diameter 1–2 cm had developed, the animals were killed.

Cyclic AMP accumulation in slices.—The rats were anaesthetized with ether, and the livers and tumours were rapidly excised and transferred to ice-cold buffer. The hepatomas were trimmed of connective tissue and necrotic areas were removed. The tissues were cut into 0.4–0.5 mm-thick slices weighing 20–80 mg. The slices were transferred to 10-ml Erlenmeyer flasks, and preincubated 45–60 min at 37°C in 1.5 ml Krebs–Ringer bicarbonate buffer containing 119.2 mM NaCl, 3 mM KCl, 1.2 mM MgSO₄, 2.4 mM KH₂PO₄, 24.9 mM NaHCO₃, 2.0 mM CaCl₂ and 10 mM glucose, under continuous gassing with 95% O₂ and 5% CO₂. The incubation was initiated by the addition of the agents to be tested dissolved in 0.5 ml of the Krebs–Ringer bicarbonate buffer with 8 mM theophylline, to give a final volume of 2 ml, and concentrations as indicated in the figure legends. Stock solutions of prostaglandins were dissolved in 96% ethanol (2 mg/ml) and diluted in buffer immediately before addition. The incubation was terminated by rapidly transferring the slice to liquid N₂ after blotting on filter paper.

The frozen slices were homogenized in 5% trichloroacetic acid. Cyclic AMP was determined as described earlier (Christoffersen *et al.*, 1973) using the protein-binding assay of Gilman (1970).

Assay of adenylate-cyclase activity.—Incubations were carried out at 30°C in 200 µl of a mixture containing: 1 mM [^α32P]ATP (~10⁶ ct/min tube), 1 mM cAMP, 4 mM MgCl₂, 30 mM KCl, 50 mM Tris–HCl, pH 7.5, 1 mM methylisobutylxanthine, 1.5 i.u. pyruvate kinase, 2.5 mM phosphoenol pyruvate, and homogenate equivalent to about 500 µg protein. The reaction was terminated by the addition of 400 µl 150 mM ZnSO₄. 50 µl [³H]cAMP (~2500 ct/min) for recovery determination during the purification step and 400 µl 150 mM Na₂CO₃ were added to each tube. After centrifugation at 2000 *g* for 5 min, the labelled cAMP in the supernatants was purified by the Dowex-50/alumina double-column method described by Salomon *et al.* (1974), and counted by liquid scintillation.

Determination of protein.—The method of Lowry *et al.* (1951) was used.

RESULTS

Hormone and prostaglandin effects on cAMP levels in slices from liver and hepatomas

Table I shows the levels of cAMP in slices from various hepatomas after exposure to adrenalin, glucagon or PGE₁. In one series of experiments, MH₁C₁-derived tumours were compared with liver from tumour-bearing animals. In the liver slices neither adrenalin nor PGE₁ showed any effect, while glucagon increased the cAMP level about 5-fold in 2 min. In contrast, the slices prepared from subcutaneous MH₁C₁-derived hepatomas or mesenterial metastases from these tumours responded strongly to adrenalin (4–5-fold) and to PGE₁ (10-fold), but only slightly to glucagon (40% increase).

Similarly, primary hepatomas, induced by AAF or DAB feeding, responded very strongly to adrenalin or PGE₁, compared to the slight effects of these agents on the cAMP content in slices from the adjacent

uninvolved liver tissue. These primary hepatomas differed from the MH₁C₁-derived tumours in that most of them were markedly responsive to glucagon.

Dose-response curves for various prostaglandins on cAMP levels in slices from liver and MH₁C₁-derived tumours are given in Fig. 1. The figure shows the striking difference between the liver and the hepatoma in responsiveness to PGE₁ and PGE₂. PGF_{1α} and PGF_{2α} had no significant effect on any of the tissues. The cAMP content of the tumour slices did not reach a plateau in the range of prostaglandin concentrations used.

Evidence for increased activity of PGE₁-sensitive adenylate cyclase in the hepatomas

The adenylate cyclase in homogenates of hepatomas responded more strongly to PGE₁ than did the enzyme from liver (Table II). The effect of PGE₁ was greater in the hepatoma homogenates (113% increase over basal activity, *vs* 63% in liver homogenates), but this effect was much less pronounced than on the slices from the same tumours (Table I). We have no explanation for this discrepancy, which was seen also for the adrenalin

TABLE I.—*Effect of prostaglandins and hormones on levels of cyclic AMP in slices of rat liver and hepatomas*

Tissue	No. of animals	pmol cAMP/mg wet wt. $\pm$ s.e.			
		No addition	PGE ₁ (28 μM)	Adrenalin (50 μM)	Glucagon (1.4 μM)
Buffalo rats:					
Liver ^a	8	0.22 $\pm$ 0.04	0.32 $\pm$ 0.08	0.28 $\pm$ 0.10 ^f	1.22 $\pm$ 0.28
MH ₁ C ₁ hepatoma ^b	10	0.31 $\pm$ 0.08	3.00 $\pm$ 0.29 (<i>P</i> < 0.001 ^g)	1.35 $\pm$ 0.20 (<i>P</i> < 0.05)	0.44 $\pm$ 0.09 (<i>P</i> < 0.001)
Metastatic tumours ^c	6	0.24 $\pm$ 0.09	3.11 $\pm$ 0.67 (<i>P</i> < 0.005)	1.77 $\pm$ 0.38 (<i>P</i> < 0.05)	0.30 $\pm$ 0.15 (<i>P</i> < 0.005)
Wistar rats:					
Liver ^a	3	0.25 $\pm$ 0.12	0.33 $\pm$ 0.10	0.48 $\pm$ 0.10	1.92 $\pm$ 0.53
AAF hepatoma ^d	5	0.64 $\pm$ 0.06	2.93 $\pm$ 0.87 (<i>P</i> < 0.05)	1.85 $\pm$ 0.25 (<i>P</i> < 0.05)	1.41 $\pm$ 0.22 (<i>P</i> < 0.5)
DAB hepatoma ^e	1	0.52	1.13	2.00	3.62

^a From tumour-bearing animal.

^b Growing subcutaneously from inocula of MH₁C₁ cells.

^c Abdominal metastases from s.c. MH₁C₁ hepatomas.

^d Primaries induced by 2-AAF feeding.

^e Primary induced by DAB feeding.

^f This value represents only 4 livers.

^g The significance levels of the comparison of percentage stimulation over basal in the tumour slices and the corresponding stimulation in the liver slices, calculated by the Wilcoxon's two-sample test for the unpaired case.

FIG. 1.—Dose-response curves for the effects of prostaglandins on cAMP accumulation in slices from MH_1C_1 -derived hepatoma (left) and liver (right) incubated as in Methods, in the presence of the various prostaglandins and 2mM theophylline in a final volume of 2 ml. Symbols: —■— PGE_1 ; —□— PGE_2 ; —▼— $PGF_{1\alpha}$; —▽— $PGF_{2\alpha}$. Each point represents mean $\pm$ s.e. of determinations on 3–6 slices.

TABLE II.—Adenylate-cyclase activity in homogenates from liver and MH_1C_1 -derived hepatoma tissue

		pmol cAMP/mg protein/min $\pm$ s.e.				
	No. samples	No addition	PGE_1 (28 μM)	Adrenalin (50 μM)	Glucagon (14 μM)	NaF (10mM)
Liver ^a	6	4.1 $\pm$ 0.4	6.7 $\pm$ 0.9	5.1 $\pm$ 0.5	10.6 $\pm$ 1.1	27.6 $\pm$ 3.3
Hepatoma ^b	6	3.9 $\pm$ 0.7	8.3 $\pm$ 1.0 ($P < 0.005^c$)	8.1 $\pm$ 1.4 ($P < 0.05$)	4.8 $\pm$ 0.8 ($P < 0.005$)	42.3 $\pm$ 2.9 ($P > 0.1$)

^a From tumour-bearing Buffalo rats.

^b s.c. hepatomas developed from inocula of MH_1C_1 cells.

^c The significance levels calculated as for Table I.

response (Table I vs Table II; cf. also Christoffersen *et al.*, 1974). It is a well-known phenomenon that a large part of the adenylate-cyclase response towards hormones may be lost on tissue homogenization (Achar *et al.*, 1977), possibly owing to membrane derangement or non-optimal composition of the incubation mixture in broken-cell preparations.

In the present case, additional evidence that the very strong effect of PGE_1 on cAMP accumulation in the hepatoma

tissue is actually due to adenylate-cyclase activation, was obtained in experiments (Table III) showing that the increase in cAMP content in slices after PGE_1 could be amplified by theophylline, and even more so by the more potent phosphodiesterase inhibitor, methylisobutylxanthine.

Hormone and PGE_1 responsiveness during AAF-carcinogenesis

The development of the hormone and

TABLE III.—*Effect of theophylline and methylisobutylxanthine on PGE₁-stimulated cAMP accumulation in hepatoma slices prepared from s.c. growing hepatomas developed from inoculates of MH₁C₁ cells as in Materials and Methods*

Addition	pmol cAMP/mg wet wt. (Mean $\pm$ s.e. from 3 tumours)
None	0.23 $\pm$ 0.04
Theophylline (2mM)	0.31 $\pm$ 0.04 ^a
Methylisobutylxanthine (0.5mM)	0.41 $\pm$ 0.08 ^a
PGE ₁ (28 μ M)	1.70 $\pm$ 0.66 ^b
+ theophylline (2mM)	2.78 $\pm$ 0.29 ^{b,c}
+ methylisobutylxanthine (0.5mM)	6.87 $\pm$ 0.62 ^{b,c}

^a $P > 0.1$ vs basal level (no addition).

^b $P < 0.03$ vs basal level.

^c $P < 0.03$ vs slices exposed to PGE₁ alone.

Statistical evaluation based on Wilcoxon's two-sample test for the unpaired case.

PGE₁ responsiveness in slices during AAF-carcinogenesis is shown in Fig. 2. As

shown previously (Christoffersen *et al.*, 1974), the adrenalin response increased rapidly during the first weeks, reaching a maximum at about 8 weeks. It then declined, as has also been found during 3'-methyl-DAB carcinogenesis (Boyd *et al.*, 1974, 1976), but in the final hepatoma the response to adrenalin was still greater than in normal liver tissue (Fig. 2).

The PGE₁ response increased slowly during the carcinogen treatment, but was greatly increased in the hepatomas. This is in accordance with the observations of Chayoth *et al.* (1973), who found increased response to PGE₁ during ethionine carcinogenesis, before and after hepatoma development. However, in the present work we show that the changes in adrenalin and PGE₁ response take completely different courses during the carcinogen treatment (Fig. 2).

FIG. 2.—Accumulation of cAMP in slices from liver during AAF carcinogenesis and from resultant hepatomas in response to PGE₁, glucagon and adrenalin in the presence of 2mM theophylline. Symbols —○— no addition; —●— adrenalin (50 μ M); —▲— glucagon (1.4 μ M); —■— PGE₁ (28 μ M). Each point represents mean $\pm$ s.e. of determinations on separate livers or tumours, the number of which is given in parentheses above the abscissa. Slices from control rats showed no age-related changes in the hormone response during the treatment period. At 8 weeks control values were: no addition 0.16 $\pm$ 0.02, adrenalin 0.40 $\pm$ 0.10, glucagon 1.77 $\pm$ 0.03, PGE₁ 0.38 $\pm$ 0.08, ($n = 3$). At the tumour stage control values were: no addition 0.22 $\pm$ 0.05, adrenalin 0.29 $\pm$ 0.04, glucagon 1.57 $\pm$ 0.32, PGE₁ 0.20 $\pm$ 0.01 ($n = 5$).

FIG. 3.—Effects of PGE₁ and glucagon added alone or in combination on liver slices from rats treated with AAF. Inset; the dose-response curve for PGE₁ and glucagon. The slices were incubated in the presence of 2mM theophylline as described in Methods.

The glucagon response rose temporarily but less markedly than the adrenaline response during carcinogenesis.

Combination of glucagon and PGE₁

In an attempt to get information on whether PGE₁ acts on the same cell population as glucagon, we tried combinations of these two agents, using a supramaxial concentration of glucagon. Although additive effects would not exclude the possibility that the two agents act on separate adenylate cyclases in a common cell population, a lack of additivity would make it improbable that they have separate target cells. These experiments were done on slices from AAF-treated animals. In these livers, before

tumour development, PGE₁ added alone increased the cAMP content 170%, and glucagon gave about a 10-fold increase. The cAMP content in slices exposed to a combination of the two agents, however, was lower than in slices exposed to glucagon alone. Inhibition by PGE₁ of the effect of glucagon on cAMP has previously been demonstrated by De-Rubertis *et al.* (1974) in liver *in vivo*. We have seen that PGE₁ also inhibits the effect of adrenalin on cAMP in hepatocytes (Brønstad & Christoffersen, unpublished).

DISCUSSION

The present results show that 3 different kinds of hepatomas, and several metastases from one of them, have greatly increased capacity to accumulate cAMP in response to E-prostaglandins, apparently due to the increased effect of these prostaglandins on adenylate cyclase. Increased effect of PGE₁ on cAMP levels or adenylate cyclase has also been observed in primary hepatomas and liver from ethionine-treated animals (Chayoth *et al.*, 1973). In our experiments we found that PGE₂ was also strongly stimulatory, while PGF_{1α} and PGF_{2α} had no significant effect on the cAMP level.

The data presented here also show that the responsiveness to adrenalin and PGE₁ develops very differently during the carcinogenic process. It has previously been shown that during liver carcinogenesis the adrenalin responsiveness increases during the first weeks of carcinogen treatment (Christoffersen *et al.*, 1972, 1974; Boyd *et al.*, 1974, 1976). After 7–12 weeks the responsiveness decreases (Boyd *et al.*, 1974, 1976), but in the final hepatomas the adrenalin responsiveness is still greater than in normal liver. This pattern was confirmed in the present study (Fig. 2). In contrast, the increased PGE₁ effect appeared much later and was not maximal until hepatomas had developed (Fig. 2). We do not know exactly at which stage the increased

PGE₁ responsiveness appears. In general, it is still not known whether changes in cell properties seen after malignant transformation result from continuous alterations during the process of carcinogenesis.

The interpretation of the present results, in terms of understanding the role of prostaglandins in the biology of hepatomas, is difficult at present. One problem is that although all the hepatomas investigated in the present study were strongly responsive to PGE₁ and PGE₂, we do not know so far whether this is a property of the hepatoma cells or of some other cell type in the tumour. We investigated whether the effects of PGE₁ and glucagon on premalignant liver were additive. The interpretation of these experiments, however, was hampered by the fact that PGE₁ inhibited the glucagon effect on cAMP accumulation, as has previously also been seen in normal rat liver *in vivo* (DeRubertis *et al.*, 1974). Therefore, these experiments did not give a final answer to the important question of the cellular localization of the increase in prostaglandin responsiveness in liver tissue during hepatoma development. The role of a prostaglandin-mediated increase of cAMP in hepatoma cells is also unclear. Cyclic AMP levels are high in various hepatomas (Thomas *et al.*, 1973; Chayoth *et al.*, 1972) as well as in liver tissue under conditions of rapid proliferation, such as in the neonatal period (Christoffersen *et al.*, 1973) and during regeneration (McManus *et al.*, 1972). Under appropriate conditions, cAMP administration leads to initiation of liver DNA synthesis *in vivo* (Short *et al.*, 1975). Prostaglandins have been proposed as positive as well as negative regulators of cell proliferation (Sykes, 1976; Jimenez de Asua *et al.*, 1975; Fehér & Gridali, 1974; Thomas *et al.*, 1973). In foetal hepatocyte cultures, PGE₁ has been shown to enhance incorporation of ³H-thymidine into DNA (Leffert *et al.*, 1976). Increased responsiveness to prostaglandins could therefore play a role in the phenotypic expression of the malignant state of some cancers.

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