The Effect of Cytosine Arabinoside and Bromodeoxyuridine on the Appearance of Tyrosine Aminotransferase in Cultured Foetal Hepatocytes of the Rat

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1. The acquisition of dexamethasone-inducibility of tyrosine aminotransferase activity by hepatocytes cultured from 15-day-foetal rat liver is blocked in the presence of cytosine arabinoside. 2. Similar results are obtained in the presence of bromodeoxyuridine. 3. No effects on steroid-inducibility of tyrosine aminotransferase are obtained with either of the above compounds in hepatocytes cultured from 19-day-foetal liver. 4. The inhibitory effects of the agents are substantially reversed after their removal from the culture medium. 5. The effects of bromodeoxyuridine suggest that cell differentiation, with respect to tyrosine aminotransferase-inducibility, occurs in cultures of 15-day-foetal hepatocytes. 6. The effects of cytosine arabinoside suggest that such an event is dependent on mitosis.

It is possible to study the differentiation of a variety of cells in culture. The transition from the respective precursor cell to its corresponding differentiated cell is usually characterized by the appearance of cell-specific markers such as the muscle isoenzyme of creatine kinase in myogenesis (Turner *et al.*, 1974), haemoglobin in erythropoiesis (Chui *et al.*, 1971) and chondroitin sulphate in cartilage (Marzullo & Lash, 1967).

In culture, it has been possible to test the requirement of cell division for cellular differentiation by using mitotic inhibitors such as cytosine arabinoside or fluorodeoxyuridine. It is also possible to investigate the effects of bromodeoxyuridine, which has been shown to block differentiation of several cell types (Rutter *et al.*, 1973).

Such experiments cannot be performed *in vivo* as the nucleoside analogues are usually lethal when given to the embryo.

By use of the mitotic inhibitors, it has been shown that cell division is a prerequisite for the differentiation of a variety of cell types in culture. These include muscle (Bischoff & Holtzer, 1969), erythrocytes (Campbell *et al.*, 1974), mammary cells (Stockdale & Topper, 1966), cartilage (Abbott & Holtzer, 1968) and even plant cells (Shininger, 1975). The incorporation of bromodeoxyuridine into the DNA of precursor cells of muscle (Bischoff & Holtzer, 1970), pancreas (Rutter *et al.*, 1978), erythrocytes (Miura & Wilt, 1971) and cartilage (Mayne *et al.*, 1973) has been shown to be effective

in blocking terminal differentiation of the respective precursor cells.

It has previously been reported that hepatocytes obtained from 15-day-foetal rat liver acquire the ability to synthesize tyrosine aminotransferase in response to glucocorticoids after 3 days of culture (Yeoh *et al.*, 1979). Since no enzyme is detected on day 1 of culture, it is proposed that the hepatocytes differentiate over the next 2 days of culture. In the present study, the effects of cytosine arabinoside and bromodeoxyuridine on such hepatocytes in culture were followed by assay of the liver-specific enzyme marker tyrosine aminotransferase. These experiments were performed to clarify whether the time interval necessary for the appearance of the enzyme was a maturation or aging period, or alternatively involved activation of the gene by a mechanism that was dependent on cell division. Experiments were also performed to determine whether enzyme accumulation can be prevented by the incorporation of bromodeoxyuridine into the DNA of the enzyme-deficient precursor cell.

Materials and Methods

Materials

Wistar albino rats were used. Gestational age was determined from the time of detection of spermatozoa in the vaginal tract and is accurate to ± 8 h.

[6-³H]Thymidine and L-[3,5-³H]tyrosine were obtained from The Radiochemical Centre, Amer-

sham, Bucks., U.K. L-Tyrosine and sodium diethyl-dithiocarbamate were from Merck, Darmstadt, Germany. Pyridoxal 5'-phosphate, 2-oxoglutarate, NADH, cytosine arabinoside, bromodeoxyuridine and dexamethasone were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. CM-Sephadex (C-50) was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden, and diaminobenzoic acid by Eastman Kodak Co., Rochester, NY, U.S.A. Collagenase was from Boehringer Mannheim, Mt. Waverley, Vic., Australia. Eagle's minimal essential medium was obtained in powder form from Flow Laboratories, Annandale, N.S.W., Australia, and foetal calf serum from Commonwealth Serum Laboratories, Parkville, Vic., Australia. Fungizone, penicillin/streptomycin and glutamine were purchased from Grand Island Biological Co., Grand Island, NY, U.S.A.

Assays

Aspartate aminotransferase was assayed by using a 'glutamate-oxaloacetate transaminase' single-vial reagent kit obtained from Calbiochem-Behring (Aust.) Pty. Ltd., N.S.W., Australia. Tyrosine aminotransferase was assayed by a radiochemical method described previously (Yeoh *et al.*, 1979), and 1 unit of enzyme activity is defined as the amount of enzyme that produced 1 nmol of *p*-hydroxyphenylpyruvate/h at 37°C. The two enzymes were assayed in fractions eluted from a CM-Sephadex column to which extracts from cultured hepatocytes had been applied (Yeoh *et al.*, 1979). Phosphoenolpyruvate carboxykinase was assayed spectrofluorimetrically as previously described (Bulanyi *et al.*, 1979). Protein content of samples was determined by using the dye-binding method of Bradford (1976), incorporating the modifications of Bearden (1978), against bovine serum albumin as a standard. DNA was assayed by a fluorimetric procedure (Hinegardner, 1971), with highly polymerized calf thymus DNA (Sigma) as a standard.

Hepatocyte culture

Cells were isolated from 19-day-foetal rat livers by the use of collagenase as described by Yeoh *et al.* (1979). Livers from 15-day fetuses were disrupted by aspiration in a wide-bore Pasteur pipette. The hepatocytes were maintained in Eagle's minimal essential medium supplemented with 10% (v/v) foetal calf serum, penicillin/streptomycin (57 units/ml and 570 µg/ml respectively), fungizone (28 µg/ml) and glutamine (2.4 mM) on 90 mm culture dishes (Sterilin Products, Teddington, Middx., U.K.) previously coated with collagen. Cultures were maintained at 37°C in a water-saturated atmosphere of 5% CO₂ in air.

Fresh medium was given to cultures 24 h after plating and 48 h thereafter. Dexamethasone dis-

solved in propylene glycol was added to a final concentration of 10 nM. Cytosine arabinoside and bromodeoxyuridine were dissolved in balanced salts solution (Hanks & Wallace, 1949), sterilized by Millipore filtration and added to cultures at the time of plating and after addition of fresh medium.

Preparation of extracts

Cells were harvested after removal of culture medium and a wash with 10 ml of balanced salts solution. Monolayer sheets of cells were scraped off the dishes into about 2 ml of balanced salts solution by using a Teflon 'policeman'. The cells were then transferred to centrifuge tubes by using a Pasteur pipette. The procedure was then repeated, the samples were pooled and the cells were harvested by centrifugation (1600 *g* for 5 min). For tyrosine aminotransferase assay, the pellet was suspended in 150 µl of 50 mM-potassium phosphate buffer, pH 6.8, then sonicated for 15 s at 2 A d.c. in a Branson model L5 75 Sonifier. The sample was then centrifuged in a Beckman Airfuge at 210 kPa (165 000 *g*_{max}) for 12 min in the A100 rotor. For phosphoenolpyruvate carboxykinase assay, the pellets were suspended in 50 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-dithiothreitol and homogenized in a Teflon/glass homogenizer, and the sample was centrifuged as before.

Radioautography

To determine the relative numbers of cells that were synthesizing DNA, cultures were exposed to [³H]thymidine (1 µCi/ml of culture medium) for 16 h, then washed with balanced salts solution six times and fixed *in situ* with 2.5% glutaraldehyde in 10 mM-cacodylate buffer, pH 7.4, at 5°C for a minimum of 24 h. The fixed cells were washed six times with balanced salts solution, then coated with Kodak NTB-S nuclear track emulsion. After a 2-week exposure at 5°C, the radioautographs were developed (Kodak D-19B) and fixed.

Results and Discussion

Effects of cytosine arabinoside and bromodeoxyuridine on DNA synthesis and cell proliferation

In control cultures, the cell numbers, as judged by the yield of DNA, increased by about 2.5-fold between day 1 and day 3 of culture. When these cultures are exposed to [³H]thymidine for 16 h, then processed for radioautography, counting of radioactivity reveals that 65–75% (range of three experiments) of hepatocytes are labelled. In the presence of 5 µg of cytosine arabinoside/ml (final concentration), no increase in DNA content of cultures was observed; in fact the cell yield on day 3 varied between 60 and 70% of day-1 controls. It should not

be construed from these data that the mitotic inhibitor was killing between 30 and 40% of the hepatocytes, since some fibroblasts are present in these cultures and it has been shown that cytosine arabinoside will kill and hence remove fibroblasts from cultures (Rubinstein *et al.*, 1976). In addition, since the mitotic inhibitor was added from the time of plating out the cells, it may also have affected the plating efficiency.

At a final concentration of $7.5\mu\text{g/ml}$, cytosine arabinoside diminished the extent of [^3H]thymidine-labelling of hepatocyte nuclei, as measured by radioautography, to 4 and 6% respectively in two experiments. Lower concentrations of inhibitor ($1\text{--}2\mu\text{g/ml}$) resulted in near-control extents of labelling, and no effect was observed on either DNA concentrations or tyrosine aminotransferase activities. High concentrations of the mitotic inhibitor ($10\mu\text{g}$ or more/ml) resulted in extensive cell death.

Bromodeoxyuridine ($150\mu\text{g/ml}$) elevated cell yields by 10–15% over controls after 3 days' exposure. From this observation it was assumed that DNA synthesis proceeds at a slightly elevated rate.

Effect of cytosine arabinoside and bromodeoxyuridine on tyrosine aminotransferase activity

Since it was previously shown that 10nm -dexamethasone in the culture medium maximized the yield of tyrosine aminotransferase (Yeoh *et al.*, 1979), all experiments were carried out in the presence of the steroid analogue. This optimized the detection of tyrosine aminotransferase-positive cells when they were present. The yields of tyrosine aminotransferase in cultured hepatocytes derived from 15-day foetuses exposed to cytosine arabinoside ($7.5\mu\text{g/ml}$) and bromodeoxyuridine ($150\mu\text{g/ml}$) were 59 ± 20 units/mg of protein and 113 ± 33 units/mg of protein respectively compared with that of controls, which was 736 ± 54 units/mg of protein (means $\pm$ S.E.M. for six experiments). The results show mean decreases in enzyme activity of 92% with cytosine arabinoside and of 85% with bromodeoxyuridine. It should be noted that in two experiments with cytosine arabinoside and in one with bromodeoxyuridine the enzyme activity was not detectable. As the limit of sensitivity of the assay was about 20 units/mg of protein, these samples were assumed to display 20 units/mg of protein and calculations of means and S.E.M. were performed accordingly. The mitotic inhibitor therefore largely prevented the appearance of enzyme activity, but the accumulation of enzyme was not completely abolished. This may be due to incomplete blockade of DNA synthesis at the dose of cytosine arabinoside used. Alternatively it may be explained by the presence of a few hepatocytes in 15-day-foetal liver that have already differentiated with respect to

inducibility of tyrosine aminotransferase, but have not accumulated sufficient enzyme activity on day 1 of culture to be detected. Such a minor population, after a further 2 days of culture in the presence of dexamethasone, might accumulate the very low enzyme activity that was observed, in spite of mitotic arrest. This alternative explanation is favoured by the fact that accumulation of enzyme activity was also largely but not completely suppressed in cultures grown in the presence of bromodeoxyuridine. Thus the steroid induction of enzyme activity in a few hepatocytes that are already differentiated in this function would not be prevented in the presence of bromodeoxyuridine. Clarification of this problem will require an assay method capable of detecting the enzyme in individual hepatocytes.

There are other possible explanations for the results obtained above that can be ruled out by other experiments. Inactivation of enzyme as a consequence of a direct interaction between the nucleoside analogues and tyrosine aminotransferase was eliminated by performing assays in the presence of cytosine arabinoside or bromodeoxyuridine. It is possible that the additives had a general cytotoxic effect on the hepatocytes, thus decreasing their ability to synthesize proteins or enzymes. To test this, aspartate aminotransferase and phosphoenolpyruvate carboxykinase were assayed in hepatocytes from 15-day foetuses cultured in the presence or absence of cytosine arabinoside or bromodeoxyuridine for 3 days. There was no diminution in the activities of these enzymes.

To show that these compounds were not specifically affecting tyrosine aminotransferase synthesis, experiments identical with those performed on 15-day-foetal hepatocytes were repeated with 19-day-foetal hepatocytes. The latter have previously been shown to be capable of tyrosine aminotransferase synthesis from day 1 of culture, in contrast with the former (Yeoh *et al.*, 1979). After 3 days of culture in the presence of cytosine arabinoside or bromodeoxyuridine, enzyme activities were determined to be 1593 ± 230 and 1835 ± 230 units/mg of protein respectively, which, when compared with the control value of 1640 ± 209 units/mg of protein (means $\pm$ S.E.M. for three experiments), provides evidence contrary to the view that cytosine arabinoside and bromodeoxyuridine have specific effects on tyrosine aminotransferase synthesis.

Finally, it could be argued that the nucleoside analogues selectively kill those hepatocytes that are destined to commence tyrosine aminotransferase synthesis during the 3 days of culture. To test this possibility, hepatocytes from 15-day foetuses were cultured in the presence of cytosine arabinoside or bromodeoxyuridine for 3 days and then returned to control medium for a further 3 days. The results

Table 1. Tyrosine aminotransferase activity in 15-day-foetal hepatocytes exposed to cytosine arabinoside or bromodeoxyuridine for 3 days, then shifted to control medium for 3 days

Hepatocyte cultures from 15-day-foetal rat liver were prepared and maintained in 10nM-dexamethasone as previously described (Yeoh *et al.*, 1979). At the time of plating cytosine arabinoside (7.5 µg/ml) or bromodeoxyuridine (150 µg/ml) was added to respective cultures. Cultures were given fresh medium the following day with appropriate supplements, and half the cultures were harvested on day 3, for enzyme assay. The remainder were subsequently maintained in control medium, supplemented with fresh medium on day 5 and then harvested on day 6 for enzyme assay.

	Tyrosine aminotransferase activity (units/µg of DNA)		
	Control	Cytosine arabinoside	Bromodeoxyuridine
Expt. I			
Day 3	700	42	55
Day 6	460	270	283
Expt. II			
Day 3	893	<20	93
Day 6	808	516	836

(Table 1) show that, whereas enzyme activity was suppressed by 90% or more on day 3, 60–100% of the control activity was obtained after returning the cells to control medium. The diminished enzyme activity that occurs between day 3 and day 6 in controls may be due to an increase in fibroblasts in the older cultures, lowering the specific activity of the enzyme as assayed.

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