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Induction of haem synthesis in Hep G2 human hepatoma cells by dimethyl sulphoxide

A transcriptionally activated event

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Exposure of cultured human hepatoma cells (Hep G2) to medium containing 2% (v/v) dimethyl sulphoxide resulted in an approximate doubling in the activity of δ-aminolaevulinate dehydratase, an increase in the haem content and a decreased growth rate; induced enzyme activity was decrease by 50% after treatment with α-amanitin. The findings are strikingly similar to those seen in murine Friend-virus-transformed erythroleukaemia cells.

INTRODUCTION

Treatment of Friend-virus-transformed murine erythroleukaemia (MEL) cells with DMSO is well known to lead to induction of haem-biosynthetic enzymes, haem, haemoglobin and terminal differentiation (Friend *et al.*, 1971; Sassa, 1976; Marks & Rifkind, 1978; Chang & Sassa, 1984). In human cells, DMSO induces phagocytosis and terminal differentiation of the leukaemia cell line HL60 (Collins *et al.*, 1978), but has no effect on the Philadelphia-chromosome-positive leukaemia cell line K562, despite the fact that erythroid maturation is induced in the latter cell type by other inducers such as sodium butyrate (Anderson *et al.*, 1979; Hoffman *et al.*, 1979). It seemed of interest to determine whether other human cell lines might also respond to DMSO and display responses similar to those observed in MEL cells. All three cell lines (MEL, HL60, K562) are derived from precursors in the bone marrow, a rich site of haem synthesis, and all have the potential to undergo differentiation through erythroid or granuloid pathways.

The liver is another major site of haem synthesis *in vivo*, but adult liver cells do not grow in culture. Although non-proliferating cultures of rat hepatocytes can be maintained *ex vivo*, they rapidly lose detectable activity of the second enzyme in the haem biosynthetic pathway, ALAD (Guzelian *et al.*, 1984). Hep G2 cells, isolated by Knowles *et al.* (1980) from a human hepatoblastoma, retain well-differentiated hepatocyte functions such as the secretion into the medium of albumin and 22 other plasma proteins that are known to be synthesized by normal liver (Knowles *et al.*, 1980; Gordon *et al.*, 1984; Wilson *et al.*, 1984; Rosner *et al.*, 1984; Murata *et al.*, 1985; Povoa *et al.*, 1985). They have also been used as model cells to study glycoprotein secretion (Bauer *et al.*, 1985), the glucose transporter (Haspel *et al.*, 1985), glycogen metabolism and insulin receptors (Verspohl *et al.*, 1984), 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and low-density-lipoprotein receptors (Cohen *et al.*, 1984) and malaria–hepatocyte interactions (Aikawa *et al.*, 1984). We therefore chose this cell line as the best available model of normal human liver.

This report describes for the first time the DMSO-mediated induction of haem synthesis and the haem-biosynthetic enzyme ALAD, as well as deceleration of cell growth in human hepatoma cells.

MATERIALS AND METHODS

Materials

DMSO (Spectro grade) was purchased from Eastman–Kodak, Rochester, NY., U.S.A., and α-amanitin was from Sigma, St. Louis, MO, U.S.A.; all tissue-culture materials were purchased from Gibco, Grand Island, NY, U.S.A., except for fetal-calf serum, which was purchased from Sterile systems, Logan, UT, U.S.A. All other reagents were of the highest purity commercially available.

Cell cultures

Hep G2 cells were generously given by Dr. Barbara Knowles of the Wistar Institute. Cells were routinely grown in 150 cm^2 flasks with 20 ml of minimal essential medium with Earles salts supplemented with 10% (v/v) fetal-calf serum, 100 units of penicillin/ml, 0.1 mg of streptomycin/ml and 2 mM-glutamine. Routine subculture was made weekly at 1:3, which maintained cells in the exponential growth phase; cells were fed with fresh medium every 2–3 days. Treatments with chemicals (e.g., DMSO or α-amanitin) were made directly to the flasks as indicated in the Figure legends. Cells were harvested by trypsin treatment for 5 min at 37 °C, washed and resuspended in Earles salts and, after 15 passages through a Pasteur pipette to break up clumps, their numbers were counted in a model-F_N Coulter counter.

Biochemical assays

ALAD assays and haem assays were performed in triplicate using 10^6 cells and 10^5 cells per assay respectively as previously described (Sassa, 1982; Chang & Sassa, 1984). Statistical analyses of data were performed by using Student's unpaired one-tail *t* test.

Abbreviations used: DMSO, dimethyl sulphoxide; MEL, murine erythroleukaemia; ALA(D), δ-aminolaevulinate (dehydratase); PBG, porphobilinogen.

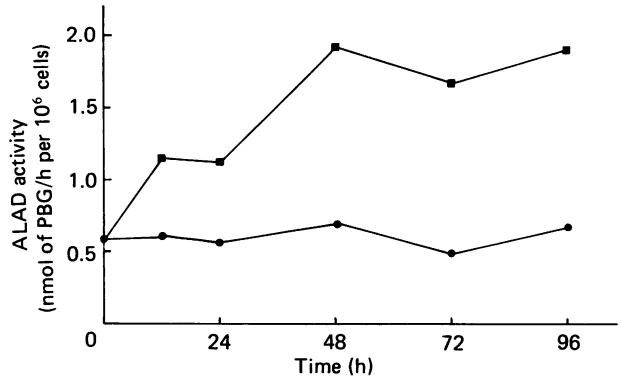

Fig. 1. Time course of the effect of DMSO on ALAD activity

Hepatoma cells were incubated with (■) or without (●) 2% DMSO from zero time, harvested at the indicated times, counted, and ALAD activity measured as described in the Materials and methods section. Two to three flasks were used to estimate each point, and assays were run in triplicate. Data are means ± S.E.M.; S.E.M. in all cases was less than 2% (i.e., less than the size of the symbol depicting the means). At each time point, DMSO-treated cells had significantly higher ALAD activity than control cells ($P < 0.001$).

RESULTS

Addition of DMSO to Hep G2-cell cultures for 96 h was found to cause an increase in ALAD activity compared with untreated controls. The maximum effect was elicited by 2% (v/v) DMSO, which consistenty resulted in a doubling of ALAD activity (0.75 ± 0.06 as against 1.61 ± 0.11 nmol of PBG/h per 10^6 cells; means for 10 expts., $P < 0.01$). A representative time course of ALAD activity after exposure to DMSO is illustrated in Fig. 1. The effect of 2% DMSO was both detectable and statistically significant within 12 h of treatment and rose to a maximum at 48 h, after which it was unchanged up to 96 h. Increased activity of ALAD was not a direct effect of DMSO, as direct addition of various concentrations of DMSO to the assay system *in vitro* addition were without effect on enzyme activity (results not shown). In order to determine whether the mechanism of DMSO action was mediated by an increased rate of synthesis of the specific mRNA coding for ALAD, we tested the effect of transcriptional inhibitors. Actinomycin D, even at very low concentrations (0.1 μg/ml), was toxic and inhibited cell growth almost totally. The inhibitor α-amanitin proved non-toxic and its effects are depicted in Fig. 2. Treatment of control cells with α-amanitin was without significant effect on ALAD activity, but diminished the increase observed in DMSO-treated cells by approx. 50%, strongly suggesting that this effect was dependent on mRNA transcription. α-Amanitin at the concentration used (0.2 μg/ml) did not significantly affect the cells' growth (results not shown).

Cells treated with 2% DMSO for 96 h also displayed small, but reproducible, increments in haem content (28.3 ± 3.03 as against 36.1 ± 3.37 pmol of haem/10^6 cells for control and DMSO-treated cells respectively; $n = 8$, $P < 0.01$). However, the same treatment inhibited cell growth such that DMSO-treated cultures contained 40% less cells than did corresponding control cultures

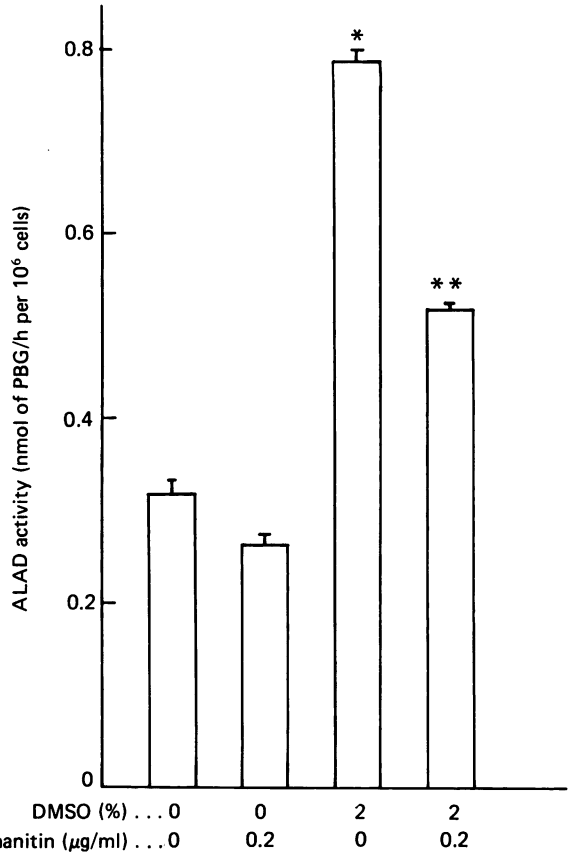

Fig. 2. Effect of α-amanitin on DMSO-induced ALAD activity

All cells were pre-exposed to 0.2 μg of α-amanitin/ml for 24 h. Medium was then aspirated, the cells washed with 20 ml of fresh medium, and fresh medium added for a further 48 h with or without DMSO (2%) or α-amanitin (0.2 μg/ml) as indicated at the bottom of the Figure. Bars represent means ± S.E.M. of triplicate determinations from two flasks per variable. *$P < 0.001$ versus control and versus 2% DMSO + α-amanitin; **$P < 0.001$ versus controls (with and without α-amanitin).

($7 \times 10^6 \pm 0.18 \times 10^6$ as against $4.81 \times 10^6 \pm 0.59 \times 10^6$ cells for control and DMSO-treatment cells respectively; $n = 6$), although protein content per cell was unchanged (0.34 ± 0.03 mg/10^6 cells against 0.35 ± 0.05 mg/10^6 cells for control and DMSO-treated cells respectively; $n = 6$).

DISCUSSION

We have demonstrated a readily detectable level of ALAD activity in human hepatoma cells and its induction in cells treated with DMSO. This finding is significant in that the level of ALAD in isolated rat hepatocytes rapidly decreases to undetectable levels in culture (Guzelian *et al.*, 1984). In contrast with ALAD activity, ALA synthase activity was undetectable in Hep G2 cells despite use of an assay that readily detects the enzyme activity in cultured chick-embryo liver cells (Sassa & Kappas, 1977). In contrast, PBG deaminase and uroporphyrinogen decarboxylase activities were detectable in Hep G2 cells, but not increased after exposure to DMSO (results not shown). The increased activity of ALAD in response to DMSO in Hep G2 cells

is diminished by treatment with α-amanitin at 0.2 μg/ml, a dose that has been shown to inhibit polymerase II specifically and hence inhibit mRNA synthesis, without significant effects on tRNA or rRNA synthesis (Swaneck *et al.*, 1979). This dose of α-amanitin was found to be non-toxic to cells, as judged from the rate of cell growth and cellular protein content, and did not affect the level of ALAD activity in untreated cells. Our findings suggest, therefore, that the increased activity of ALAD and the resultant increase in haem content in DMSO-treated hepatoma cells is transcription-dependent. DMSO has been shown to stimulate melanin synthesis in human melanoma cells (Huberman *et al.*, 1979), to stimulate albumin synthesis in murine and rat hepatocellular carcinoma cells (Higgins & Borenfreund, 1980) and to decrease alkaline phosphatase content and collagen substrate attachment of human pancreatic tumour cells (McIntyre & Kim, 1984). However, to our knowledge, effects of DMSO on the haem pathway have not been previously reported, except in MEL cells, wherein the changes bear a striking resemblance to those described above (Friend *et al.*, 1971; Sassa, 1976; Chang & Sassa, 1984).

It is also of interest that DMSO inhibits the rate of cell growth in human hepatoma cells, as shown in our experiments, which is in good agreement with the findings reported in MEL cells (Friend *et al.*, 1971; Sassa, 1976; Marks & Rifkind, 1978; Chang & Sassa, 1984) and various other tumour cells (Huberman *et al.*, 1979; Higgins & Borenfreund, 1980; McIntyre & Kim, 1984). It has been reported that the level of microsomal haemoproteins, especially cytochrome *P*-450, and mitochondrial cytochromes ($a+a_3$, b and c_1) are inversely related to the rate of growth of hepatomas and regenerating and normal liver (Sugimura *et al.*, 1966; Hagihara *et al.*, 1973). Haem is the prosthetic group of those cytochromes which are suppressed in fast-growing tumours, raising the possibility that haem synthesis might be inversely proportional to, or reflective of, cellular growth or differentiation.

It is possible that DMSO alters cell-cycle kinetics in a way analogous to that reported in MEL cells in which the G_1 phase is prolonged by DMSO treatment, with concomitant commitment to undergo erythroid differentiation (Gambari *et al.*, 1979), and in mouse hepatomas, in which DMSO leads to an increased percentage of cells in the G_2 phase with increased synthesis of albumin (Higgins, 1967). However, in the latter instance, albumin synthesis is known to be cell-cycle-dependent (Tsukada & Hirai, 1967), whereas ALAD, to our knowledge, is not.

It has been reported by other investigators that Hep G2 cells are capable of activating cyclophosphamide (Dearfield *et al.*, 1983) and benzo[*a*]pyrene (Diamond *et al.*, 1980) to genotoxic metabolites, chemical reactions generally ascribed to microsomal cytochrome *P*-450. More recently, Dawson *et al.* (1985) demonstrated the presence in Hep G2 cells of *O*-de-ethylation of 7-ethoxycoumarin, which was induced 24% after phenobarbital administration and 20–30-fold after 3-methylcholanthrene treatment. Western blots utilizing specific antibodies to liver cytochrome *P*-450 isoenzymes revealed the existence of a *P*-450 isoenzyme that was induced after phenobarbital and decreased after 3-methylcholanthrene. Thus not only haem synthesis, but also synthesis of haem-dependent cytochrome *P*-450 and its associated hydroxylase activities, are inducible in Hep G2 cells. This experimental system should provide a valuable means for studying the control of gene expression not only of individual human haem-pathway enzymes and cytochrome *P*-450s in liver, but also their co-ordinated induction in response to changing chemical environments.

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