Dimethyl sulphoxide induces a reduced growth rate, altered cell morphology and increased epidermal-growth-factor binding in Hep G2 cells

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Culture of Hep G2 cells in medium containing 2% (v/v) dimethyl sulphoxide (DMSO) resulted in a slowing of growth and a marked change in morphological appearance. By day 6, cultures containing DMSO had one-third the number of cells compared with parallel control cultures. Measurement of ^{125}I -epidermal-growth-factor (EGF) binding to DMSO-treated cells revealed a striking time-dependent elevation in specific EGF binding to their cell surface. Increased binding was detectable within 24 h of the start of DMSO treatment, reaching, by 6 days, levels almost 25 times greater than those for control cells. Addition of EGF to DMSO-treated cells caused a rapid down-regulation of the EGF receptor, but did not alter their proliferation rate. Slowing of growth by other means, such as serum starvation, growth to confluence or culture in the presence of sodium butyrate, did not affect ^{125}I -EGF binding, indicating a specific effect of DMSO on these cells.

INTRODUCTION

The Hep G2 cell line, derived from a human hepatoblastoma, retains many synthetic functions of normal hepatocytes [1]. More differentiated features, such as the preferential synthesis of albumin compared with α -foetoprotein and the induction of haem synthesis, can be induced by culturing at high cell density or in the presence of dimethyl sulphoxide (DMSO), conditions which slow the growth of this cell line [2,3]. Other experimental procedures to slow growth, such as culture without refeeding, modulate surface characteristics of these cells, exemplified by a 4-fold increase in insulin-receptor density [4].

These observations parallel findings on primary cultures of rat hepatocytes, where a reciprocal relationship between hepatocyte growth and the expression of differentiated functions has been observed [5]. Growth responses can be diminished by high density culture, by use of an extracellular matrix, and by phenobarbital, sodium butyrate and DMSO [5–11]. The expression and response of the epidermal-growth-factor (EGF) receptor at the cell membrane has received attention because of the evidence implicating this receptor in hepatic growth control *in vivo* [12,13]. DMSO diminishes the proliferative response of primary hepatocyte cultures to EGF at doses which do not affect EGF-receptor affinity [5]. We therefore studied the effect of DMSO on the interaction between EGF and Hep G2 cells. Reduced Hep G2 cell proliferation induced by DMSO was associated with a striking increase in EGF binding without acquisition of a proliferative response to EGF.

MATERIALS AND METHODS

Materials

[6- ^3H]Thymidine (sp. radioactivity 25 Ci/mmol) and carrier-free Na^{125}I were obtained from Amersham International, Amersham, Bucks., U.K. EGF was purified from male-mouse

submaxillary glands [14], and Collaborative Research tissue-culture-grade mouse EGF was obtained from Universal Biologicals, London S.W. 18, U.K. DMSO (spectrophotometric grade) was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Collagenase (from *Clostridium histolyticum*) was purchased from Boehringer-Mannheim, Lewes, East Sussex, U.K. Tissue-culture medium and supplements were from Gibco, Paisley, Renfrewshire, Scotland, U.K. Sephadex G-50 (superfine grade) was from Pharmacia-LKB, Milton Keynes, Bucks., U.K. Other reagents and chemicals were from Sigma Chemical Co. or BDH Chemicals, both of Poole, Dorset, U.K. Male August rats (250 g) were obtained from the National Institute of Medical Research, Mill Hill, London N.W.7, U.K., and kept with access to food and water *ad libitum*.

Cell culture

Hep G2 cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) heat-inactivated foetal-bovine serum (FBS), glutamine (2 mM), Hepes (18 mM), fungizone (1.25 $\mu\text{g}/\text{ml}$), penicillin (200 i.u./ml) and streptomycin (200 i.u./ml). Cells were seeded at $(0.5\text{--}1.0) \times 10^5$ cells/well of 24-well Falcon/Nunc tissue-culture plates and allowed to attach for 24 h. Adult rat hepatocytes were isolated by collagenase perfusion *in situ* similar to that described by Berry & Friend [15] as previously described [16]. Viability was greater than 80%. Cells were seeded at 1.5×10^5 /well on rat tail collagen in 24-well plates and cultured in 0.5 ml of William's medium E supplemented with 5% (v/v) FBS, dexamethasone (10^{-8} M), and antibiotics as described above. Cells were maintained at 37 °C in air/ CO_2 (19:1) with > 95% humidity. Under these conditions cells reach confluence at 6–7 days, and the cell layers start detaching from the wells at 7–8 days.

Scanning electron microscopy

Cells were grown on 15 mm-diameter Thermanox tissue-culture coverslips for 5 days with or without DMSO (2%, v/v).

Abbreviations used: DMSO, dimethyl sulphoxide; EGF, epidermal growth factor; FBS, foetal-bovine serum.

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They were then fixed in glutaraldehyde and OsO_4 , freeze-dried, coated in gold and examined with a Cambridge S600 scanning electron microscope.

Iodination of EGF

EGF was iodinated by using chloramine-T [17]. EGF, 10 μg in 50 μl of 0.4 M-sodium phosphate buffer, pH 7.6, was combined with 0.5–0.8 mCi (in 5–8 μl) of carrier-free Na^{125}I . Chloramine-T (5 μg in 10 μl of 0.04 M-sodium phosphate buffer) was added and mixed for 14 s at room temperature. The reaction was terminated by the addition of sodium metabisulphite (25 μg in 100 μl of 0.04 M-buffer) and NaI (100 μg in 50 μl of water). This mixture was then diluted with 115 μl of 0.04 M-phosphate buffer containing 10% (w/v) sucrose. The total sample was applied to a Sephadex G-50 (superfine grade) column (1.1 cm $\times$ 32 cm) pre-equilibrated with phosphate/EDTA buffer containing BSA [54 mM- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ /7 mM- KH_2PO_4 /10 mM-EDTA/0.3% (w/v) BSA, pH 7.2] and eluted with the same buffer. The first peak contained ^{125}I -EGF, which accounted for 40–60% of the radioactivity added to the column. Fractions in which greater than 95% of the radioactivity was precipitable with cold 10% (w/v) trichloroacetic acid were pooled. The sp. radioactivity of the label, as determined by radioimmunoassay, was between 15 and 60 $\mu\text{Ci}/\mu\text{g}$. It was stored in 0.2 ml aliquots at -20°C .

^{125}I -EGF-binding assay

Cells were washed twice in Williams' medium E containing 0.1% (w/v) BSA and 20 mM-Hepes (binding medium) and cooled on ice. Incubations were in 0.5 ml of binding medium containing ^{125}I -EGF (4×10^5 c.p.m.) for 2.5 h on ice. For Scatchard analysis, increasing amounts of ^{125}I -EGF (50–3000 fmol; sp. radioactivity 15–25 $\mu\text{Ci}/\mu\text{g}$) were used. Cells were washed four times with ice-cold Hanks balanced-salt solution containing BSA (0.1%) and treated twice with 0.6 ml of 0.2 M-acetic acid, pH 2.5, containing NaCl (0.5 M) for 6 min [18]. Acid washes were combined and the radioactivity was determined. Non-specific binding was determined in the presence of excess (1 $\mu\text{g}/\text{ml}$) unlabelled EGF and subtracted from total radioactivity bound to obtain specific binding. Cells pre-treated with EGF were washed twice (2×1 min) with a solution containing glycine (50 mM) and NaCl (0.1 M), pH 4, to remove surface-bound EGF before the binding medium washes and binding assay [19].

Estimation of DNA synthesis

After culturing the cells for 3 days with or without DMSO (2%), cells were transferred to serum-free medium for 24 h ($\pm$ DMSO). At the end of this period, FBS (to 5%) or EGF (to 40 ng/ml) were added to the wells (in triplicate) and the cells incubated for a further 14 h. [^3H]Thymidine (2 $\mu\text{Ci}/\text{well}$) was added to the cells for the last 2 h of culture. Trichloroacetic acid-insoluble radioactivity was then determined [20]. Aliquots (50 μl) of the final hydrolysate were counted in a β -scintillation counter. [^3H]Thymidine incorporation was expressed per mg of total cell protein.

DNA measurement

Cells were washed three times with PBS (1 ml) and solubilized in 20 mM-Hepes buffer, pH 7.5, by sonication for 20 s. Samples were stored at -20°C before assay. DNA was estimated by fluorescence at 454 nm after forming a complex with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) [21]. Calf thymus DNA (0–200 ng/ml) was used as a standard.

Protein measurement

Cells were washed three times with PBS (1 ml) and solubilized in 0.5 ml of a $\text{NaCO}_3/\text{NaOH}$ solution (10%/0.5 M) by sonication

for 20 s. Protein was estimated by a modification of the method of Lowry [22], with BSA as a standard. Samples were stored at -20°C before assay.

RESULTS

Cell numbers, protein and DNA

The cells were allowed to adhere for 24 h in the absence of DMSO. The medium was replaced with fresh medium with or without DMSO and subsequently replaced daily with similar medium. Every 24 h, cells from four wells were trypsin-treated and counted in medium containing Trypan Blue (0.02%) with a haemocytometer. Initial experiments demonstrated that DMSO caused a dose-related reduction in cell proliferation. At doses of 3% DMSO or less there was no reduction of cell viability ($> 95\%$ in both cultures), but at higher concentrations increasing numbers of dead cells were present. Routinely further experiments were therefore performed with 2% DMSO. There was a significant reduction in the number of cells present in wells containing 2% DMSO compared with control cells at 48 h (Fig. 1a). After 6 days there were 3-fold more cells in control cultures than in 2% DMSO-treated cultures. The changes in DNA and protein per well in DMSO-treated and control Hep G2 cells paralleled changes in cell numbers (Figs. 1b and 1c). DNA per cell did not differ between DMSO and untreated cultures at any time point (approx. 10 $\mu\text{g}/10^6$ cells).

Microscopic examination

The optical-microscopic appearance of the cells in 2% DMSO were similar to that of control cells. Only slight changes could be observed under a phase-contrast microscope. Treated cells formed a more uniform monolayer and appeared to have a firmer attachment to the dish. Although DMSO treatment reduced the cell numbers, visually there was not such a striking difference in confluence after 6 days. However, control cells piled up on each other forming a thicker final cell layer and began to detach from the wells after 7 days. Scanning electron microscopy confirmed that DMSO-treated cells were flatter and spread to a greater extent (Fig. 2).

^{125}I -EGF binding

Cells cultured in 2% DMSO showed a time-dependent increase in binding of ^{125}I -EGF (Fig. 1d). After 6 days cultures in DMSO, cells bound almost 25 times more labelled EGF than control cells (8767 as against 355 c.p.m./ 10^6 cells respectively). Further binding studies were carried out on cells cultured for 5 days with or without 2% DMSO, the binding being measured in the absence of DMSO. Untreated Hep G2 cells bound a very small proportion of the radioactivity added (and typically non-specific binding accounted for 60–90% of radioactivity bound). The binding data from control Hep G2 cells was therefore not analysed by Scatchard analysis. Scatchard plots for DMSO-treated Hep G2 cells and primary rat hepatocytes are shown in Fig. 3. DMSO-treated cells had a K_d of 0.24 nM and expressed 1.6×10^4 receptors per cell, whereas primary hepatocytes after 27 h in culture had a K_d of 0.71 nM and expressed 1×10^5 receptors per cell. To show that this increase in ^{125}I -EGF binding was not just due to non-specific growth arrest, binding of ^{125}I -EGF to cells in serum-free culture over a 7 day period was measured (results not shown). No increase in EGF binding was observed. Likewise increases in binding were not seen when cell growth was slowed due to confluence or when sodium butyrate (2–4 mM) was used to slow the growth of Hep G2 cells (results not shown). The effect of increasing concentrations of DMSO on ^{125}I -EGF binding and cell number is demonstrated in Fig. 4.

Fig. 1. Effect of DMSO on (a) the growth rate, (b) cell protein, (c) cell DNA and (d) binding of ^{125}I -EGF to the surface of Hep G2 cells

Cells were grown for 6 days with (○) or without (●) 2% (v/v) DMSO. Every 24 h cells were counted, DNA and protein determined and ^{125}I -EGF binding measured. The points represent the mean of triplicate measurements. All panels represent the results from typical experiments (the bars represent S.E.M.). In each case at least two further experiments gave similar results.

Fig. 2. Scanning electron micrographs of Hep G2 cells (a) Control cells; (b) cells cultured in the presence of DMSO (2%, v/v) for 5 days. (The bar represents 40 μm .)

Fig. 3. Analysis of EGF binding to DMSO-treated Hep G2 cells (○) and primary hepatocytes (●)

(a) Binding of increasing concentrations of ^{125}I -EGF by 5-day DMSO-treated Hep G2 cells compared with primary hepatocytes after 27 h in culture. (b) Scatchard analysis of data. Linear regression analysis was performed; $r^2 = 0.89$ for primary cells; $r^2 = 0.95$ for Hep G2 cells.

EGF-receptor down-regulation

EGF (20 ng/ml) caused a down-regulation of the DMSO-induced increase in EGF binding. Cells were cultured for 5 days

Higher doses of DMSO (up to 4%) induced a more dramatic effect on EGF binding and cell proliferation, but, as previously mentioned, cell viability was compromised above 3%.

Fig. 4. Dose-response effect of DMSO on ^{125}I -EGF binding and final cell density

Cells were cultured for 5 days with the indicated concentrations of DMSO. Cell monolayers were washed twice, and cell numbers and ^{125}I -EGF binding were determined as described in the Materials and methods section.

Table 1. DNA synthesis in Hep G2 cells in response to EGF

Cells were treated with or without DMSO in the presence of 5% FBS for 3 days. At this time medium was replaced with serum-free medium with or without DMSO. After 24 h medium was replaced. Half of the cells treated with DMSO were cultured in medium without DMSO. The factors indicated were added, EGF (to 40 ng/ml) or FBS (to 5%), and cells cultured for 14 h. ^3H Thymidine (2 μCi /well) was added for the last 2 h of culture. Data represent means $\pm$ S.D. for triplicate wells in a typical experiment. Two further experiments gave similar results.

Treatment	^3H Thymidine incorporation (d.p.m./mg of protein)		
	Untreated cells	DMSO-treated cells	
		+DMSO	-DMSO
Control	308 $\pm$ 36	284 $\pm$ 49	331 $\pm$ 26
FBS	997.7 $\pm$ 148	2818 $\pm$ 120	3397 $\pm$ 620
EGF	326 $\pm$ 62	364 $\pm$ 70	347 $\pm$ 25

in 2% DMSO followed by a 2 h incubation with EGF (20 ng/ml). Binding studies were carried out using 2 nM- ^{125}I -EGF. Cells treated with EGF bound 1895 ± 51 (mean $\pm$ S.E.M.) c.p.m./ 10^6 cells. The magnitude of this effect after 2 h was comparable with the down-regulation seen in primary hepatocytes under similar incubation conditions (results not shown).

Proliferative response to EGF

Before addition of EGF, cells were cultured under serum-free conditions for 24 h. EGF (40 ng/ml) did not stimulate thymidine incorporation in either control or DMSO-treated cells, even after removal of DMSO and despite stimulation of DNA synthesis by re-addition of serum (Table 1).

DISCUSSION

EGF-receptor protein is present in detergent extracts of Hep G2 cells [23], but the data reported here indicate that, under conventional culture conditions, only very low levels of surface ^{125}I -EGF binding occur. This concurs with the observation that EGF binding to Hep G2 cells was the lowest observed among a panel of six hepatocellular carcinoma-derived cell lines [24], and

the low level of binding of an anti-(EGF receptor) monoclonal antibody to this cell type [23]. However, after culture in the presence of 2% DMSO, EGF binding to Hep G2 cells is dramatically enhanced.

Enhanced binding could result from either an increase in the affinity of the receptor for EGF or an increase in the number of receptors present at the cell surface. The options could not be distinguished, since untreated cells specifically bound such small amounts of ^{125}I -EGF as to render assessment of receptor affinity inaccurate. The increase in EGF binding reflects a specific action of DMSO, rather than merely a general slowing of the growth rate, since slowing proliferation by transfer to serum-free medium or culture with sodium butyrate did not enhance EGF binding. As DMSO is highly polar and freely diffusible through the cell membrane, actions at the level of protein synthesis or gene expression may occur; alterations in haem synthesis induced in this cell line by DMSO were attributed to changes in transcription [3].

There are a number of observations on the effect of DMSO on EGF binding to normal rat hepatocytes in primary culture. When exposed to 2% DMSO for 2 h, there was no effect on EGF binding [5], although concentrations of 5–7.5% reduced receptor affinity [25]. Over longer time periods (80 h), the gradual reduction in EGF binding normally observed in culture is prevented by DMSO [5], due in part to preservation of a high-affinity EGF-receptor subpopulation [26]. This clearly differs from the progressive increase in EGF binding to Hep G2 cells over time.

Recently Chan *et al.* [27] reported that removal of DMSO from primary hepatocyte cultures while retaining EGF in the medium could initiate rapid proliferation in primary hepatocytes, suggesting that receptor internalization and initiation of DNA synthesis occur normally once the DMSO is removed. Intriguingly, once this cycle of proliferation was exhausted, further exposure to DMSO followed by withdrawal of DMSO again induced a proliferative response. This observation could possibly reflect an action of DMSO permitting re-expression of the EGF receptor in adult primary cells. It is possible that our observations on Hep G2 cells illustrate a similar phenomenon.

Despite the development of increased binding of EGF to Hep G2 cells, they did not develop a proliferative response to EGF, even after removal of DMSO. Under the conditions of culture used, however, in the absence of DMSO we have not found the low level of proliferation in response to EGF that some authors have reported [24] and, like others, we regard Hep G2 cells as being non-responsive to EGF [28]. The failure of EGF to modulate growth under conditions in which EGF binding is markedly enhanced and EGF-receptor down-regulation occurs confirms that proliferation in this tumour-derived cell line is not controlled by EGF-receptor-mediated stimulation. However, the ability to modulate EGF binding in a readily available cell line provides a valuable model in which cell-surface events relevant to growth stimulation may be studied.

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