Regulation of epitectin production in a malignant cell line

SCOTT A. BADER and HENRY HARRIS

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, England

Summary

RT112 cells, a line derived from a human bladder carcinoma, produce epitectin at very low levels in standard culture media; but production and secretion of this mucin are greatly increased when the cells are exposed to hyperosmotic conditions. It appears that hyperosmolarity, by inducing an increase in intracellular sodium, entrains an increase in intracellular free calcium. Evidence is presented for the view that it is the increase in intracellular free calcium that provides the more direct stimulus for the enhanced production of epitectin.

Key words: epitectin, malignancy, calcium.

Introduction

Epitectin is a mucin that is found on the surface of a wide range of malignant human cells and certain specialized normal epithelia (for reviews, see Harris, 1984, 1987). It can be detected in cell cultures or in sections of tissue by means of the Ca group of monoclonal antibodies (Ashall et al. 1982; Bramwell et al. 1985). The main structural features of the molecule have been established (Wiseman et al. 1984; Bramwell et al. 1986), and it has been shown to be subject to a genetically determined polymorphism (Swallow et al. 1987). Because this mucin has been found on the luminal surface of the urothelium and of the ducts of sweat glands, where the epithelial cells confront fluids of pH and osmolarity far outside the normal physiological range, it has been suggested that its function might be to protect these cells from extracellular conditions that would otherwise be destructive.

The presence of very high concentrations of lactate in sweat (Kaiser et al. 1974) suggested the idea that lactate might be involved in regulating the synthesis of epitectin. The effect of added lactate was therefore tested in a range of malignant human cell lines. Most of these synthesized epitectin constitutively and were unaffected, or only marginally affected, by the addition of lactate. However, one line derived from a renal carcinoma, RT112, was found to synthesize only very small amounts of epitectin under conventional conditions, but showed a marked enhancement of epitectin synthesis in the presence of added lactate (Bramwell et al. 1983). D-Lactate was as effective as L-lactate in this respect, thus indicating that the induced synthesis did not require further metabolism of the lactate molecule. In the present paper we examine the induction of epitectin synthesis in RT112 cells in more detail.

Materials and methods

Cells

RT112/84 is a clonal derivative of the cell line originally derived from a bladder carcinoma by Marshall et al. (1977). All the experiments described in the present paper were done with this clonal line. It was routinely tested and shown to be mycoplasma-free.

Chemicals

Ouabain octahydrate, ethyleneglycolbis[(S-aminoethyl)ether]-N,N′-tetraacetic acid (EGTA), 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester hydrochloride (TBM8), amiloride, nigericin, calcium ionophore A23187 and the [acetoxymethyl]ester of Quin 2 (Quin 2-AM) were from Sigma. Monensin sodium salt was from Calbiochem.

Media

Except where stated otherwise, the cells were grown in Dulbecco’s modified minimum essential medium (DMEM). A standard stock of hyperosmolar DMEM was made by adding 1 ml of racemic lactic acid to 100 ml of DMEM. The pH was adjusted to 7.4 by the addition of 4-n sodium hydroxide; the final concentration of sodium lactate in the solution was 133 mM. This stock was diluted with DMEM to give lower concentrations of lactate, as required.

Monoclonal antibodies

The Cal antibody (Ashall et al. 1982) was used to detect epitectin. M27, an antibody that recognizes an antigen
Assays for epitectin

In cell extracts. Cell pellets were extracted with deoxycholate and reacted with the Cal antibody. The immunoprecipitates were resolved by electrophoresis in gradient gels and the epitectin located in the gels by affinity labelling with 125I-labelled wheatgerm agglutinin. Details of the methodology are given by Ashall et al. (1982).

On the cell surface. The relative amounts of epitectin present on the surface of the cells were estimated by the indirect saturation binding assay described by Williams (1977). The assays were done in duplicate in microtitre wells and the epitectin located on the cells by affinity labelling with 125I-labelled rabbit F(ab')2 anti-mouse immunoglobulin G (IgG) as the second antibody. The results are expressed as net mean cts min\(^{-1}\) of labelled antibody bound after deduction of the background given by the negative control.

In tissue sections. Epitectin can be detected in routine paraffin sections by standard immunoperoxidase techniques. The procedure described by McGee et al. (1982) was used in the present experiments.

In single cells. The cells were grown on coverslips, fixed in methanol containing 10% (v/v) hydrogen peroxide and then stained by the same immunoperoxidase procedure as that used for tissue sections.

Examination of tumours

Inocula of 10\(^6\) RT112 cells were injected subcutaneously into the flanks of young adult nude mice and the resulting tumours excised after intervals of 7, 15 and 33 days. The tissues were fixed in formol-saline and standard paraffin sections prepared. These were then stained by the immunoperoxidase procedure as described.

Quin 2 spectrofluorimetry

The method used was essentially that described by Tsien (Tsien, 1980; Tsien, Pozzan & Rink, 1982; Grynkiewicz et al. 1985). The cells were harvested in phosphate-buffered saline containing 0·02% (w/v) sodium azide. They were stored at —20°C.

Background fluorescence was calculated from measurements on a cell suspension incubated with 10 \(\mu\)l of dimethylsulphoxide but no Quin 2. The addition of MnCl\(_2\) had no effect on background fluorescence, but Triton X-100 produced a slight increase. This increase was measured in each experiment and subtracted from the \(F_{\text{max}}\) value obtained on cells loaded with Quin 2. Since, in the present experiments, comparative and not absolute values for [Ca\(^{2+}\)]\(_{\text{cyt}}\) were of interest, no adjustment was made for possible quenching of the Quin 2 signal as a consequence of intracellular heavy-metal chelation. Appropriate checks established that any possible leakage of Quin 2 into the extracellular compartment was too small to affect the intracellular fluorescence measurements.

Results

Induction of epitectin by lactate

Racemic D-L-lactate at a concentration of 133 \(\text{mM}\) induced a dramatic increase in the amount of epitectin produced by the RT112 cells. Within 48 h the amount of epitectin immunoprecipitated from cell extracts by the Cal antibody increased by a factor of about 4. Epitectin was also shed into the medium. An accurate time course for the accumulation of epitectin in the medium has not yet been established, but in a confluent cell culture, maximally induced, there is about as much epitectin in the medium as in the cells. The increase in the amount of epitectin on the cell surface was much greater than the increase in the total cellular epitectin: as measured by the indirect binding assay, 10- to 20-fold increases in surface epitectin were regularly observed. Maximal induction was usually seen between 48 and 72 h after the addition of the lactate. At equimolar concentration, D-lactate was as effective as L-lactate. Cells continued to grow in
Fig. 2. A. RT112 cells grown in DMEM and stained for epitectin by the immunoperoxidase procedure. Only an occasional cell shows a positive immunoperoxidase reaction. B. RT112 cells grown in 133 mM-sodium lactate. Virtually all cells show a strong immunoperoxidase reaction. C. Section of a tumour-produced by RT112 cells. The tissue has been stained for epitectin by the immunoperoxidase procedure. The yellow tint at the degenerating centres of the nodules indicates the presence of epitectin. D. A later stage in which the centres of the nodules have undergone necrosis. Epitectin has accumulated in the necrotic areas and especially in the rim of living cells surrounding them.
133 mM-sodium lactate, but at about half their normal rate in DMEM. These findings confirm and extend observations previously reported (Bramwell et al. 1983; Harris, 1987).

The effect of sodium lactate in inducing epitectin production was dose-dependent and reversible. As shown in Fig. 1, there was a progressive increase in surface epitectin with increasing concentrations of added sodium lactate, reaching a maximum at a concentration of about 150 mM. If cells exposed to lactate for 48 h were returned to medium without lactate, the amount of intracellular and surface epitectin slowly decreased, regaining the uninduced level after 5 or 6 days. Examination of cell populations by immunocytochemistry with the Cal antibody revealed that in uninduced populations of RT112 cells between 5 and 10% of the cells gave a positive immunoperoxidase reaction. In a fully induced culture, more than 90% of the cells gave a positive reaction. The intensity of staining showed marked variation from cell to cell (Fig. 2A, B). It thus appears that the lactate effect is due at least in part to the induction of epitectin synthesis in cells that, in DMEM alone, do not synthesize the molecule or do so at an undetectable level. The few cells that give a positive immunoperoxidase reaction in uninduced populations are not a genetically stable subpopulation. Re-cloning the culture generates subclones that continue to produce small numbers of immunoperoxidase-positive cells.

The effect of increased osmolarity of the medium

Since D-lactate, which is not metabolized, was as effective as L-lactate in inducing epitectin in the cells, it was clear that the induction did not require metabolic events involving lactate. In the light of this absence of steric specificity and of the very high concentrations of lactate required to achieve epitectin induction, it was suggested by S. J. Goss that the lactate might be acting in a completely non-specific way by increasing the osmolarity of the medium: the addition of 113 mM-sodium lactate to DMEM increases the osmolarity from about 330 mosmol kg\(^{-1}\) to 590 mosmol kg\(^{-1}\). This possibility was explored by examining the effects of comparable increases in osmolarity produced by the addition of other agents to the medium.

NaCl (133 mM) and sucrose (266 mM) were added to DMEM to produce an increase in osmolarity equivalent to the addition of 133 mM-sodium lactate, and the effects of these additives on epitectin induction were monitored. Sucrose at 266 mM was too toxic to permit a 48 h experiment to be satisfactorily completed, but at 200 mM this was possible. The results are shown in Fig. 3. It will be seen that NaCl and sucrose are about as effective as sodium lactate in inducing the appearance of epitectin on the surface of the cell. Similar results were obtained with sodium sulphate and with sodium pyruvate. Dose–response curves were established for NaCl and sucrose and were found to be closely similar to that given by sodium lactate (Fig. 1).

If the NaCl content of DMEM was reduced by 66 mM and 66 mM-sodium lactate was substituted, a solution was produced that was isosmotic with DMEM but contained enough sodium lactate to induce epitectin if it had simply been added to DMEM. Fig. 4 shows that the addition of sodium lactate is effective in inducing epitectin only if this results in a hyperosmotic medium; the same concentration of lactate in an isosmotic medium is without effect.
If the NaCl content of DMEM was reduced by 66 mM and water substituted, the osmolarity of the medium was reduced from about 330 mosmol kg\(^{-1}\) to about 150 mosmol kg\(^{-1}\). As shown in Fig. 5, hypotonic medium does not induce epitectin. It can therefore be concluded that the addition of lactate does indeed act non-specifically through an increase in the osmolarity of the extracellular phase; a decrease in osmolarity is ineffective.

The effect of electrolyte movements

Sodium. The cell responds to an increase in the osmolarity of the extracellular fluid by raising the intracellular Na\(^+\) concentration. If the induction of epitectin by hyperosmolarity is mediated by an increase in intracellular Na\(^+\) concentration, one might expect that other procedures that increase intracellular Na\(^+\) concentration might induce epitectin. The effects of ouabain, monensin, nigericin and amiloride were therefore studied. Ouabain produces an increase in intracellular Na\(^+\) concentration by inhibiting Na\(^+\)/K\(^+\) exchange across the cell membrane; monensin produces it by stimulating Na\(^+\)/H\(^+\) exchange. At the maximum concentration that permitted a 48 h experiment to be completed (3\(\times\)10\(^{8}\) M), ouabain produced only a marginal increase in surface epitectin. When added together with sodium lactate, it enhanced the effect of sub-maximal concentrations of lactate (33 mM and 66 mM) by a factor of about 2. Monensin was a little more effective: at concentrations of 0·5 and 2·0 \(\mu\)M it produced a two- to fourfold increase in surface epitectin, but no dose-response curve could be established.

Nigericin stimulates efflux of intracellular K\(^+\) and influx of H\(^+\), thus producing a fall in intracellular pH. This, however, stimulates Na\(^+\)/H\(^+\) exchange, so that a secondary consequence of the action of nigericin is an increase in intracellular Na\(^+\). Fig. 6 shows the effect of increasing concentrations of nigericin on epitectin production.

Fig. 4. The effect of adding sodium lactate to the medium and of replacing some NaCl with sodium lactate. a. DMEM; b, DMEM in which 66 mM-NaCl has been replaced by 66 mM-sodium lactate; c, DMEM + 66 mM-sodium lactate; d, DMEM + 66 mM-NaCl.

Fig. 5. The effects of hyperosmolarity and hypoosmolarity compared. a. DMEM; b, DMEM + 66 mM-NaCl; c, DMEM in which 66 mM-NaCl has been replaced by water.

Fig. 6. The effect of increasing concentrations of nigericin on epitectin production.
Fig. 7 shows that amiloride at 0.5 mM greatly reduces the effect of 66 mM-lactate in inducing epitectin and also produces some reduction in the basal amount of epitectin produced in the cells in DMEM alone.

These experiments do, indeed, indicate that an increase in intracellular Na⁺ is involved in some way in mediating the induction of epitectin by extracellular hyperosmolarity; but the evidence is indirect and the overall effects of stimulating the movement of Na⁺ into the cell by other agents are not striking.

**Calcium.** There is evidence for interaction between Na⁺ and Ca²⁺ across cell membranes (al-Shaikhaly et al. 1979; Smith et al. 1982; Brass, 1984; Kaczorowski et al. 1985; Nedergaard, 1984), and a rich literature testifies to an important role for free Ca²⁺ in the regulation of many intracellular events (Campbell, 1983). It was therefore of interest to see whether procedures that increased or reduced the flux of Ca²⁺ within the cell had any effect on the induction of epitectin. Increase in the concentration of intracellular Ca²⁺ was produced by the Ca²⁺ ionophore, A23187; decrease by chelation of the extracellular calcium with EGTA or by the use of the inhibitor TMB8. Fig. 8 shows the dose–response curve for the Ca²⁺ ionophore, A23187. It will be seen that at ionophore levels between 5 × 10⁻⁷ and 10⁻⁶ M there is a concentration-dependent induction of epitectin, which, at its peak, results in an approximately 10-fold increase in the amount present on the surface of the cell. At concentrations of ionophore greater than 10⁻⁶ M, increasing toxicity reduces the response. On the other hand, if EGTA is added to the medium at a concentration sufficient to chelate out all unbound calcium (2.7 mM), the induction of epitectin produced by 66 mM-lactate is completely inhibited (Fig. 9). Addition of 200 μM-CaCl₂ to the medium containing EGTA relieves the inhibition. Although the precise mode of action of TMB8 has not yet been resolved, this compound has been shown to inhibit a variety of cellular responses mediated by an increase in cytoplasmic free Ca²⁺ (Chiou & Malagodi, 1975; Rittenhouse-Simmons & Deykin, 1978; Smith & Iden, 1979; Owen & Villereal, 1982; Grier & Mastro, 1985). At a concentration of 40 μM, TMB8 reduced by about half the induction of epitectin produced by the addition of 133 mM-sodium lactate. Higher concentrations of TMB8 were too toxic to permit the experiment to be completed.

**Direct measurement of intracellular free Ca²⁺ levels**

The substantial and concentration-dependent induction of epitectin by the Ca²⁺ ionophore A23187 made it seem worthwhile to investigate by more direct
measurements whether hyperosmolarity of the extracellular fluid did in fact produce an increase in intracellular free Ca\(^{2+}\) levels. Quin 2 fluorescence measurements were made, as described, on 14 samples of RT112 cells grown in DMEM and 28 samples of cells grown for 48 h in DMEM + 133 mM-sodium lactate. For cells grown in DMEM, the mean value for intracellular Ca\(^{2+}\) was 74 nM with a standard deviation of 15 nM; for cells grown in 133 mM-lactate the mean value was 561 nM with a standard deviation of 353 nM. While there are uncertainties in the relationship between Quin 2 fluorescence and absolute values of intracellular free Ca\(^{2+}\), it is clear that in the hyperosmotic medium, the intracellular free Ca\(^{2+}\) levels are much higher than in DMEM.

**Induction of epitectin in vivo**

RT112 cells grow in vivo as small nodules separated by a well-developed stroma. In the earliest tumours excised, no immunoperoxidase staining of epitectin was seen. The tumours removed 15 days after inoculation, however, were stained by the immunoperoxidase procedure, but not uniformly. The epitectin first makes its appearance in the central areas of the larger nodules where the cells, under the stress of an inadequate nutrient supply, are beginning to show early degenerative changes. These central areas undergo necrosis as the nodules enlarge further, and the immunoperoxidase staining is heaviest in the necrotic regions and especially in the rim of surviving cells that surround them (Fig. 2C,D). While no measurements appear to have been made of the osmolarity of the extracellular fluid in the central areas of such tumour nodules, it is known that in such areas lactate may accumulate and a substantial fall in the extracellular pH may occur (Vaupel et al. 1981). It is therefore not unreasonable to suppose that where the nutrient supply is exceeded the tumour cells become exposed to extracellular fluids of highly abnormal composition. If so, the synthesis of epitectin in the tumour cells may reflect the operation of the same regulatory mechanisms as control its synthesis in urinary or sweat gland epithelia where the cells also confront a physicochemical environment far outside the limits of normal extracellular fluid. The relationship between the phenomena observed in RT112 cells in vitro and the regulation of epitectin synthesis in vivo is being further investigated.

**Discussion**

The present experiments show decisively that the production of epitectin by RT112 cells is dramatically increased when the osmolarity of the extracellular fluid is raised. This increase in epitectin production is achieved, at least in part, by the recruitment of cells that either do not produce epitectin under standard conditions, or produce it in amounts too small to be detected by immunocytochemical procedures. The ability of high concentrations of sodium lactate to induce epitectin synthesis is a non-specific effect mediated by the concomitant hyperosmolarity of the extracellular fluid. Evidence is presented for the view that the extracellular hyperosmolarity acts by raising the level of intracellular Na\(^{+}\) which, in turn, produces an increase in intracellular free Ca\(^{2+}\). It appears probable that it is the Ca\(^{2+}\) flux that is more directly responsible for the induction of epitectin synthesis. Some preliminary experiments with 4-\(\beta\)-phorbol-12,13-didecanoate (PDD) and with mezerein, activators of protein kinase C, suggest that the increased intracellular Ca\(^{2+}\) flux might exert its effect through this well known pathway.

Since virtually nothing is known about the molecular mechanisms that regulate the production of mucins, the findings presented here may serve as a modest introduction to a subject that should prove to be of interest. Experiments based on cell fusion are at present being done to explore the regulation of epitectin synthesis at the genetic level.

We thank Sarah Grindley, Helen Coulson and J. H. D. Kent for skilful technical assistance. S.A.B. was the holder of a Medical Research Council Scholarship for Training in Research Methods. The work was supported by the Cancer Research Campaign.

**References**


CHOU, C. Y. & MALAGODI, M. H. (1975). Studies on the mechanism of action of a new Ca\(^{2+}\) antagonist, 8-(N,N-


(Received 30 December 1986 – Accepted 19 January 1987)