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QUARTERLY

Globotriaosyl ceramide (Gb_3) expression in human tumour cells: Intracellular trafficking defines a new retrograde transport pathway from the cell surface to the nucleus, which correlates with sensitivity to verotoxin*°

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The verotoxin receptor globotriaosyl ceramide (Gb3) is overexpressed in an ovarian tumour resistant to chemotherapy. An overlay of frozen tumour sections shows extensive staining of the tumour cells with verotoxin B subunit. In addition, blood vessels within the tumour mass are stained. The sensitivity of ovarian tumour cells *in vitro* to verotoxin can be modulated by culturing the cells in sodium butyrate to obtain an approximatly 5000-fold increase in susceptibility. This increased susceptibility is correlated with the intracellular targeting of verotoxin as monitored by using FITC-VT B subunit, in that prior to sodium butyrate treatment the toxin is internalized to a juxtanuclear (likely) Golgi location whereas, following butyrate treatment the intracellular toxin is distributed around the nucleus, consistent with endoplasmic reticulum and nuclear envelope location. This perinuclear location is similar to that found for drug-resistant variants of ovarian tumour cell lines. These results suggest that intracellular targeting of verotoxin to the perinuclear area results in increased cytotoxicity. Potentially such targeting may also occur in other human tumours.

While the synthesis, sorting and secretion of glycoproteins have been well studied [1-3], the equivalent pathways for glycolipids are less well understood [4]. Moreover, it might be stated that since glycolipids exist predominantly on the cell surface, the demands for such regulation are less restrictive for glycolipids.

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Abbreviations: Gb_3, globotriaosyl ceramide; FITC, fluorescein isothiocyanate; RPMI, Roswell Park Memorial Institute Medium H1640; VTEC, verotoxin producing *Escherichia coli*; HUS, Hemolytic Uremic Syndrome; LPS, lipopolysaccharide; VT, verotoxin.

Retrograde intracellular traffic is less well understood but is a focus of much current interest [5]. The concept that glycolipids, lacking the potential to carry targeting signals, could play a significant role in the retrograde traffic pathway has received little attention. However, the demonstration that both verotoxin [6] and cholera toxin [7, 8], both of which bind to glycolipid receptors (globotriaosyl ceramide-Gb_3 and monosialylganglioside-G_{M1}, respectively), are intracellularly targeted from the cell surface to the Golgi apparatus, has awakened a realization that glycolipids may be key components in this sorting process.

Because of the potential use of verotoxin (VT) as an antineoplastic agent [9], particularly against ovarian carcinoma cells, we have examined the Gb_3 content of primary and secondary ovarian tumour tissue [10] and found a marked elevation relative to normal ovary, especially for metastases. We have also been attempting to explain the differential cytotoxicity of verotoxin towards certain tumour cells *in vitro* which have, on gross analysis, approximately equal levels of the receptor glycolipid, globotriaosyl ceramide [11]. These studies have led us to compare the intracellular fate of surface-bound verotoxin and resulted in the proposition of a new intracellular glycolipid routing pathway for cells highly sensitive to verotoxin-induced cytopathology. This pathway may be upregulated drug resistant in tumours to increase tumour susceptibility to verotoxin *in vivo*.

MATERIALS AND METHODS

Recombinant VT1 and VT1 B subunit [12] were purified by affinity chromatography as earlier described [13]. The purified B subunit was labelled with fluorescein isothiocyanate [14]. Tumour samples were snap frozen, and FITC frozen sections stained with FITC-VT1B as previously [9].

Human malignant astrocytoma cell lines, SF-539 and XF-498, were kindly provided by Dr. J. Rutka (Hospital for Sick Children, Toronto) [15, 16]. SKOV3 and SF-539, the ovarian carcinoma lines, were grown in monolayers in α-MEM (GIBCO) plus nonessential amino acids, glutamine, gentamycin, and 10% heat-inactivated fetal bovine serum. XF-498 was grown in RPMI (Roswell Park Memorial Institute Medium H1640) medium and SKVLB which is a multi-drug resistant variant of the parental SKOV3 ovarian carcinoma cell line [17], was grown in α-MEM containing 1 μg/ml vinblastine.

XF-498 and SKOV3 cells were cultured in the presence of 2 mM sodium butyrate prior to assay for VT sensitivity and FITC-VT1 B intracellular targeting.

Cell viability was monitored by staining viable cells with crystal violet [18] 72 h after verotoxin addition.

For internalization studies cultured cells were treated with 10 μg/ml FITC-B at 4°C for 30 min. After extensive washing, cells were transferred to 37°C for 1 h, fixed with 1% paraformaldehyde and the endocytosed B subunit was detected using a fluorescence microscope under incident UV illumination.

RESULTS

FITC-VT1B staining of drug resistant ovarian tumour

In our analysis of about 40 ovarian tumours [10] we have found that the Gb_3 content is most markedly elevated for ovarian metastases and for primary tumours which are clinically resistant to drug therapy. VT1 B staining of differentiated tumour samples was less distinct than that of undifferentiated samples. Figure 1 shows the staining of a drug resistant, undifferentiated primary ovarian serous carcinoma. The tumour cells are highly toxin reactive. In addition blood vessels within the tumour mass are stained.

Cell sensitivity and intracellular routing of verotoxin

We have previously found that multidrug resistant variants of an ovarian carcinoma cell line show a 1000-fold hypersensitivity to verotoxin associated with a small increase in Gb_3 expression [9] as compared with the parental cell. This is of interest in relation to the increased expression of Gb_3 in the ovarian metastases (Fig. 1).

In studies of the VT sensitivity of other tumour cell lines we found a similar discrepancy in the VT sensitivity of several astrocytoma cell lines [19]. The CD_{50} for verotoxin 1 for the astrocytoma cell line XF498 is > 50 ng/ml, whereas the CD_{50} for the cell line SF539 is

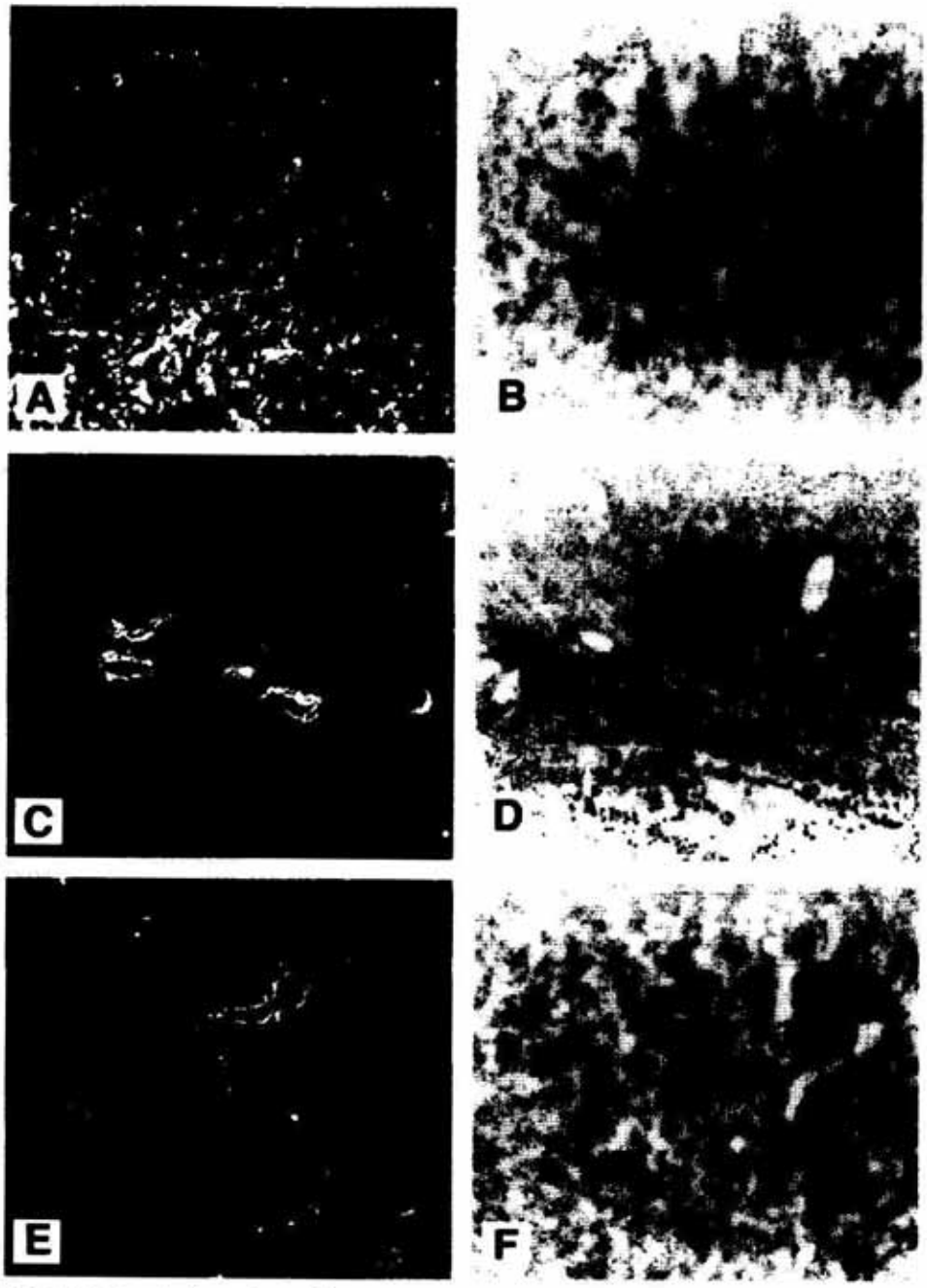

Figure 1. FITC-VT1 B staining of ovarian tumour.

A. Poorly differentiated ovarian papillary serous carcinoma. Extensive staining of tumour cells by FITC- VT1B. B. Corresponding H&E. C, E. Omentum metastasis (from tumour in A) showing toxin binding to blood vessels within the tumour. D, F. Corresponding H&E staining. Magnification × 45.

< pg/ml. Thus, these cells exhibit a > 5000-fold difference in VT sensitivity. Despite the fact that they have similar levels of the Gb_3 receptor [11]. Cell culture in the presence of sodium butyrate has been shown to increase cell sensitivity to verotoxin in many cells by increasing Gb_3 synthesis [20, 21]. Culture of XF498 cells in the presence of 2 mM sodium butyrate resulted in a major increase in sensitivity to VT, so that these cells became as, or even more, sensitive to verotoxin than the SF539 cells [11].

We have shown in Daudi cells [22], and other workers in HeLa cells [6, 21], that internalization of cell surface verotoxin can result in the intracellular targeting of the toxin to the rough endoplasmic reticulum and nuclear envelope. We therefore investigated the intracellular targeting of FITC (or RITC) labeled VT1B in these cells differing in verotoxin sensitivity. The difference in VT sensitivity between the ovarian drug resistant *vs* parental cell, the different astrocytoma cell lines and the effect of sodium butyrate was found to correlate with intracellular targeting of the VT following receptor mediated endocytosis. In the less sensitive cells FITC-B accumulated in the Golgi apparatus, whereas in the highly sensitive cells, the toxin accumulated around the nucleus, in the endoplasmic reticulum and nuclear envelope.

In order to further verify the importance of intracellular targeting in determining cell sensitivity to verotoxin, we cultured the drug sensitive ovarian carcinoma cell line SKOV3 in the presence of sodium butyrate and measured VT1 sensitivity and intracellular routing.

Sensitivity to VT1 was markedly increased following butyrate treatment (Fig. 2) and the CD_{50} was reduced by approximately 5000-fold.

We monitored the internalization of FITC VT1B in the SKOV3 cells with or without sodium butyrate (Fig. 3) and compared this to the astrocytoma cells previously demonstrated to show differential VT1 susceptibility and intracellular toxin targeting [11].

The results show that VT1B is targeted to a juxtanuclear area in SKOV3 cells (Fig. 3A, a) similar to that observed for XF498 astrocytoma cells (Fig. 3B). In contrast, the drug resistant SKVLB cells showed a ring of staining around the nucleus (Fig. 3A, b) in a manner similar to the highly VT sensitive astrocytoma cell line SF539 (Fig. 3B). The internalization of toxin was changed in SKOV3 cells cultured in the presence of butyrate such that the toxin was also now localized to the nucleus (Fig. 3B). Similarly butyrate treated XF498 cells also showed VT1 B labelling around the nucleus (Fig. 3A, c) [11]. The nuclear staining

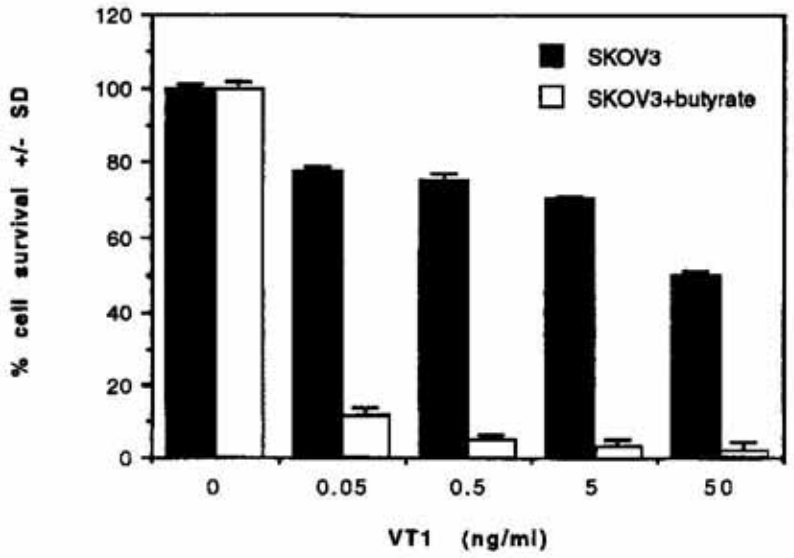

Figure 2. Effect of sodium butyrate on sensitivity of ovarian carcinoma cell line SKOV3 to verotoxin.

Cells were cultured with or without 2 mM sodium butyrate for 24 h, VT1 was added to triplicate cultures and the cells remaining after 2 days were quantitated by crystal violet staining.

was more diffuse for butyrate treated SKOV3 as compared to the ring of staining seen around the nucleus for XF498 cells. Nevertheless a clear difference in intracellular targeting of FITC-B is seen following butyrate treatment.

genesis. Thus VT may have both an antineoplastic and antiangiogenic effect.

Our evidence indicates that differential retrograde transport is a newly recognized component in determining the cell sensitivity to

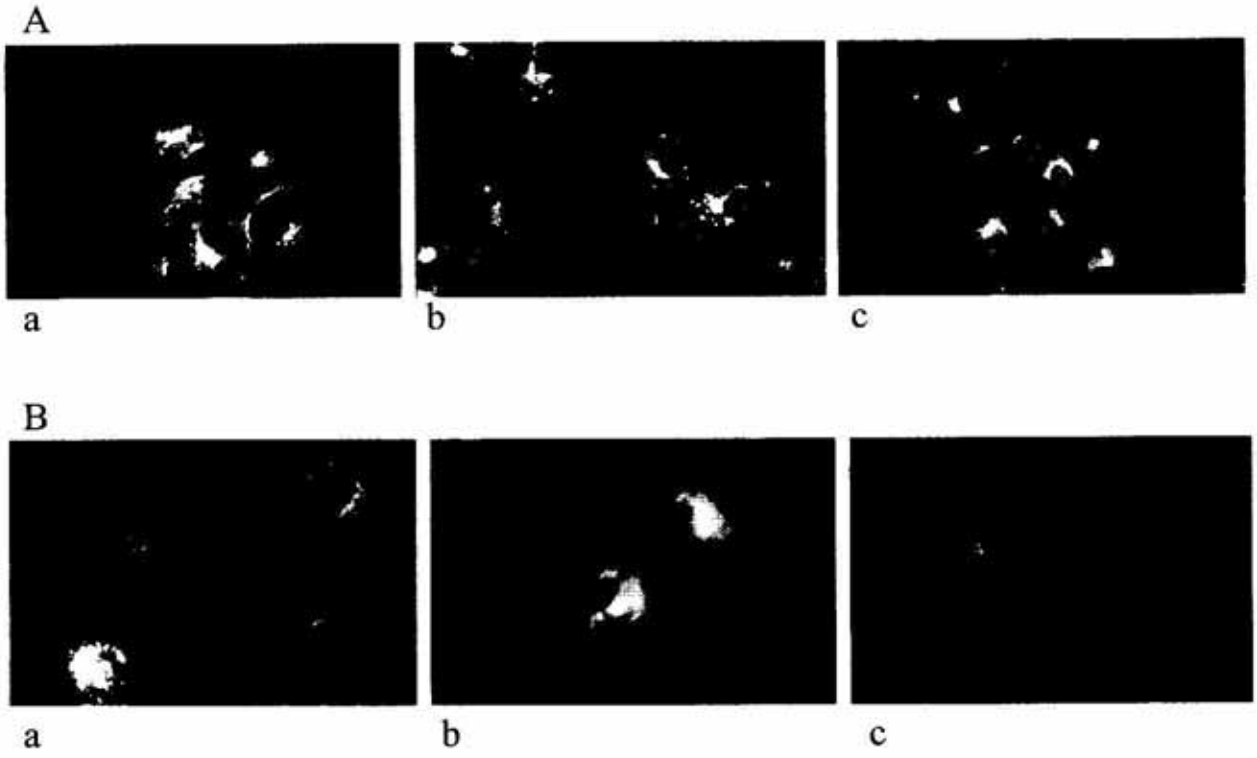

Figure 3. Intracellular targeting of FITC- VT1 B subunit.

A. Ovarian carcinoma cell lines: juxtanuclear (Golgi) staining is seen for SKOV3 (a) whereas perinuclear and nuclear staining is seen for SKVLB (b) and butyrate treated SKOV3 cells (c), respectively. B. Astrocytoma cell lines: juxtanuclear (Golgi) staining is seen for XF498 cells (b). Perinuclear staining is seen (plasma membrane is not detectable) for SF539 (a) and butyrate treated XF498 cells (c). Magnification × 250.

DISCUSSION

Elevated Gb_3 expression has been reported for a variety of tumours [23–26]. In addition Gb_3 expression varies during the cell growth cycle [27, 28]. Thus it is possible that Gb_3 plays a role in cell growth. The finding that blood vessels within the ovarian tumour (Fig. 1) are also VT reactive, suggests that Gb_3 expression may be an activation marker for endothelial cells during tumour-induced angio-

verotoxin *in vitro*. We have now extended our studies which demonstrated that an ovarian tumour cell line selected for multiple-drug resistance showed increased sensitivity to verotoxin concomitant with a change in intracellular routing of the toxin from the Golgi apparatus to the endoplasmic reticulum and nuclear envelope. Similarly, an astrocytoma cell line derived from a tumour which was drug resistant *in vivo* showed a similar increase in toxin sensitivity and change in intracellular target-

ing compared with other tumour derived astrocytoma cell lines. The present results confirm that this differential intracellular toxin localization can be mimicked by treatment of cells with sodium butyrate. Treatment of the drug-sensitive ovarian tumour cell line SKOV3 with butyrate resulted in a more than 5000-fold increase in sensitivity to verotoxin, such that the toxin sensitivity was now similar to that we previously reported for the multi-drug resistant variant of SKOV3 (SKVLB). This increase in sensitivity following culture in sodium butyrate correlated with a change in the intracellular targeting of verotoxin in these cells such that the FITC VTB formed a ring around the nucleus, a typical staining pattern for the endoplasmic reticulum/nuclear envelope. We have previously shown that this staining pattern correlates with an increased synthesis of Gb_3 containing shorter chain fatty acid isoforms, primarily C:16 and C:18 [11]. Similarly, we have shown there is a marked increase in Gb_3, particularly Gb_3 isoforms which migrate more slowly on thin-layer chromatography (probably containing shorter fatty acid species) in metastases and chemotherapy-resistant primary ovarian tumours. Thus the change in intracellular targeting of VT may be a feature of drug resistant tumours *in vivo* and thus the increase in sensitivity of cells in culture may be reflected *in vivo*, in which case verotoxin may be an important new addition to current cancer chemotherapy.

The initial concept that Gb_3 targeting of verotoxin to kidney endothelial cells was the reason for the development of hemolytic uremic syndrome, is likely an over simplification. We have shown that adult glomerular endothelial cells do not express the toxin receptor *in vivo* [14], even in kidney samples from the elderly, who can develop HUS following gastrointestinal VTEC infection. We therefore hypothesize that a concomitant stimulus of Gb_3 synthesis within the renal glomerular endothelial cells is required for the development of HUS and this may be provided by various cytokines which have been shown to induce Gb_3 synthesis in endothelial cells *in vitro* [29–33]. Recent studies have shown that monocytes respond to verotoxin in a unique manner in that they are not killed but rather stimulated to produce a cytokine response [34]. Such a cytokine response may well be a component in the development of HUS. It is possible that an aberrant cytokine response, for example as a result of systemic LPS from the VTEC infection, might stimulate renal endothelial cell Gb_3 synthesis and thus prime cells for systemic verotoxin-induced cytopathology. Thus, it is possible that the sterile toxin may not induce such pathology. We have shown the toxin receptor is present in the endothelial cells of renal glomeruli of young children [14]. However, the biopsies analyzed were from minimum lesion nephrotic syndrome patients, and such patients are pretreated with steroids prior to biopsy. It is possible that steroids may have induced renal receptor expression and thus, even in young children, it is possible that a cytokine stimulation of renal endothelial Gb_3 synthesis is necessary prior to the development of HUS. This remains an open question.

The finding that sodium butyrate treatment can increase the expression of the P glycoprotein [35] responsible for multi-drug resistance, opens the possibility that butyrate induced nuclear targeting of verotoxin may result from a mechanism similar to that responsible for the nuclear targeting in multiple-drug resistant ovarian carcinoma cell lines. The P glycoprotein has been shown to be a lipid flipase [36] able to translocate short chain fatty acids from one side of the plasma membrane to the other.

It is conceivable that such a relocation might result in altered intracellular trafficking such that the glycolipid (and it's ligand?) is now sorted to the nucleus.

Depending on the cell line, verotoxin is a potent initiator of apoptosis [19, 37–39]. We have proposed that the B cell differentiation antigen CD19 also binds to Gb_3 as a result of

N-terminal amino acid similarity with VT B [40] in the region of the Gb_3 binding site [41]. Ligation of cell surface CD19 also induces apoptosis [42] in B cells, but only in Gb_3+ve cells [43]. The ligated CD19 is internalized to the nuclear envelope only in Gb_3+ve cells, suggesting that Gb_3 binding may be responsible for nuclear targeting and that nuclear targeting may be required for the induction of apoptosis.

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