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Author(s) :ERICA L. LOWTHERS, CYNTHIA L. RICHARD, JONATHAN BLAY
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DIFFERENTIAL SENSITIVITY TO SHORT-CHAIN CERAMIDE ANALOGUES OF HUMAN INTESTINAL CARCINOMA CELLS GROWN IN TUMOR SPHEROIDS VERSUS MONOLAYER CULTURE

ERICA L. LOWTHERS, CYNTHIA L. RICHARD, AND JONATHAN BLAY[1]

Department of Pharmacology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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SUMMARY

The cytotoxic activity of short-chain (C_2) ceramide was evaluated in human intestinal carcinoma cells grown as multicellular tumor spheroids versus the same cells cultured as monolayers under closely comparable conditions. A decrease in cell number was seen in monolayer cultures of HT-29, Caco-2, and HRT-18 cells, with an EC_{50} (concentration for half-maximal toxicity) of between 13 and 23 μM. However, when the same cells were grown in the multicellular spheroid format, C_2 was markedly less potent in reducing cell number, with an EC_{50} of between 44 and 63 μM, representing a 1.9- to 4.9-fold decrease in its potency. The chemotherapeutic agents 5-fluorouracil and cisplatin were equally potent against spheroids and monolayer cultures, indicating that although drug access is a problem in conventionally grown tumor spheroids it is not a problem for spheroids grown under the conditions used in this study. Our results suggest that although ceramide is capable of inducing cell death in intestinal carcinoma cells grown in spheroid culture, its cellular toxicity is constrained by influences that are independent of drug access and may be the consequence of the altered cellular relationships. Carcinoma cell populations show an intrinsically decreased responsiveness to the effects of ceramide when they are grown in a three-dimensional culture format.

Key words: multicellular spheroids; cytotoxicity; chemotherapy; potency.

The effectiveness of antitumor therapies is affected profoundly by the abnormal microenvironment that is generated within the tumor (Brown and Giaccia, 1998). The packed, three-dimensional nature of the tumor cell population produces a milieu that may indirectly enhance tumor cell proliferation and impede the antitumor immune response (MacKenzie et al., 2002; Mujoomdar et al., 2003). Multicellular tumor spheroids reproduce the three-dimensional nature of tumor tissues and retain much of the regional changes in oxygen, metabolic products, and pH that are characteristic of solid tumors (Sutherland, 1986, 1988; Vaupel et al., 1989; Raleigh et al., 1998). Such spheroids have been used to study the distribution (Wartenberg and Acker, 1996) and efficacy (Frankel et al., 1997; Oloumi et al., 2000) of conventional chemotherapeutic agents, as well as the responses of tumor cells to novel immunotoxins (Chignola et al., 1995) or natural product medicines (Wartenberg et al., 2003). However, the chemosensitivity of the cells within spheroids and their susceptibility to apoptotic signals has not been well studied.

One class of mediators involved in the action of chemotherapeutic drugs is that of ceramides, which are endogenous lipid molecules generated intracellularly by certain cytotoxic stimuli to provoke apoptosis (Perry and Hannun, 1998; Pettus et al., 2002). It has been suggested that it may be possible to enhance the cell killing ability of conventional chemotherapeutic drugs by increasing ceramide levels, either by stimulating ceramide production or by inhibiting its removal (Hannun, 1994; Radin, 2001; Kolesnick, 2002). However, this approach presumes that our understanding of ceramide action, obtained predominantly using monolayer or isolated cell systems, will be applicable in an in vivo context.

The ceramide response of tumor cells grown in three dimensions is not clear. We have therefore used cultured tumor spheroids to examine the effects of short-chain ceramides on human intestinal carcinoma cells, which undergo apoptosis in response to the same agents when in monolayer culture (Ahn and Schroeder, 2002). We used three human intestinal carcinoma cell lines (HT-29, Caco-2, and HRT-18) grown either in monolayer culture or as multicellular tumor spheroids on a base layer of 50 μl of 0.5% agarose under closely comparable culture conditions (Dulbecco modified Eagle medium containing 0.5 g/L glucose and 1% newborn calf serum, a seeding density of 5×10^4 cells/well in 96-well plates, and a final culture volume of 200 μl). The HT-29 cells and Caco-2 cells are both relatively well-differentiated carcinoma cells derived from colonic primary tumors; HRT-18 cells originate from a tumor at an ileocecal site.

We examined the sensitivity of these three carcinoma cell lines to exogenous short-chain (C_2) ceramide. The C_2-ceramide used here is a cell-permeable ceramide analogue that induces characteristic apoptotic changes in cellular targets, including HT-29 and other colorectal carcinoma cells (Kolesnick et al., 2000; Ahn and Schroeder, 2002). All three cell lines grown in monolayer culture showed

[1] To whom correspondence should be addressed at Department of Pharmacology, Faculty of Medicine, Sir Charles Tupper Building, Dalhousie University, 1459 Oxford Street, Halifax, Nova Scotia, Canada B3H 4R2. E-mail: jonathan.blay@dal.ca

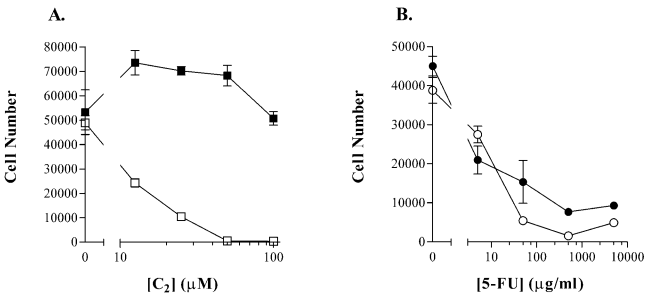

FIG. 1. Effect of short-chain (C_2)–ceramide (*panel A*) or 5-fluorouracil (5-FU) (*panel B*) on HRT-18 cells grown in monolayers or as tumor spheroids. The *points* show responses in monolayer (□, ○) or spheroid (■,●) cultures to C_2 (□, ■) or 5-FU (○,●). Values are mean cell number ± standard error of mean, n = 6 (monolayer) or n = 4 (spheroids) from representative experiments.

TABLE 1

EC_{50} VALUES FOR CYTOTOXICITY DUE TO CERAMIDE OR CHEMOTHERAPEUTIC DRUGS IN HUMAN INTESTINAL CARCINOMA CELL LINES CULTURED EITHER IN MONOLAYERS OR AS MULTICELLULAR TUMOR SPHEROIDS.[a]

Cytotoxic agent	Cell line	Monolayer EC_{50}[b]	Spheroid EC_{50}
C_2	HT-29	20.2 ± 2.9 μ*M*	49.9 ± 4.7 μ*M****
	Caco-2	23.2 ± 1.0 μ*M*	43.6 ± 3.9 μ*M***
	HRT-18	12.8 ± 2.4 μ*M*	62.8 ± 9.3 μ*M***
5-FU	HRT-18	20.1 ± 10.1 μg/ml	20.1 ± 9.1 μg/ml (NS)
Cisplatin	HRT-18	1.1 ± 0.1 μg/ml	2.5 ± 1.4 μg/ml (NS)

[a] For monolayer cultures, cytotoxic agents were added on day 1 and cells counted on day 4. For spheroids, drug additions were made on day 7, and cells were counted on day 10. Data are mean ± SEM values for three to six independent experiments.

[b] EC_{50}, concentration for half-maximal toxicity; 5-FU, 5-fluorouracil; NS, not significant.

***, Statistically significant difference between EC_{50} values $P < 0.001$; **, $P < 0.01$.

typical toxicity curves for C_2 (e.g., Fig. 1*A*) with EC_{50} values (Effective Concentration for 50% cytotoxicity) for each cell line ranging between 13 and 23 μ*M* (Table 1). Dihydro-C_2, an inactive derivative of C_2 that lacks the 4,5-*trans* double bond and has no cytotoxic activity, did not affect cell numbers at concentrations up to 100 μ*M* (data not shown).

In contrast to these results with monolayer cultures, the same cell lines grown as spheroids under comparable culture conditions showed a much-reduced sensitivity to the effects of C_2 (Fig. 1*A*). The respective EC_{50} values for Caco-2, HT-29, and HRT-18 cells in spheroid cultures were 1.9-, 2.5-, and 4.9-fold greater (i.e., lower potency) than for monolayer cultures (Table 1). Figure 1*A* shows the comparison for HRT-18 cells. The differences in actual EC_{50} values were 20.4 ± 4.0 μ*M* (Caco-2), 29.7 ± 5.3 μ*M* (HT-29), and 50.0 ± 10.8 μ*M* (HRT-18), all of which were statistically significant (*P* values, 0.0072, 0.0003, and 0.002). In spheroids, as for monolayer culture, the inactive analogue dihydro-C_2 was without effect on cell number (data not shown).

We assumed initially that the diminished activity of C_2 in spheroids was the result of decreased access of C_2 into the spheroid mass. However, our spheroids are grown under conditions that were different (i.e., low serum, low glucose) from those in most spheroid studies. We therefore examined the effects of two other cytotoxic agents that are known to be hindered in their passage through packed cell multilayers (Tunggal et al., 1999; Tannock et al., 2002) and would thus be expected to show decreased effectiveness in spheroid cultures compared with monolayer cultures if access were compromised. However, both 5-fluorouracil (5-FU), a pyrimidine antimetabolite, and cisplatin, a cross-linking platinum drug, were equally potent and efficacious against both monolayer and spheroid cultures in our experiments. The comparison is shown for HRT-18 cells (Fig. 1*B*; Table 1). This finding indicates that, under the conditions used in this study, both 5-FU and cisplatin had adequate access to cells in the spheroids. Thus, the differential response to ceramide is unlikely to be the result of impeded access into the spheroid but is more likely the result of intrinsic differences in sensitivity of the different cell populations.

The exact mechanisms by which intestinal carcinoma cells in this context show diminished ceramide sensitivity remain to be explored. The cellular actions of ceramide are mediated through a variety of cellular effectors, including ceramide-activated protein phosphatase, ceramide-activated protein kinase, protein kinase Cζ, the stress-activated protein kinase / jun N-terminal kinase pathway, and the Bcl-2 family of proteins (Perry and Hannun, 1998; Senchenkov et al., 2001). Presumably, one or more of these pathways is modulated by signals derived from the spheroid microenvironment, leading to the observed changes. It has been shown that cells within spheroids may differ in their expression, phosphorylation, and/or function of different proteins, including those involved in apoptotic processes (Oloumi et al., 2000; Poland et al., 2002; Brown et al., 2003). Studies of drug resistance in spheroids have shown that the cellular relationships within the structure play a part in determining that phenomenon (Wartenberg and Acker, 1996; Oloumi et al., 2000). It may be that the cell–cell interactions within a three-dimensional tissue are able to modulate certain steps in the pathways of drug metabolism and sensitivity.

The ability of ceramide to act against colorectal cancer cells is of direct practical interest. Sphingomyelin, the substrate from which ceramide is derived, is present in the diet and may influence the fate of intestinal neoplasms by increasing the availability of cellular ceramide. Mice fed dietary sphingomyelin show a reduced number of aberrant colonic crypts (precursors of tumors) and a lower incidence of colon tumors in response to treatment with the carcinogen 1,2-dimethylhydrazine (Dillehay et al., 1994; Schmelz et al., 1996, 2000). It is therefore possible that endogenous ceramide might play a part in enhancing tumor cell death by chemotherapeutic drugs. However, understanding the role of ceramide in drug-induced cytotoxicity requires a clear knowledge of ceramide sensitivity in a proper biological context. Our finding adds a note of caution for in vitro assays of these and other cytotoxic activities: using monolayer cultures to evaluate chemotherapeutic drug responses may prove to be misleading.

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References

Ahn, E. H.; Schroeder, J. J. Sphingoid bases and ceramide induce apoptosis in HT-29 and HCT-116 human colon cancer cells. Exp. Biol. Med. 227:345–353; 2002.

Brown, J. M.; Giaccia, A. J. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 58:1408–1416; 1998.

Brown, L. A.; Arterburn, L. M.; Miller, A. P.; Cowger, N. L.; Hartley, S. M.; Andrews, A.; Silber, P. M.; Li, A. P. Maintenance of liver functions in rat hepatocytes cultured as spheroids in a rotating wall vessel. In Vitro Cell. Dev. Biol. 39A:13–29; 2003.

Chignola, R.; Foroni, R.; Franceschi, A., et al. Heterogeneous response of individual multicellular tumour spheroids to immunotoxins and ricin toxin. Br. J. Cancer 72:607–614; 1995.

Dillehay, D. L.; Webb, S. K.; Schmelz, E.-M.; Merrill, A. H. Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1 mice. J. Nutr. 124:614–620; 1994.

Frankel, A.; Buckman, R.; Kerbel, R. S. Abrogation of taxol-induced G2-M arrest and apoptosis in human ovarian cancer cells grown as multicellular tumor spheroids. Cancer Res. 57:2388–2393; 1997.

Hannun, Y. A. The sphingomyelin cycle and the second messenger function of ceramide. J. Biol. Chem. 269:3125–3128; 1994.

Kolesnick, R. The therapeutic potential of modulating the ceramide/sphingomyelin pathway. J. Clin. Invest. 110:3–8; 2002.

Kolesnick, R. N.; Goñi, F. M.; Alonso, A. Compartmentalization of ceramide signaling: physical foundations and biological effects. J. Cell. Physiol. 184:285–300; 2000.

MacKenzie, W. M.; Hoskin, D. W.; Blay, J. Adenosine suppresses $\alpha_4\beta_7$ integrin-mediated adhesion of T lymphocytes to colon adenocarcinoma cells. Exp. Cell Res. 276:90–100; 2002.

Mujoomdar, M.; Hoskin, D.; Blay, J. Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism. Biochem. Pharmacol. 66:1737–1747; 2003.

Oloumi, A.; MacPhail, S. H.; Johnston, P. J.; Banáth, J. P.; Olive, P. L. Changes in subcellular distribution of topoisomerase Iiα correlate with etoposide resistance in multicell spheroids and xenograft tumors. Cancer Res. 60:5747–5753; 2000.

Perry, D. K.; Hannun, Y. A. The role of ceramide in cell signaling. Biochim. Biophys. Acta 1436:233–243; 1998.

Pettus, B. J.; Chalfant, C. E.; Hannun, Y. A. Ceramide in apoptosis: an overview and current perspectives. Biochim. Biophys. Acta 1585:114–125; 2002.

Poland, J.; Sinha, P.; Siegert, A.; Schnölzer, M.; Korf, U.; Hauptmann, S. Comparison of protein expression profiles between monolayer and spheroid cell culture of HT-29 cells revealed fragmentation of CK18 in three-dimensional cell culture. Electrophoresis 23:1174–1184; 2002.

Radin, N. S. Killing cancer cells by poly-drug elevation of ceramide levels. A hypothesis whose time has come? Eur. J. Biochem. 268:193–204; 2001.

Raleigh, J. A.; Calkins-Adams, D. P.; Rinkeer, L. H.; Ballenger, C. A.; Weissler, M. C.; Fowler, W. C.; Novotny, D. B.; Varia, M. A. Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. Cancer Res. 58:3765–3768; 1998.

Schmelz, E.-M.; Dillehay, D. L.; Webb, S. K.; Reiter, A.; Adams, J.; Merrill, A. H. Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with 1,2-dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis. Cancer Res. 56:4936–4941; 1996.

Schmelz, E.-M.; Sullards, M. C.; Dillehay, D. L.; Merrill, A. H. Colonic cell proliferation and aberrant crypt foci formation are inhibited by dairy glycosphingolipids in 1,2-dimethylhydrazine-treated CF1 mice. J. Nutr. 130:522–527; 2000.

Senchenkov, A.; Litvak, D. A.; Cabot, M. C. Targeting ceramide metabolism—a strategy for overcoming drug resistance. J. Natl. Cancer Inst. 93:347–357; 2001.

Sutherland, R. M. Importance of critical metabolites and cellular interactions in the biology of microregions of tumors. Cancer 58:1668–1680; 1986.

Sutherland, R. M. Cell and environmental interactions in tumor microregions: the multicell spheroid model. Science 240:177–184; 1988.

Tannock, I. F.; Lee, C. M.; Tunggal, J. K.; Cowan, D. S. M.; Egorin, M. J. Limited penetration of anticancer drugs through tumor tissue. A potential cause of resistance of solid tumors to chemotherapy. Clin. Cancer Res. 8:878–884; 2002.

Tunggal, J. K.; Cowan, D. S. M.; Shaikh, H.; Tannock, I. F. Penetration of anticancer drugs through solid tissue: a factor that limits the effectiveness of chemotherapy for solid tumors. Clin. Cancer Res. 5:1583–1586; 1999.

Vaupel, P.; Kallinowski, F.; Okunieff, P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res. 49:6449–6465; 1989.

Wartenberg, M.; Acker, H. Induction of cell death by doxorubicin in multicellular spheroids as studied by confocal laser scanning microscopy. Anticancer Res. 16:573–580; 1996.

Wartenberg, M.; Budde, P.; de Mareés, M., et al. Inhibition of tumor-induced angiogenesis and matrix-metalloproteinase expression in confrontation cultures of embryoid bodies and tumor spheroids by plant ingredients used in traditional Chinese medicine. Lab. Invest. 83:87–98; 2003.