Indole-3-carbinol-induced death in cancer cells involves EGFR downregulation and is exacerbated in a 3D environment

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Published online: 9 March 2006

Indole-3-carbinol (I3C) is a promising anticancer dietary compound, which inhibits breast cancer in animal models. The objective of the current study was to characterize I3C-induced cell death in a panel of human breast tumorigenic cells (MCF7, MDA-MB-468, MDA-MB-231 and HBL100) in comparison with normal fibroblasts. Since epithelial cells are protected from cell death by a three-dimensional environment, 3D cell culture (collagen I gel and spheroids) was employed to investigate susceptibility to I3C. Cell viability in the presence of 256 µM I3C, a concentration close to the physiologically achievable range, was in the order fibroblasts = HBL100 > MDA-MB-231 > MCF7 > MDA-MB-468 in monolayer culture. However, 3D culture conditions increased the susceptibility of MCF7 and MDA-MB-468 cancer cells towards I3C. I3C induced cell death in breast cancer MCF7, MDA-MB-468 and MDA-MB-231 cells via the mitochondrial apoptotic pathway. I3C significantly reduced levels of epidermal growth factor receptor (EGFR) in MDA-MB-468 after 6 h and in MDA-MB-231 and HBL100 cells after 30 h. Downregulation of EGFR in MDA-MB468 and MDA-MB-231 cells using an EGFR inhibitor resulted in apoptosis. EGFR modulation using EGF or an EGFR inhibitor markedly influenced viability and response to I3C in MDA-MB-468 cells in 3D conditions. EGFR expression was modulated by 3D conditions. Therefore, I3C-induced EGFR reduction in these cells is likely to be responsible for I3C-induced apoptosis.

Keywords: apoptosis; breast cancer; epidermal growth factor receptor; indole-3-carbinol; 3D cell culture.

Introduction

Breast cancer has the highest incidence in women in the Western world compared to other forms of cancer. In England and Wales, the number of breast cancer cases between 1988–1990 exceeded the number of cases of lung and colon cancer taken together. Current estimates indicate that one in eight women will be diagnosed with breast cancer in her lifetime. Epidemiological studies and analysis of diet suggest that consumption of some foods, e.g. those containing genistein, coincides with the reduced risk of breast cancer. Indole-3-carbinol (I3C), derived from vegetables of the Cruciferae family, such as broccoli, brussel sprouts and cabbage, has been shown to inhibit development of tumors in a variety of tissues in animal models (thoroughly reviewed in4). It reduces or delays development of mammary tumors induced by carcinogens and viruses, as well as growth of spontaneous mammary tumors in animal models. Therefore, it is regarded as a promising chemopreventive agent. Two clinical phase II trials indicated that I3C causes significant regression of cervical intraepithelial neoplasia and recurrent respiratory papillomatosis in patients.5,6

The chemopreventive efficacy of I3C can be partially attributed to its effect on estradiol metabolism, since it increases 2-hydroxylation and decreases proliferative activity of endogenous estrogens in volunteers and patients, as well as in animal models.3,4,5,7,8 However, both estrogen receptor (ER)-negative MDA-MB-231 and MDA-MB-468 cells and ER-positive MCF7 breast cancer cells are affected by I3C. I3C has been reported to inhibit growth of MDA-MB-231 cells and induce apoptosis in MDA-MB-468 and MCF7 cells.9–13 Therefore, the mechanisms of I3C-induced growth inhibition or apoptosis are not related to ER status and remain obscure.

Data on the mechanism of I3C-induced apoptosis are contradictory. I3C-induced apoptosis was related to increased death receptors, DR4 and DR5, in prostate cancer LNCaP cells, which points to an extrinsic apoptotic pathway.14 Conversely, upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2 and Bcl-xL preceded or coincided with the onset of apoptosis in prostate cancer PC3 and breast cancer MDA-MB-435 cells.15–17 These data imply involvement of an intrinsic apoptotic pathway. There is also some confusion regarding the sequence of apoptotic events induced by I3C. Loss of mitochondrial membrane potential and cytochrome release preceded downregulation of Bcl-2 and translocation of Bax into mitochondria in breast cancer cells MCF10CA1a.18 In breast cancer cells MDA-MB-468, modulation of Bcl-xL and Bcl-2 occurred.
as post-apoptotic events. Furthermore, several studies showed that I3C upregulates expression of pro-apoptotic proteins, e.g. DR4, DR5, caspase-1 and Bax, at transcriptional and protein levels. Therefore, we investigated which initiator caspase was involved in the apoptotic pathway in breast cancer cells in more detail. We also examined epidermal growth factor receptor (EGFR), which is required for maintaining viability of EGFR-dependent solid tumours, as a primary I3C target. EGFR signaling plays an important role in cancer development and progression.

Cells in tissues are involved in three-dimensional interactions, and it is well known that their physiology and fate is influenced by a 3D environment. Modulation of cell adhesion in laminin-based 3D culture dramatically influences morphology and function of breast cancer cells. Laminin-based and spheroid 3D cultures protect breast cells from apoptosis or increase resistance to cytotoxic agents compared to monolayer culture.

The aim of this study was to investigate whether 3D culture altered the susceptibility of breast cancer cells to I3C. Collagen-based 3D culture is more relevant for our investigation, because breast cancer is associated with basement membrane fragmentation, increased collagen synthesis and accumulation. Aggressive breast cancer induces a strong fibroproliferative response with synthesis of type I collagen. Metastatic spreading of breast cancer cells in vivo occurs along collagen filaments. Moreover, drug resistance tests using collagen gel culture have proved to be accurate and highly predictive. All cell lines used in this study express α2β1 integrin, responsible for adhesion to collagen I. The other 3D model we investigated was spheroid culture, because in some cases cells in spheroids are more resistant to cytotoxic agents than in monolayer culture.

We investigated I3C-induced cell death in human breast cancer cells in comparison to the transformed breast cell line HBL100 and normal fibroblasts. A panel of cancer cell lines (MCF7, MDA-MB-468 and MDA-MB-231) was chosen to represent cells with a wide range of tumorigenicity. They produce tumors in nude mice with efficiencies of 22, 90 and 100%, and are known as non-metastatic, poorly metastatic and aggressively metastatic, respectively. The breast cell line HBL100, derived from normal human breast epithelium and containing integrated defective SV40 virus, was also included in the study. This transformed cell line is able to form colonies in soft agar and produce adenocarcinomas in nude mice.

**Methods**

**Cells**

Human breast cell lines MCF7 (ER−ve, p53wt), MDA-MB-468 (ER−ve, p53mutated), MDA-MB-231 (ER−ve, p53mutated) and HBL100 (ER−ve, p53wt), originating from the American Type Culture Collection, were kindly provided by Prof. Rosemary Walker (University of Leicester, UK). All cells were cultured as described previously. Normal human fibroblasts GM05399 were obtained from Coriell Cell Repositories (Camden, NJ, USA) and used in passages 12–16. For culture in collagen gels, collagen I (Invitrogen) was diluted in DMEM to the concentration of 1 mg/ml (on ice), the pH adjusted to within physiological range, mixed with cells and dispensed in Packard Viewplates-96 (2500 cells/100 µl per well). After 1 h incubation at 37°C to set collagen gels, an equal volume of the medium containing 20% FBS was added. In some experiments an equal amount (1 mg/ml) of growth factor-reduced matrigel (BD Biosciences) was added to collagen I before mixing with cells. Spheroids were generated by seeding 2.5 × 10^4 cells/ml on to 0.8% agarose-coated 24-well tissue culture plates and overnight incubation. The DMSO amounts were equal in all wells in all experiments and did not exceed 0.1%.

Cell viability and apoptosis-related studies

The viability of cells in response to I3C was determined by measuring ATP levels, since this assay detects the number of cells in a wide range from 5 × 10^3 to 5 × 10^4. The number of adherent cells was examined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer’s recommendations, in a FluoStar plate-reader (BMG). Cells were seeded in white Packard Viewplates-96 (2500 cells/well) overnight in DMEM containing 10% FBS, followed by I3C treatment in the same medium for 48 h.

The following adjustments were made for 3D cultures. To measure ATP levels in cells grown in collagen gels, time periods for cell extraction and ATP assay were extended from 15 min to 90 min to obtain the highest measurements. Formed spheroids were treated with I3C for 48 h and transferred to Packard Viewplate-96 plates in aliquots/well, equivalent to 2.5 × 10^3 cells of the starting spheroid culture, for measuring ATP amounts as described above. In some experiments cells were treated with a highly specific EGFR inhibitor PD153035 (Calbiochem, UK) or EGF (Sigma).

Apoptosis was measured using the Annexin V-FITC kit (Bender Medsystems) as described previously. The percentage of live, apoptotic or necrotic cells in the whole population was determined using a FACScan flow cytometer. In some experiments cycloheximide (Sigma), Z-YVAD-FMK (Sigma) or Z-VAD-FMK (Promega) was added to the cell culture medium 60 min prior to I3C. The effect of I3C on mitochondrial potential was measured via tetramethylrhodamine ethyl ester (TMRE) binding to I3C-treated adherent cells using a FACScan flow cytometer as described previously. TMRE is a cationic lipophilic dye that accumulates in the negatively charged mitochondrial matrix.