Regulation of the Expression of E-Cadherin on Human Cancer Cells by γ-Linolenic Acid (GLA)1

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ABSTRACT

E-cadherin is a cell to cell adhesion molecule which acts as a suppressor of metastasis. This study examined the effect of γ-linolenic acid (GLA), a n-6 polyunsaturated fatty acid, on the expression of E-cadherin in human cancer cells. Western blotting studies demonstrated that treatment of cells with GLA for 24 h increased the expression of E-cadherin in lung, colon, breast, melanoma, and liver cancer cells, but not in endothelial cells and fibroblasts. The results were confirmed by immunocytochemistry. In contrast, two other n-6 fatty acids, linoleic acid and arachidonic acid, failed to induce these changes. The increased expression of E-cadherin was correlated with reduced in vitro invasion and increased aggregation, indicating that the increased E-cadherin expression induced by GLA was biologically active. These data add GLA to the short list of E-cadherin up-regulatory factors. The up-regulation of E-cadherin expression in human cancer cells may contribute to the anticancer properties of GLA.

INTRODUCTION

Certain n-6 PUFAs3 have been shown to have in vitro antiproliferation and cytotoxic effects on breast, prostate, pancreatic cancer, and hepatoma cells (1–7). Inhibition of tumor cell growth by some cytokines is also dependent on the presence of PUFAs (4). These anticancer effects are seen particularly with GLA, dihomo-GLA, and EPA. We have recently reported another property of GLA that is demonstrable on human colon cancer cells, namely, the inhibition of motility and in vitro invasive properties (8).

E-cadherin is a calcium-dependent transmembrane cell to cell adhesion molecule. E-cadherins bind to one another by homophilic interactions and help to maintain tissue/organ structure. It appears that E-cadherin is capable of inhibiting invasion and metastasis because (a) the metastatic potential of cell lines is inversely related to E-cadherin expression; (b) high E-cadherin levels in certain tumors are related to a less metastatic prognosis; (c) blocking E-cadherin function by antibody or other material, or complete removal of E-cadherin by deletion of its gene, increases tumor cell motility, invasion, and metastatic potential, while up-regulation of E-cadherin in breast cancer cells by insulin-like growth factor I (9) inhibits invasion and motility; and (d) transfection of highly metastatic cells with E-cadherin produces cells with a low metastatic potential. Loss of E-cadherin expression in some human cancers, or its mutation to an inactive form has been reported in gastric, gynecological, pancreatic, esophageal, lung, bladder, and breast cancers and other tumor types (10–15; for reviews, see Refs. 16–19). Recent work (20) also shows that the expression of E-cadherin mRNA (in situ hybridization) in colorectal cancer is decreased, and that this is correlated with long-term survival of the patients.

These relationships between E-cadherin and tumor progression prompted us to examine further the effects of GLA on E-cadherin expression in a broader range of tumor cells. In this article, we report that GLA increased expression of E-cadherin in a range of human cancer cells. This has been demonstrated by both Western blotting and immunohistochemical studies. The raised levels of E-cadherin were associated with increased cell aggregation and reduced in vitro invasion, suggesting that the expressed protein was functional.

MATERIALS AND METHODS

Cell Lines. Human hepatoma PLC/PRF/5, colon cancer HT29, HRT18, and HT115, lung cancer SK LU 1, Cor L23, Cor L47, Cor L51, Cor L677, and Cor L88, melanoma G361, endothelial ECV304, and fibroblasts MRC5 cells were obtained from the European Collection of Animal Cell Culture (Salisbury, UK). Human breast cancer cells MCF-7, MDA-MB-231, and ZR-751, and the monocytic cell line U937 were obtained from American Type Culture Collection. The M19 human melanoma cells were a gift from D. Jones (Department of Surgery, University of Wales College of Medicine).

Materials. Matrigel (reconstituted basement membrane) was purchased from Collaborative Research Product (Bedford, MA). A transwell plate equipped with a porous insert (pore size, 8.0 μm) was obtained from Becton Dickinson (Oxford, UK) for the in vitro invasion study. A mouse antihuman E-cadherin (HECD-1) mAb was obtained from British Biotechnology (Oxford, UK), peroxidase-conjugated rabbit antimouse IgG for immunohistochemical study was obtained from DAKO Ltd., and sheep antimonoe IgG for Western blotting was obtained from Amersham International (Little Chalfont, Buckinghamshire, UK). Recombinant human HGF/SF was a generous gift from Dr. T. Nakamura (Osaka, Japan). The cells were passaged three to five times before assays were undertaken. Hoescht 33258, GLA, LA, AA, and EPA were obtained from Sigma. Fatty acids were dissolved in ethanol and stored in liquid nitrogen. They were added to cells after dilution in complete medium containing albumin. All other materials were purchased from Sigma (UK) unless otherwise stated.

Cell Invasion Assay. This was based on the methods of Albini et al. (21) and Parish et al. (22). Transwell chambers (Costar, Cambridge, MA) equipped with 6.5-mm diameter polycarbonate membrane (pore size, 8 μm) were precoated with a solubilized tissue basement membrane-Matrigel (50 μg/membrane). After gel rehydration, 50,000 cells were added to each membrane with or without treatment. HGF/SF (20 ng/ml) was used in the lower chamber to induce invasion. In selected cultures, HECD-1 or EGTA was included. After 72 h of culture, the noninvasive cells were removed with a cotton swab, and the cells that had migrated through the membrane and stuck to the lower surface were fixed and stained with crystal violet. After extraction with 10% acetic acid, the absorbance was measured at 540 nm with a Titertek multisecanner. Invasion is shown here as invasion index: cell with treatment/control (8).

Tumor Cell Aggregation Assay. Cells treated with fatty acids or medium only (control) were first briefly washed with balanced salt solution and then incubated in HCMF buffer (160 mM NaCl, 0.6 mM Na2HPO4, 0.1% w/v glucose, and 0.01% HEPES, pH 7.4) containing 0.01% trypsin and 2 mM CaCl2 for 30 min at 37°C. Cells were washed and resuspended in HCMF with 2 mM CaCl2, and the concentration was adjusted to 105 cells/ml. They were then added into a tube and put on a shaker. In selected cultures, HECD-1 or EGTA was included. The proportion of this cell suspension were removed at 15, 30, and 45 min and fixed in a 1.5 volume of 5% glutaraldehyde, a procedure which did not cause artificial aggregation or disaggregation as previously reported (23). The total numbers of particles (cells or cell aggregates) were counted, and the aggregation index was calculated by (Nf − Nt)/Nf, where Nt is the number of particles before the experiment started, and Nf is the number at the chosen time (23, 24).

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3The abbreviations used are: PUFA, polyunsaturated fatty acid; GLA, γ-linolenic acid; EPA, eicosapentaenoic acid; HGF/SF, hepatocyte growth factor/scatter factor; LA, linoleic acid; AA, arachidonic acid; ECL, enhanced chemiluminescence.

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Fig. 1. Expression of E-cadherin by Western blotting. Cells were treated with medium only (control) or with LA, GLA, AA, or EPA for 24 h. HRT18 colon cancer (A) and PLC/PRF/5 liver (B) show increased E-cadherin after GLA treatment. Tops, E-cadherin band stained with HECD-1 antibody and visualized with ECL; bottoms, relative density of the corresponding band for that gel.

Fig. 2. Kinetic changes in GLA-induced E-cadherin expression demonstrated by Western blotting. A, PLC/PRF/5 cells; and B, Cor L23 cells. Tops, E-cadherin band stained with HECD-1 antibody and visualized with ECL; bottoms, relative density of the corresponding band for that gel. Cells were treated with GLA for 4, 12, 24, and 48 h. GLA increased expression slightly at 12 h, with maximum changes seen after 24 h.

Table 1 Effects of GLA on the expression of E-cadherin and aggregation of the cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Western blotting</th>
<th>Immunohistochemical</th>
<th>Aggregation index&lt;sup&gt;a&lt;/sup&gt; (t = 30 min)</th>
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</thead>
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<tr>
<td></td>
<td>Control</td>
<td>With GLA</td>
<td>Control</td>
</tr>
<tr>
<td>HRT18</td>
<td>15</td>
<td>52</td>
<td>Positive</td>
</tr>
<tr>
<td>HT29</td>
<td>4</td>
<td>7</td>
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<tr>
<td>ZR751</td>
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<td>7</td>
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</tr>
<tr>
<td>G361</td>
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<td>8</td>
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</tr>
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<tr>
<td>U9377</td>
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<td>0</td>
<td>No stain</td>
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</table>

<sup>a</sup> Western blotting results shown as relative density of E-cadherin band scanned by a densitometer. ND: not done.

<sup>b</sup> Aggregation was determined in a calcium-containing buffer (HCMF, with 2 mM Ca<sup>2+</sup>). Results shown are aggregation indices in 30 min.
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Fig. 3. Immunohistochemical staining of E-cadherin. A and B, HRT18 cells; C and D, PLC/PRF/5 cells; and E and F, ZR751 cells. Cells were treated with GLA (50 μM; B, D, and F) for 24 h and E-cadherin was stained with HEC1-1 antibody. Cells cultured with medium were used as controls (A, C, and E). Cells with GLA showed increased staining of E-cadherin on the surface, particularly in the cell to cell junctions.

SDS-PAGE and Western Blotting. Cells were pelleted and lysed in HCMF buffer containing 0.5% SDS, 0.5% Triton X-100, 2 mM CaCl₂, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 30 min. They were then boiled at 100°C for 5 min before clarification at 13,000 × g for 10 min. Protein concentrations were measured using fluorescamine and quantified by using a multifluoroscanner (Denly, Sussex, UK). Equal amounts of protein from each cell sample (controls and treated) were added onto an 8% polyacrylamide gel. Following electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% skimmed milk for 60 min. Protein bands were visualized with an ECL system (Amersham, UK). The band densities of E-cadherin on the photographic film were analyzed with a densitometer and are shown here as relative values.

Immunohistochemical Study. Cells were fixed with 4% formaldehyde and endogenous peroxidase blocked by methanol (0.3% H₂O₂) before blocking with 2% BSA for 60 min. Cells were then incubated with HEC1-1 (0.5 μg/ml) for 60 min and extensively washed. For the immunohistochemical study, peroxidase-conjugated secondary antibody was added, and this was followed by color-developing agents (dimethylaminobenzene, 60 μg/ml). Slides were mounted with Sterilyte.

Cell Growth Monitoring. To eliminate the possibility that the effects seen in the above assays might arise from the cytotoxicity of GLA, we choose low GLA concentrations (<75 μM) in this study. Such concentrations have been
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**RESULTS**

**GLA Increased E-Cadherin Expression.** Cells exposed to GLA (50 μM) for 24 h had increased expression of E-cadherin. Fig. 1 shows E-cadherin levels by Western blotting of HRT18 colon cancer (Fig. 1A) and PLC/PRF/5 liver (Fig. 1B) cancer cells in which expression of E-cadherin was increased. A kinetic study revealed that the increased E-cadherin expression appeared as early as 12 h, but that maximum changes were seen only after 24 h. The responses of PLC/PRF/5 and Cor L23 cells are shown in Fig. 2. The rest of the data are summarized in Table 1. Although most of the cells that express detectable E-cadherin, e.g., HRT18, HT29, MCF-7, ZR751, G361, Cor L23, Cor L51, Cor L88, and PLC/PRF/5, showed a positive response to GLA, those with undetectable expression, including MDA MB 231 breast cancer, HT115 colon, SK LU 1, Cor L47 and Cor L677 lung, and M19 melanoma cells, showed no response. Endothelial cells ECV304 and fibroblasts MRC5 also had no detectable E-cadherin with this particular antibody (HECD1), and they did not show any increase with GLA.

**Effects of Other Fatty Acids on E-Cadherin Expression.** Other fatty acids in the n-6 series (LA and AA, and a n-3 fatty acid, EPA) were tested for their effects on E-cadherin (Fig. 1). LA, AA, and EPA caused either no significant increase in E-cadherin expression or very small increases. It is worth noting that in the MCF-7 cells, there was a reduced expression of E-cadherin with LA.

**Immunohistochemical Detection of E-Cadherin.** To determine the location of the expression of E-cadherin, immunohistochemical detection was carried out, and this revealed that cell surface E-cadherin staining was mainly in the cell to cell junctions, with PLC/
PRF/5, HT29, HRT18, MCF-7, Cor L23, ZR-75, and G361 cells staining strongly; Cor L51 and Cor L88 exhibiting weak staining; and M19, MDA MB 231, HT115, SK LU 1, and Cor L47 and Cor L677 cells showing no significant staining. This was consistent with Western blotting. With GLA treatment, there was a marked increased in staining in PLC/PRF/5, HT29, HRT18, MCF-7, Cor L23, ZR-75, and G361 cells. Cor L51, L88, and HT115 also showed increased staining, whereas others (M19, MDA MB 231, SK LU 1, and Cor L47 and Cor L677) showed no response. Fig. 3 shows examples of this staining, with the results for the rest of the cells summarized in Table 1.

**Effects of GLA on Aggregation.** An aggregation assay was performed to test the function of E-cadherin on the cell surface. Cells were cultured with GLA for 24 h, and their aggregation ability was tested in a calcium-containing buffer (HCME plus 2 mM Ca²⁺). After GLA treatment, cells with increased expression of E-cadherin showed increased aggregation compared with their control counterparts. Fig. 4 shows examples of these cells at different time points (15, 30, and 45 min). Inclusion of anti-E-cadherin antibody (HECD-1) or calcium-chelating agent (EGTA) abolished GLA-induced aggregation of HRT18 cells and significantly reduced GLA-induced aggregation of MCF-7 cells (Fig. 3). Table 1 summarizes the results from all of the cell types at t = 30 min. There was a correlation between the expression of E-cadherin and aggregation in different cell types.

**Effects of GLA on Invasion.** It has been proposed that the level of E-cadherin on the tumor cell surface is inversely correlated with their invasive behavior. We tested whether a change in E-cadherin expression correlated with invasion by using a Matrigel invasion assay. In this study, HGF/SF (20 ng/ml) was placed in the lower chamber of the culture system as a chemoattractant (8). A marked reduction in cell invasion into Matrigel was observed with those cells displaying an increase in E-cadherin (Fig. 5). Both HECD-1 and EGTA prevented the inhibitory effect of GLA on invasion. Furthermore, both HECD-1 and EGTA increased invasion in unstimulated and HGF/SF-treated cells (Fig. 5).

Cell growth in the presence of GLA (75 μM) was determined by measuring cellular DNA quantities with a Hoescht 33258 assay. This experiment demonstrated that GLA, at the highest concentration used in this study, did not change the quantity of DNA in the cultures, indicating that GLA at such concentrations was not toxic to the cells.

**DISCUSSION**

In this study, we have shown that GLA enhanced the expression of E-cadherin, an effect which was correlated with increased aggregation and reduced *in vitro* invasion of these cells. Furthermore, increased aggregation and decreased invasion were dependent on extracellular calcium and prevented by anti-E-cadherin antibody, demonstrating that GLA induced E-cadherin was functionally active. Other *n*-6 PUFAs showed little or no effects.

Metastasis is a key factor in determining the prognosis of the patients with cancer. To establish a distant metastasis, a tumor cell must detach from the primary tumor, migrate through the basement membrane and extracellular matrix, invasavate, and travel in the circulation to the new site before reattachment, extravasation, or the development of a new focus or neovascularization occurs (25-28). Essential and early events if a tumor is to initiate this “metastatic cascade” (28, 29) are the loss of cell to cell adhesion and modified cell-matrix interactions, which allow the tumor cells to become motile and enhance their invasive potential. E-cadherin is a cell to cell adhesion molecule which is linked to the cytoskeleton by a group of proteins now known as catenins (17, 30). E-cadherin is now widely accepted as a tumor/metastasis suppressor. Reduced expression, deletion, or mutation of E-cadherin has been shown in a variety of human cancers, and levels of the molecule are inversely correlated with disease stage, tumor invasiveness, and long-term prognosis of the patients (10-12, 16-20).

Such a relationship has raised interest in elucidating ways of regulating E-cadherin expression in the hope that this may inhibit the motile and invasive nature of tumor cells. Bracke *et al.* (31) have reported that tamoxifen restores the E-cadherin function in human breast cancer MCF-7/6 cells and suppresses their invasive phenotype. Vandewalle *et al.* (32) have recently reported that a higher calcium environment may increase the expression of E-cadherin on MCF-7 cells. Relaxin (33), 17-ß estradiol (34), and retinoic acids induce the expression of E-cadherin (35). Here, our data add GLA to the short list of E-cadherin up-regulatory factors. Of the variety of cell types tested in this study, most of them responded to GLA. However, those with absent expression of E-cadherin showed no response. We currently have no evidence that the effects of GLA are either on transcription or translation levels. Further work is now required to establish the mechanism of regulation of this cell to cell adhesion molecule.

In our study of the fatty acids tested, GLA showed the strongest up-regulation of E-cadherin expression, whereas LA and AA did not. Interestingly, LA slightly inhibited E-cadherin expression in MCF-7 cells. It has been previously reported in animal studies that LA may stimulate growth and metastasis of some breast cancer models (36, 37), whereas oils containing both GLAs inhibit breast cancer growth in the nude mouse model (38). Our data may provide a partial explanation for those phenomena.

The clinical importance of this result is worth exploring. GLA has been used in pilot studies in patients with cancers, including breast, melanoma, and pancreatic cancer, as an adjuvant therapy. All of these tumor types have been shown in *vivo* to be rather sensitive to GLA in terms of cytotoxicity. Patients with other tumors such as colon cancers, which are less sensitive to GLA, are not widely considered suitable candidates for GLA cytotoxic treatment. Our study, along with previous reports on the GLA-induced inhibition of cell motility and invasion (8), suggests that the cytotoxic effects of GLA and the effects on motility/invasion can be achieved independently. The latter effects can be achieved at much lower concentrations, making GLA of possible value as an adjuvant to surgery, chemotherapy, or radiotherapy.

This study reports that GLA, a *n*-6 series essential fatty acid, increases expression of E-cadherin in a range of human cancer cells. The increased expression is associated with improved biological function as indicated by increased aggregation and reduced invasion of these cells. This may have importance in the development of future clinical studies.

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