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Enhanced Tight Junction Function in Human Breast Cancer Cells by Antioxidant, Selenium and Polyunsaturated Lipid

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Abstract Paracellular permeability (PCP) is governed by tight junctions (TJs) in epithelial cells, acting as cell–cell adhesion structures, the aberration of which is known to be linked to the dissociation and metastasis of breast cancer cells. This study hypothesized that the function of TJs in human breast cancer cells can be augmented by gamma linolenic acid (GLA), selenium (Se), and iodine (I) in the presence of 17-β-estradiol, as these molecules are known to increase TJ functions in endothelial cells, using assays of trans-epithelial resistance (TER), PCP, immunofluorescence, and in vitro invasion and motility models. GLA, I, and Se individually increased TER of MDA-MB-231 and MCF-7 human breast cancer cells. The combination of all three agents also had a significant increase in TER. Addition of GLA/Se/I reduced PCP of both breast cancer cell lines. GLA/Se/I reversed the effect of 17-β-estradiol (reduced TER, increased PCP). Immunofluorescence revealed that after treatment with Se/I/GLA over 24 h, there was increasing relocation to breast cancer cell–cell junctions of occludin and ZO-1 in MCF-7 cells. Moreover, treatment with GLA/Se/I, alone or in combination, significantly reduced in vitro invasion of MDA-MB-231 cells through an endothelial cell barrier ($P < 0.0001$) and reduced 17-β-estradiol induced breast cancer cell motility ($P < 0.0001$). Our previous work has demonstrated that GLA, I, and Se alone, or in combination are able to strengthen the function of TJs in human endothelial cells; this has now proved to be true of human breast cancer cells. This combination also completely reversed the effect of 17-β-estradiol in these cells. J. Cell. Biochem. 101: 155–166, 2007.

Key words: breast cancer; tight junctions; invasion; selenium; GLA (gamma linolenic acid); iodine

Cell–cell interactions as well as ECM–cell interactions are indispensable for normal tissue architecture. In epithelial tissue cell–cell interactions are mediated by junctional complexes that consist of tight junctions (TJs) and adherens junctions, desmosomes and gap junctions, each of which possess unique morphological characteristics, composition, and functions [Itoh and Bissell, 2003]. TJs are directly involved in paracellular sealing and in membrane domain differentiation. They are found at the most apical region of the cell junction complex where they occlude the extracellular space [Wong and Gumbiner, 1997]. Epithelial and endothelial cell sheets establish compositionally distinct fluid compartments [Tsukita and Furuse, 1999] and for these to function as barriers maintaining the distinct internal environment of each compartment, the TJs regulate the tight seal between the paracellular pathway between adjacent cells in the sheet, thus preventing the diffusion of solutes; the apical and basolateral membrane domains must be differentiated to allow active transport across the sheet [Tsukita and Furuse, 1999]. The TJ is thus crucial for generating chemical and electrical gradients across the cell monolayer that is necessary for vectorial transport processes such as absorption and secretion.

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Breast tumors characteristically lack TJs [Hoover et al., 1998], which is consistent with the idea that loss of cell–cell adhesion is essential for tumor invasion [Glukhova et al., 1995]. Only a relatively small number of papers have been published investigating the function of TJs in human breast epithelial cells. TJs in mammary glands have been mainly investigated in relation to lactogenesis [Itoh and Bissell, 2003]. TJs also regulate transport of small molecules between cells and therefore allow epithelial cells to form a cellular barrier that separates compartments of different composition. This intercellular barrier formed by TJs has selectivity in molecular size and ion type as well as differences in permeability depending on the cell type and condition. Recent studies have shown that several TJ components are directly or indirectly involved in breast cancer progression, such as ZO-1, ZO-2, claudin-7, claudin-1 [Hoevel et al., 2002; Kominsky et al., 2003; Martin et al., 2004; Morin, 2005; Soini, 2005; Martinez-Estrada et al., 2006]. We have recently shown that standardized transcript levels of ZO-1 were significantly lower in patients with metastatic breast disease as compared with those remaining disease-free (DF) (median follow-up: 72.2 months) [Martin et al., 2004]. Immuno-histochemistry confirmed these results, with decreased levels in ZO-1 staining. For both ZO-1 and ZO-3, staining was confined to the intercellular regions in normal tissue, whereas in tumor tissues staining was diffuse and cytosolic. It was concluded that low levels of TJ plaque molecules, such as ZO-1 and MUPP-1, in breast cancer are associated with poor patient prognosis.

A significant portion of the body of work concerning TJs has concentrated on the effect of disruptive agents, rather than those that might enhance their structure and function. This study was carried to continue investigations as to what agents may be able to augment TJ functions. Iodine is an essential component of the thyroid hormones, thyroxine and triiodothyronine, and has been thought to be used almost exclusively by the thyroid. Iodinated contrast media (ICM) are extensively employed for diagnostic imaging and should be biologically inert [Fanning et al., 2002]. Reports indicate that these agents are able to alter cell function. Selenium, an essential dietary nutrient for mammals and known antioxidant, has been found to exert a chemo-preventative activity in cancer [Ip and Ganther, 1991; Kowaltowski et al., 1998] with increased selenium level associated with decreased cancer incidence, although mechanisms by which this occurs remain unclear [Gupta et al., 1994]. It has been suggested that selenium is able to inhibit neo-angiogenesis [Jiang et al., 1999; Lu, 2001] and is anti-angiogenic in malignant breast tumors possibly due to reducing VEGF production [Streicher et al., 2004]. Selenium has been shown to be able to affect the expression of cell adhesion molecules (ICAM-1, VCAM-1), crucial in the inflammatory process [Jahnova et al., 2002].

We have previously shown that 17-β-estradiol can induce concentration- and time-related biphasic effects on TJ functions expression of occludin in endothelial cells and that this perturbation of TJ functions may have implications in the etiology of mastalgia [Ye et al., 2003]. Recently, we have demonstrated that treatment of human endothelial cells with gamma linolenic acid (GLA), selenium (Se), and iodate (I), alone or in combination, increased trans-endothelial cell resistance (TER) and reduced the paracellular permeability (PCP) to large molecules and was active against the effect of 17-β-estradiol [Martin et al., 2006], via the redistribution of claudin-5. The effects were seen without any changes in the viability of the endothelial cells. Occludin, which plays a major role in TJs, was upregulated by this fatty acid as revealed by both Western blotting and immunofluorescence [Jiang et al., 1998; Martin et al., 2006].

This current study examined how the effect that GLA has on human breast cancer cell TJs might be augmented. GLA was added to culture medium with/without the addition of selenium and/or iodine (GLA 100 μm; Se 50 ng/ml; I 10 ng/ml) [Martin et al., 2006]. We also investigated whether GLA/Se/I, alone or in combination, could inhibit the effect of 17-β-estradiol on human breast cancer cells and changes in breast cancer cell invasion and motility.

MATERIALS AND METHODS

Reagents and Antibodies

Anti-occludin and ZO-1 antibodies were purchased from PharMingen International (BD Biosciences, Erembodegem, Belgium).

Peroxidase-conjugated anti-mouse, anti-rabbit IgG for Western blotting were from Sigma-Aldrich Ltd. (Poole, Dorset, UK). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse and anti-rabbit IgG were from Santa-Cruz Biotechnologies (Santa-Cruz, CA). FITC-conjugated Dextran (10 kDa) was obtained from Molecular Probe, Inc. (Eugene, OR). Carbonate filter inserts with pore size of 0.4 μm (for 24-well plates) were from Greiner Bio-One Ltd. (Stonehouse, Glos, UK). GLA, sodium selenate (Se), iodate (I), and 17-β-estradiol were purchased from Sigma-Aldrich TD.

Cell Lines

Two human breast cancer cells lines, MDA-MB-231 and MCF-7, and the human endothelial cell line, HECV (ICLC Genova, Italy), were routinely maintained in Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich Ltd, TD) supplemented with 10 % fetal calf serum (FCS), penicillin and streptomycin (Sigma-Aldrich TD).

Trans-Epithelial Resistance (TER)

TER was measured with an EVOM voltohmmeter (EVOL, World Precision Instruments, Aston, Herts, UK), equipped with a pair of STX-2 chopstick electrodes (WPI, Sarasota, FL), as we previously reported [Ye et al., 2003; Martin et al., 2006]. Briefly, MDA-MB-231 or MCF-7 cells were seeded into the 0.4 μm pore size insert (upper chamber) and allowed to reach full confluence, after which fresh medium was replaced for further experiments. Inserts without cells, inserts with cells in medium, and inserts with cells with GLA/Se/I were tested for a period of 24 h. Electrodes were placed at the upper and lower chambers and resistance was measured with the voltohmmeter.

Trans-Epithelial Cell Permeability

This was determined using fluorescently labeled dextran FITC-Dextran-40, molecular weight being 10 kDa [Jiang et al., 1998]. Human breast cancer cells were prepared and treated as in the TER study, but with the addition of Dextran-40 to the upper chamber. Medium from the lower chamber was collected for intervals up to 24 h after addition of GLA/Se/I. The relative fluorescence from these collections was read on a multichannel fluorescence reader (Denly, Sussex, UK).

Immunofluorescent Staining of Human Breast Cancer Cells (MCF-7)

For immunofluorescence staining, cells were grown in 16-well chamber slides (LAB-TEK Fisher Scientific UK, Loughborough, Leics, UK) (30,000 cells/well) in the presence or absence of GLA/Se/I and incubated in a 37°C/5% incubator for a set period of time (0–24 h). After incubation, the culture medium was aspirated, the wells rinsed with balanced salt solution (BSS) buffer and the cells fixed in methanol for 20 min at −20°C. After fixation the cells were washed twice using BSS buffer and permeabilized by the addition of 200 μl of 0.1% Triton X-100 (Sigma Aldrich TD) detergent in phosphate buffered solution (PBS) for 5 min at room temperature. Cells were rinsed twice with BSS buffer and 200 μl of blocking buffer (10% horse serum in TBS) was added to each well and the chamber slide incubated for 40 min at room temperature on a bench rocker. The wells were washed once with wash buffer (3% horse serum in TBS buffer containing 0.1% Tween20) and 100 μl of primary antibodies prepared in wash buffer was added to the appropriate wells. The chamber slide was incubated on the rocker for further 60 min at room temperature. Wells were washed twice with TBS buffer (with 0.1% Tween20) and cells were incubated in 100 μl of secondary antibodies (FITC conjugate) (diluted in the same manner as the primary antibodies) for 50 min. The chamber slide was wrapped in foil to prevent light reaching the conjugate. Finally, the wells were rinsed twice with wash buffer, once in BSS buffer mounted with FluorSave (Calbiochem-Novabiochem Ltd, Nottingham, UK) reagent and visualized using an Olympus BX51 microscope with a Hamamatsu Orca ER digital camera at 100× using oil immersion lens.

Endothelial Cell Barrier Invasion Assay

Trans-well chambers equipped with 6.5 mm diameter polycarbonate filter (pore size 8 μm) (Becton Dickinson Labware, Oxford, UK) are pre-coated with 50 μg/membrane (100 μl) of solubilized basement membrane in the form of Matrigel (Collaborative Research Products, Bedford, MA) and dried over-night. After membrane re-hydration (100 ml complete medium), HECV cells were seeded and left to reach confluency. Following this, 30,000 breast cancer cells previously labeled with DiI were aliquoted

into each insert and treated as described. After 96 h co-culture, non-invasive cells are removed from the inner chamber with a cotton swab. Invaded cells on the underside of the insert were then fixed (4% formaldehyde). The cells are then counted using fluorescent microscopy (10 fields/insert).

Cytodex-2-Bead Motility Assay

Cells are pre-coated onto cytodex-2 carrier beads (Sigma-Aldrich TD) for 2 h in complete medium [Jiang et al., 1995]. After the medium is aspirated and the cells washed (X2 in complete medium), they are aliquoted into wells of a 96-well plate in triplicate (300 μl/well). I/Se/GLA with/without 17-β-estradiol was added and the cells incubated overnight. The beads are washed off in medium, and the cells that migrated onto the floor of each well fixed (4% formaldehyde) and stained with crystal violet. The cells are then counted microscopically (40×, 10 fields of view).

Statistical Analysis

Statistical analysis was performed by MINITAB version 14 (Minitab, Inc. State College, PA) using *t*-tests.

RESULTS

Se, I, GLA, and Combinations Increase TER and Decrease PCP in Human Breast Cancer Cells

The concentrations of each agent were chosen by previous experimentation [Martin et al., 2006] as most beneficial for enhancement of TJ function (I at 10 ng/ml, Se at 100 ng/ml, and GLA at 100 μm). Using these concentrations, combinations were then used for further experiments. Of each independent agent, GLA increased TJ function as assessed by TER over 4 h (MCF-7 control 21 ± 1, GLA 65 ± 6; MDA-231 control -34 ± 4, GLA 52 ± 1), although I had the most sustained effect in MCF-7 cells (Fig. 1A) whereas GLA and Se had similar effects in MDA-MB-231 cells (Fig. 1B). Again, when using TER, all the combinations increased TJ function over 0–4 h, with the combination of all three compounds being particularly effective (Se and I and GLA: MCF-7 101 ± 4; MDA-MB-231 84 ± 11, Fig. 1C,D). Addition of 17-β-estradiol (the most effective, water soluble form estrogen commercially available) was used to ascertain the effect of Se, I, and GLA on 17-β-estradiol-induced TER in human breast cancer cells. 17-β-estradiol significantly reduced TER over 4 h (Fig. 2), particularly in the

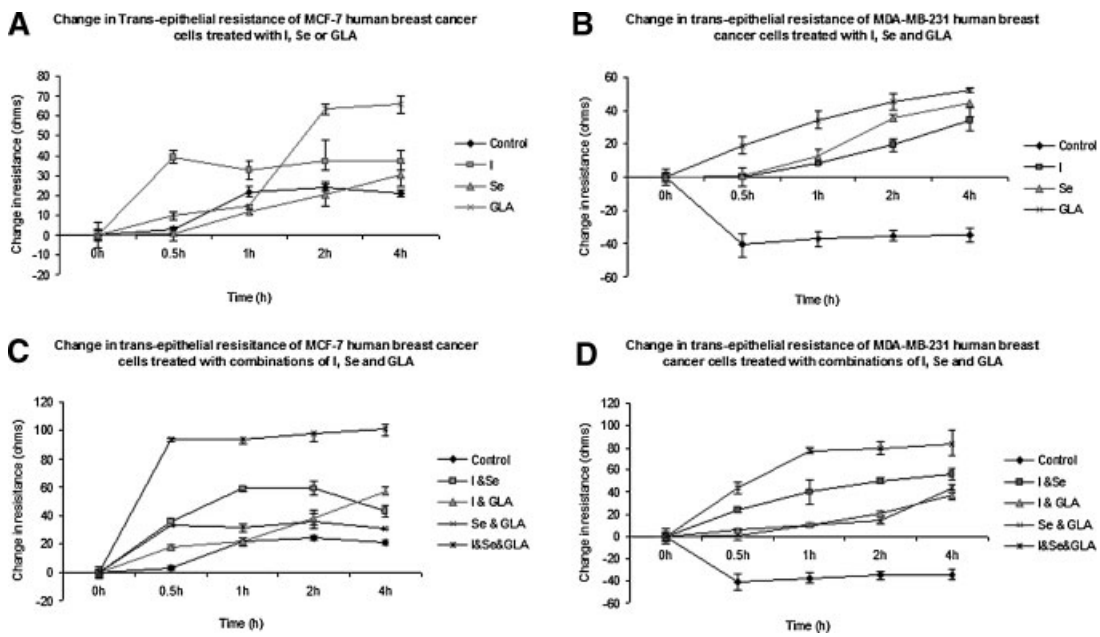

Fig. 1. Change in trans-epithelial resistance (TER) (ohms) of human breast cancer cells treated with I (10 ng/ml), Se (100 ng/ml), and GLA (100 μm) over 4 h. **A** and **B**: Effect on TER of each agent independently in MCF-7 and MDA-MB-231 cells, respectively. **C** and **D**: Effect of these agents in combination, again, MCF-7 and MDA-MB-231 cells, respectively.

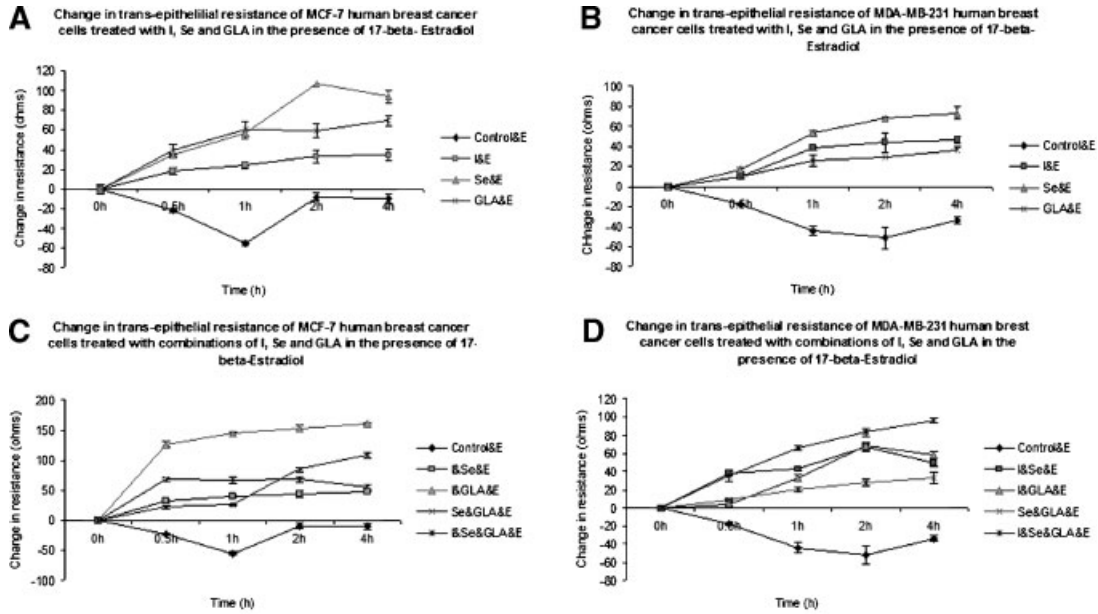

Fig. 2. Change in TER (ohms) of human breast cancer cells treated with I (10 ng/ml), Se (100 ng/ml), and GLA (100 μm) over 4 h with addition of 17-β-estradiol. **A** and **B**: Effect on TER of each agent independently against 17-β-estradiol in MCF-7 and MDA-MB-231 cells, respectively. **C** and **D**: Effectiveness of these agents in combination against 17-β-estradiol in MCF-7 and MDA-MB-231 cells, respectively.

Erβ2 expressing MDA-MB-231 cells. Individually, Se was most effective at negating any effect of 17-β-estradiol (at 25×10^{-9}M) in both breast cancer cells, although GLA was next most effective in MCF-7 cells, compared with I in MDA-MB-231 cells (at 4 h: MCF-7 control -10 ± 4; Se 94 ± 6; I 34 ± 5; GLA 69 ± 6; MDA-MB-231 control -33 ± 3; Se 73 ± 6; I 46 ± 3; GLA 36 ± 3) (Fig. 2A,B). Combinations of these were then used to assess their effect against 17-β-estradiol-induced changes at 25×10^{-9}M. Combinations of Se, I, and GLA all reversed this effect, particularly the combination of all three in MDA-MB-231 cells (17-β-estradiol with Se, I, and GLA in combination 96 ± 2) (Fig. 2D); in comparison, the I and GLA combination was most effective in MCF-7 cells (161 ± 2) (Fig. 2C). We have previously shown that 17-β-estradiol reduced TJ function in endothelial cells—these results indicate that these compounds are able (in combination) to reverse the effect of 17-β-estradiol in this cell type.

An additional test was also performed, namely change in PCP, another assessment of TJ function. When evaluating the effect of these compounds using (PCP, there was a similarly dramatic effect) (Fig. 3A,B). Permeability increases over time due to changes in the structure of the epithelial cell layer. Addition of all three substances reduced this permeability effect over 4 h, in both cell lines (at 4 h: MCF-7 control 498 ± 30 RFU; Se 84 ± 5; I 73 ± 78; GLA -549 ± 111; at 4 h: MDA-MB-231 control 549 ± 122 RFU; Se 431 ± 76; I -13 ± 316; GLA -345 ± 350). Combinations of Se, I, and GLA were also effective at reducing PCP, with a combination of all three being effective over 4 h in MDA-MB-231 cells (I and Se and GLA -580 ± 23) (Fig. 3C,D).

In contrast to our previous work on human endothelial cells, the addition of 17-β-estradiol increased PCP of both human breast cancer cell lines when compared to no 17-β-estradiol control and addition of Se, I, and GLA alone reduced this induced permeability (Fig. 4A,B). Addition of combinations of Se, I, and/or GLA were also effective at reducing permeability over 4 h with addition of 17-β-estradiol (Fig. 4C,D) with combinations including I in MCF-7 cells and all treatments in MDA-MB-231 cells particularly effective.

Immunofluorescence Reveals the Relocation of TJ Molecules in MCF-7 Cells

After treatment with the Se/I/GLA alone or in combination, over 4 h treatment, immunofluorescence was used to evaluate the staining pattern and intensity of the two TJ proteins:

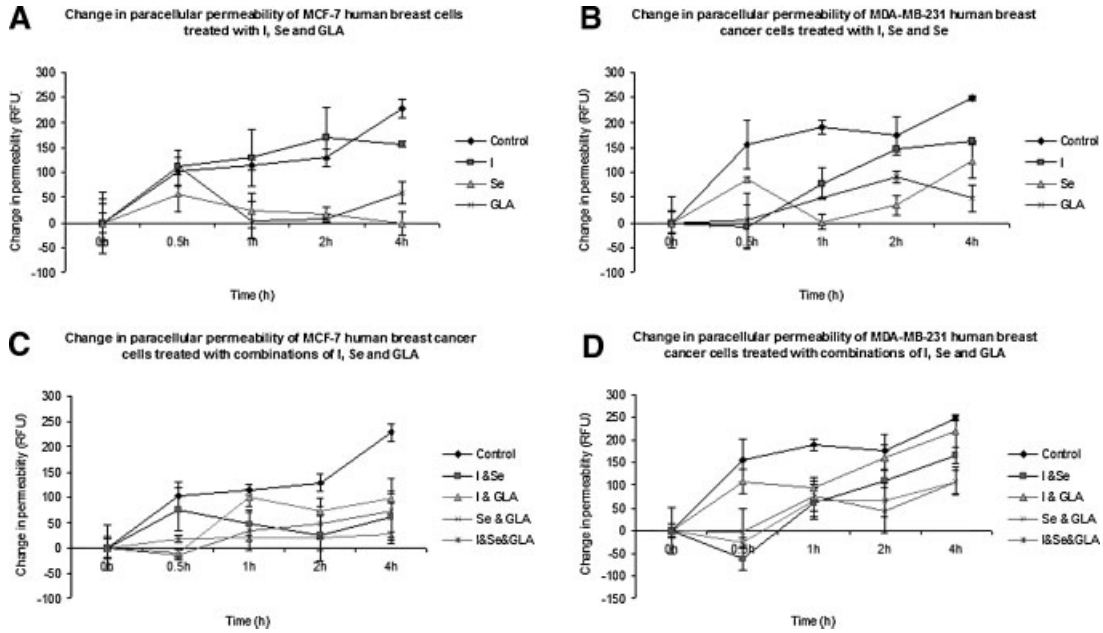

Fig. 3. Change in paracellular permeability (PCP) (RFU) of human breast cancer cells treated with I (10 ng/ml), Se (100 ng/ml), and GLA (100 μm) over 4 h. **A** and **B**: Effect on TER of each agent independently in MCF-7 and MDA-MB-231 cells, respectively. **C** and **D**: Effect of these agents in combination, again, MCF-7 and MDA-MB-231 cells, respectively.

ZO-1 (the plaque/peripheral protein responsible for maintenance of TJ structure and function) and occludin (the transmembrane protein that confers cell–cell adhesiveness and TJ functioning). Over the 4 h incubation, it was evident that the intensity of staining was increased for both occludin (Fig. 5) and ZO-1 (Fig. 6) (representative of four replicate

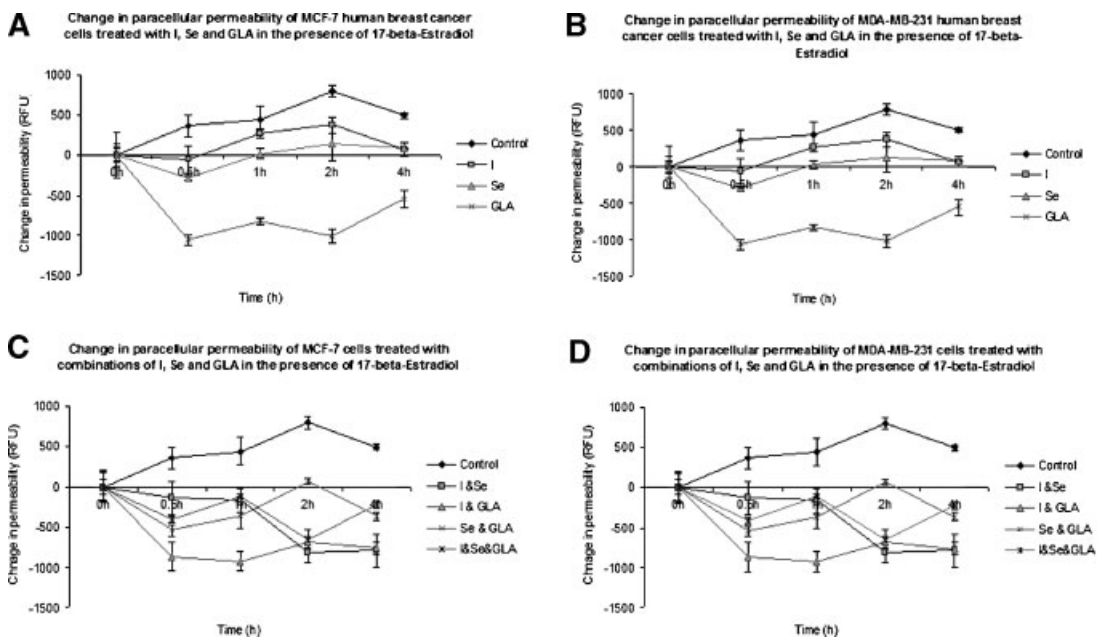

Fig. 4. Change in PCP (RFU) of human breast cancer cells treated with I (10 ng/ml), Se (100 ng/ml), and GLA (100 μm) over 4 h with addition of 17-β-estradiol. **A** and **B**: Effect on TER of each agent independently against 17-β-estradiol in MCF-7 and MDA-MB-231 cells, respectively. **C** and **D**: Effectiveness of these agents in combination against 17-β-estradiol in MCF-7 and MDA-MB-231 cells, respectively.

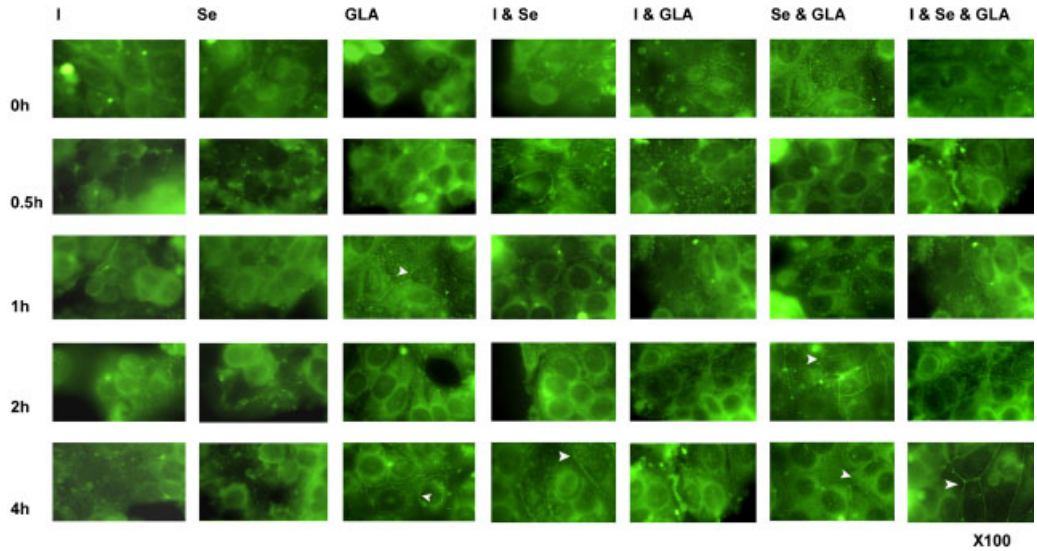

Fig. 5. Effect of I, Se, and GLA, alone and in combination, on relocation of occludin in MCF-7 human breast cancer cells treated over 4 h (X100). The arrows indicate the accumulation of ZO-1 to the cell–cell junctions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

experiments). Occludin showed increased intensity with all treatments (Fig. 5). Occludin showed relocation to the cell junctions of adjacent cell by 4 h incubation, particularly when the MCF-7 cells were treated with combinations of I and Se or all three agents together. ZO-1 also showed increased tendency to relocation at the cell membranes in cells treated with Se alone or in combination with I and/or GLA.

Inhibited Invasion of MDA-MB-231 Human Breast Cancer Cells Through an Endothelial Cell Barrier

The effect of these agents against the invasive potential of the MDA-MB-231 human breast cancer cells was assessed using an in vitro invasion model composed of a semi-permeable membrane layered with basement membrane and human endothelial cells. As can be seen in

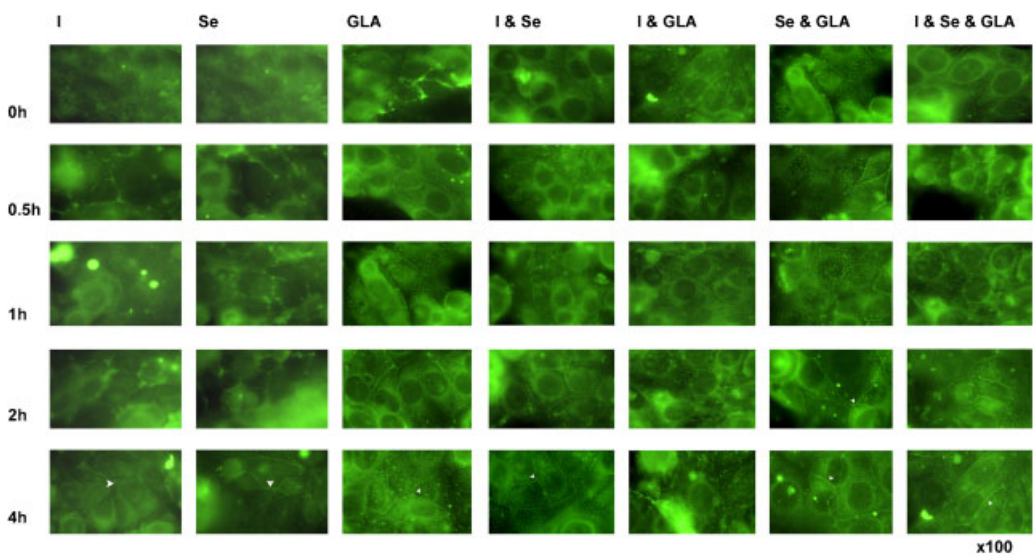

Fig. 6. Effect of I, Se, and GLA, alone and in combination, on relocation of ZO-1 in MCF-7 human breast cancer cells treated over 4 h (X100). The arrows indicate the accumulation of ZO-1 to the cell–cell junctions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

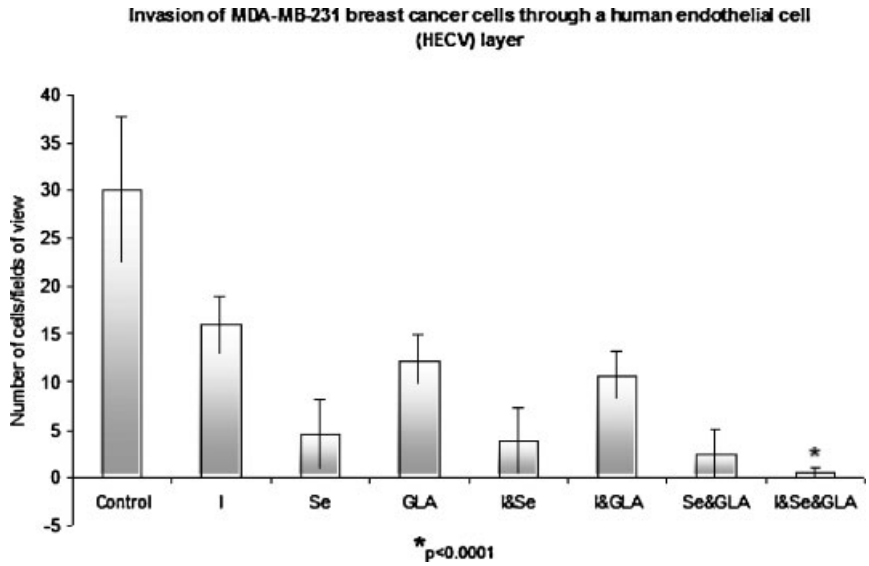

Fig. 7. Effect of I, Se, and GLA on the in vitro invasion of MDA-MB-231 human breast cancer cells through an endothelial cell layer. All these agents, alone or in combination, inhibited the invasiveness of these cells.

Figure 7, all three agents significantly inhibited invasion of the breast cancer cells, particularly treatments containing Se (control 30.1 ± 7.6; I 15.9 ± 2.9; Se 4.5 ± 3.7; GLA 12.2 ± 2.6; I and Se 3.8 ± 3.2; I and GLA 10.7 ± 2.5; Se and GLA 2.5 ± 2.6; I and Se and GLA 0.5 ± 0.5), either alone or in combination. A combination of all three agents was most effective ($P < 0.0001$).

Inhibited Motility of MDA-MB-231 Breast Cancer Cells

The motility of the MDA-MB-231 cells was investigated using a Cytodex bead motility assay to assess the effect of I, Se, and GLA. I and Se were most effective at reducing breast cancer cell motility, with Se especially effective (control 7.35 ± 1.3; Se 2.9 ± 0.9, $P < 0.0001$; Se and GLA 3.3 ± 1.3, $P < 0.0001$; I and Se 3.55 ± 0.9, $P < 0.0001$; GLA and I 4.6 ± 1.2, $P < 0.0001$; I, Se, and GLA 5.8 ± 1.2, $P = 0.003$) even in the presence of 17-β-estradiol (control 10.9 ± 1.6; Se 3.35 ± 1.1, $P < 0.0001$; I, Se, and GLA 5.35 ± 1.6, $P < 0.0001$) (Fig. 8).

DISCUSSION

This present study, a follow-up to our previous work in endothelial cells [Martin et al., 2006], reveals that I and Se, alone or in combination, can alter the function of TJs in human breast cancer cells in conjunction with GLA, as we have previously shown [Martin et al., 2000; Ye et al., 2003]. The combinations of Se, I, and GLA were also able to inhibit the effect that 17-β-estradiol had on TJ function. From immunocytochemical staining it was evident that these increases in TJ function were affected by the increased location of occludin and ZO-1 to the cell–cell junction of MCF-7 cells. Moreover, the invasion and motility of MDA-MB-231 cells were significantly reduced upon treatment with the same. This suggests that these substances may have an impact in the regulation of TJs of breast epithelial cells, thus affecting their function as regards cell–cell adhesion and permeability.

TJs are thought to be directly involved in barrier function and fence functions in epithelial and endothelial cells by sealing them to generate the primary barrier against the diffusion of solutes through the paracellular pathway and by acting as a boundary between the apical and basolateral plasma membrane domains to create and maintain cell polarity, respectively [Furuse et al., 1998]. Different tissues possess TJs with dissimilar permeability to ions and molecules [Wong, 1997] and appear to be important for the respective physiological function of each tissue. Due to the location of the TJs they are involved in a wide variety of pathological conditions where the physiological regulation of passage of ions, molecules, and inflammatory cells may be affected [Sawada et al., 2003]. These

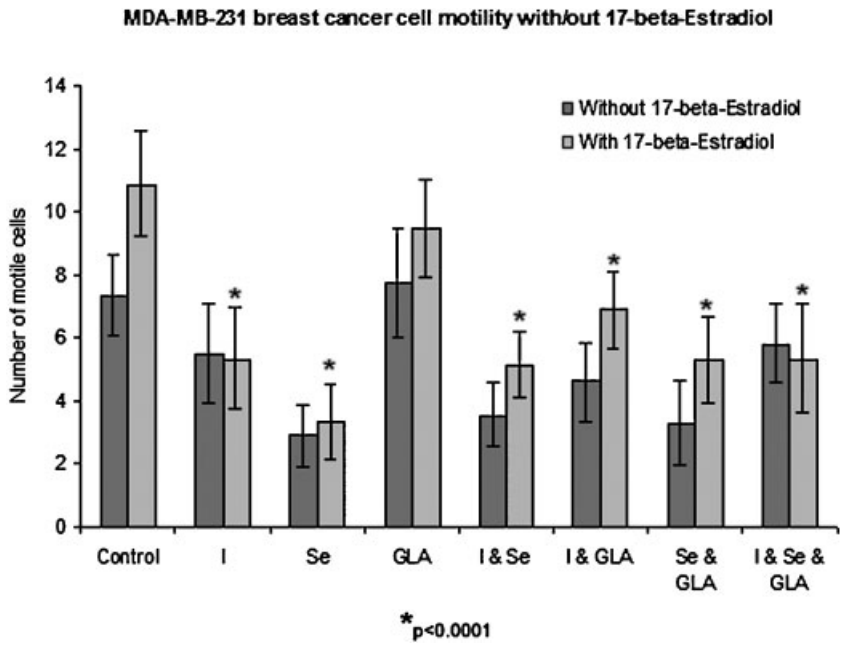

Fig. 8. Effect of I, Se, and GLA on the motility of MDA-MB-231 human breast cancer cells with/without 17-β-estradiol. Treatments containing Se were most effective in both cases.

conditions include vascular system barrier function disturbances (edema, multiple sclerosis, metastasis, cytokinemia), gastrointestinal tract diseases (Crohn's, colitis, gastritis, Coeliac disease) as well as jaundice, asthma, viral infections, etc. [Sawada et al., 2003]. During such various dynamic physiological processes such as leukocyte transmigration across endothelium, the TJs are tightly controlled. The permeability barrier is temporarily disrupted, but subsequently re-sealed, relatively quickly (usually within 1 h) with the re-sealing of the TJ accomplished by assembly of pre-existing elements, rather than re-synthesis of junctional components [Wong, 1997].

We have previously demonstrated that GLA is able to regulate the expression of occludin in human endothelial cells [Ye et al., 2003; Martin et al., 2006] and it has been shown that GLA increases TER and expression of occludin in brain capillary endothelial cells [Yamagata et al., 2003]. Our recent work has shown that Se, I, and GLA in combination can upregulate the expression of occludin, ZO-1, and claudin-5 in human endothelial cells, leading to increased TJ function [Martin et al., 2006]. Moreover, we have also previously demonstrated that 17-β-estradiol is able to reduce TJ function via reduced occludin expression in human endothelial cells [Ye et al., 2003] and that Se, I, and GLA, alone or in combination, can prevent the effect of 17-β-estradiol in human endothelial cell TJs. This lends considerable support to the evidence presented here, that GLA, I, and Se can reduce the effects of this hormone on human breast cancer cells, including reduced motility and reduced invasive potential of these cells through an endothelial cell barrier.

There were some differences in effect between the agents and the two cell lines used. The invasive human breast cancer cell line MDA-MB-231 showed greater response to Se and GLA when assessing TER, whereas I had a sustained effect in the non-invasive MCF-7 human breast cancer cells. A combination of all three was most effective, but this discrepancy in effect is highly interesting, given the difference in pathology of these two cell lines. Moreover, this remained the effect even in the presence of 17-β-estradiol. Even changes in PCP showed a disparity between the cell types, with although GLA being most effective for both, I or I combinations were most effective with MCF-7 cells. A recent study showed that selenium suppressed estrogen induction of the endogenous target gene c-myc [Lee et al., 2005]. In contrast to the effect on ERα in MCF-7 cells, selenium increased ERβ mRNA expression in MDA-MB231 human breast cancer cells. Thus, differential regulation of ERα and ERβ in breast

cancer cells may represent a novel mechanism of selenium action and provide a rationale for selenium breast cancer prevention trials. This would be an interesting line of inquiry for future work in this area, particularly in attempting to elucidate the exact mechanism by which these changes are effected.

Studies on TJs have multiplied over recent years and TJs are being increasingly associated with a number of pathological conditions. However, the majority of these studies are conducted as investigations into the mode and means of cancer and metastatic spread, concentrating on agents that are able to disrupt and dismantle TJs. Few studies have investigated agents that are able to increase the "strength" of TJs and thus prevent "leakiness" of this vital structure. This is a novel study in breast cancer cells, suggesting possible combinations of agents that may have this strengthening effect. It has long been known that GLA has a positive effect on cell–cell adhesion, but we have for the first time shown that this effect can be increased with the addition of I and Se, two dietary supplements. Blood selenium has been shown to decline as breast cancer progresses and fluctuates with estrogen [Breedlove et al., 2006]. Se has been demonstrated to be able to modulate adhesion molecule expression during the inflammatory process [Jahnova et al., 2002] and evidence has proposed that Se may possess cancer preventative activity via inhibition of angiogenesis [Lu, 2001; Jiang et al., 1999], particularly in breast cancer, where low levels of Se are linked to induction of proangiogenic growth factors such as VEGF [Streicher et al., 2004]. It has also been shown that the selenium compound methylseleninic acid (MSA) inhibits estrogen receptor alpha (ERα) signaling in ER-positive MCF-7 breast cancer cells as evidenced by decreased estradiol-dependent cell growth and gene expression. Shah et al. [2005] and Singh et al. [2005] have shown that there was a 7% lower risk of breast cancer if the level of selenium was increased by 1 unit indicating a strong association of vitamin C, vitamin E, and selenium with breast cancer in the Indian population. Interestingly, Se has also been shown to regulate permeability of mitochondrial pores [Shilo et al., 2003] and that Se deficiency in rats leads to increased permeability of the heart and eye [Demirel-Yilmaz et al., 1998].

It has been demonstrated [Menendez et al., 2005] that GLA treatment substantially reduced Her-2/neu protein levels in the Her-2/neu-overexpressing cell lines BT-474, SK-Br3, and MDA-MB-453 (breast cancer), SK-OV3 (ovarian cancer), and NCI-N87 (ga stroin-testinal tumor derived). GLA exposure led to a dramatic decrease in Her-2/neu promoter activity and a concomitant increase in the levels of polyomavirus enhancer activator 3 (PEA3), a transcriptional repressor of Her-2/neu, in these cell lines. Molecular iodine is known to inhibit the induction and promotion of *N*-methyl-*n*-nitrosourea-induced mammary carcinogenesis, to regress 7,12-dimethylbenz(a)anthracene-induced breast tumors in rats, and has also been shown to have beneficial effects in fibrocystic human breast disease. Cyto-toxicity of iodine on cultured human breast cancer cell lines, namely MCF-7, MDA-MB-231, MDA-MB-453, ZR-75-1, and T-47D, was reported [Shrivastava et al., 2006], with iodine-induced apoptosis in all of the cell lines tested, except MDA-MB-231. It has been proposed that the digestion of nutrients high in I and Se (having a synergistic effect with I) contributes to lower incidences of both benign and malignant breast disease [Cann et al., 2000]. It has been shown that I deficiency enhances mammary tissue sensitivity to 17-β-estradiol in rats [Eskin et al., 1995]. In humans, I-containing desiccated thyroid or thyroxine were effective in reducing mastalgia [Daro et al., 1964; Estes, 1981]. Moreover, I supplementation in women with benign breast disease and breast pain led to a beneficial effect [Ghent et al., 1993; Vishniakova and Murav'eva, 1966].

17-β-estradiol is known to promote permeability of cervical cells, playing a role in cervical and vaginal secretion [Gorodeski, 2000]. The hormonal regulation of TJs in mouse mammary epithelium has also been investigated [Nguyen et al., 2001]. Closure of the TJs of the mammary epithelium has been shown to accompany the onset of copious milk secretion (lactogenesis) in both goats and humans. Progesterone withdrawal was a trigger for TJ closure within 4 h. The hormonal requirements for TJ closure were similar to those required to promote lactogenesis. It appears that regulation of TJs could have a direct impact on the permeability of blood vessels within mammary tissue; it could be construed that this may be an important regulatory means of edema in the breast during the menstrual cycle, consequently

effecting changes in the breast associated with mastalgia.

In conclusion, this current study has provided evidence that I and Se, two agents previously demonstrated to influence TJ function in endothelial cells, also work together with GLA to enhance the function of TJs in human breast cancer cells. These three agents are also able to negate the 'leaky' effect of 17-β-estradiol on epithelial TJs. A possible mechanism underlying these effects is relocation of the TJ proteins occludin and ZO-1 to the cell–cell junction of these human breast cancer cells. The study has therefore provided a novel insight into the biological and physiological impact of these agents, and in particular their combination on breast cancer cell junctional functions.

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