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Genistein and quercetin increase connexin43 and suppress growth of breast cancer cells

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Connexin proteins form gap junctions, which permit direct exchange of cytoplasmic contents between neighboring cells. Evidence indicates that gap junctional intercellular communication (GJIC) is important for maintaining homeostasis and preventing cell transformation. Furthermore, connexins may have independent functions including tumor growth suppression. Most tumors express less connexins, have reduced GJIC and have increased growth rates compared with non-tumorigenic cells. The purpose of this study was to determine whether common flavonoids, genistein and quercetin, increase connexin43 (Cx43) levels, improve GJIC and suppress growth of a metastatic human breast tumor cell line (MDA-MB-231). Quercetin (2.5, 5 μg/ml) and genistein (0.5, 2.5, 15 μg/ml) upregulated Cx43 but failed to increase GJIC. Cx43 localized to the plasma membrane following genistein treatment (2.5, 15 μg/ml). In contrast, Cx43 aggregated in the perinuclear region following quercetin treatment (0.5, 2.5, 5, 15 μg/ml). Both genistein (15 μg/ml) and quercetin (2.5, 5, 15 μg/ml) significantly reduced MDA-MB-231 cell proliferation. In summary, genistein and quercetin increase Cx43 and suppress MDA-MB-231 cell proliferation at physiologically relevant concentrations. These results demonstrate that genistein and quercetin are potential anti-breast cancer agents.

Introduction

Breast tumors are the most common type of cancer in Caucasian women living in Western nations (1). It is estimated that ~1 in 8 North American females will develop breast cancer throughout their lifetime, and 1 in 30 will die from the disease. Many traditional treatments, include hormone therapy, radiation therapy, chemotherapy and surgical tumor removal, do not ensure a cure and are associated with significant physiological and psychological side effects (2). Therefore, there is a need for more effective treatments with minimal side effects.

Experimental and clinical evidence indicates that cancer is a multi-step process reflecting genetic alterations, which drive the progressive transformation of normal cells to highly malignant ones. Malignant tumors are characterized by self-sufficient growth, insensitivity to anti-growth signals, sustained angiogenesis, the ability to evade apoptosis, replicate indefinitely, and invade surrounding tissue and spread to distant organs. Cell autonomy and dysregulated homeostasis are central to many of these malignant characteristics (3).

Adjacent cells are able to exchange potential homeostatic regulators, including possible anti-growth signals and apoptotic factors, through hydrophilic channels called gap junctions. Each gap junction channel is formed by two hemichannels (connexons) embedded in the plasma membrane of adjacent cells. Each hemichannel is in turn composed of six connexin proteins (4). Presently, 21 different human connexin genes have been identified (5). Three connexin proteins have been identified in human breast tissue: Cx43, Cx26 and Cx32 (6). Cx43 is the most common connexin protein in breast tissue, and is most prominent in basal and lateral surfaces of myoepithelial cells. Cx26 has been identified between luminal cells in major ducts, and to a lesser extent in alveolar/lobular structures and in breast epithelium. Cx32 has been identified in mammary glands in the secretory luminal cells of alveoli during lactation and has been observed co-localized with Cx26 in non-pregnant human breast tissue (6,7).

Most tumors exhibit dysfunctional gap junctional intercellular communication (GJIC) and decreased Cx43 expression (8,9). Additionally, connexin proteins may be internalized and degraded by lysosomes and proteosomes, or impeded during transport to the plasma membrane (10). Alternatively, tumor promoters such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or excessive connexin phosphorylation can block the exchange of possible homeostatic regulators through gap junction pores (11). However, studies have shown that flavonoids, plant-derived molecules with estrogenic activity, can maintain GJIC, and suppress tumor growth, in the presence of tumor promoters (12–14).

Connexin proteins have also been shown to have antitumor effects independent of channel function. Transfection of Cx43 into human glioblastoma cells significantly reduced cell proliferation in monolayer culture and in nude mice without increasing GJIC (15). Also, the C-terminal region of Cx43 protein has been shown to reduce levels of S phase kinase associated protein 2 (Spk2), which in turn suppresses degradation of p27, a protein which halts the cell cycle between the G_1 and S phases (16). Furthermore, introduction of Cx26 and Cx43 into various human breast cancer cell lines (MDA-MB-231, Hs578T and HBL100) suppressed growth independent of GJIC. It was found that Cx43 protein reduced fibroblastic growth factor receptor (FGFR) expression and

Abbreviations: DAPI, 4′-6-diamidine-2-phenylindole; DMSO, dimethyl sulfoxide; ER, estrogen receptor; GJIC, gap junctional intercellular communication; LDH, Lactate dehydrogenase; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

possibly affected the expression of other proteins involved in tumor progression (17).

Asian females are 4–6 times less likely to develop breast cancer compared with North American females (18). However, when Asian females migrate to North America and adopt an American diet, their breast cancer incidence approaches that of North American women (19). This suggests that diet plays a significant role in breast cancer acquisition. Asians consume a greater quantity of soy, fruit and vegetables compared with North Americans (20). These products are rich in flavonoids (21,22). Research has shown that flavonoids have vasodilatory, antiviral, antioxidant, antiallergenic and antitumor functions (23). The most studied flavonoids with respect to antitumor functions are genistein and quercetin, which have been shown to have potent anti-proliferative effects on tumor cells by inducing apoptosis, halting the cell cycle, reducing the growth stimulatory effect of IP_3 and inhibiting tyrosine kinase activity (24–27). Genistein and quercetin can also enhance differentiation, and inhibit estrogen effects by competitively binding to estrogen receptors (ERs) (28,29). Additionally, flavonoids have been reported to upregulate connexin expression and increase GJIC in rat liver epithelial cells (30).

This study investigated the growth suppressive effects of genistein and quercetin on a human metastatic breast tumor cell line, MDA-MB-231, through gap junctional mechanisms. The results demonstrate that genistein and quercetin are potential anti-breast cancer agents since they increased Cx43 expression at physiologically relevant concentrations and suppressed MDA-MB-231 cell proliferation at concentrations which were not toxic to either non-tumorigenic (MSTV1-7 cells) or metastatic human breast cells.

Methods

Treatment chemicals

The flavonoids, genistein (98% HPLC from soybean) and quercetin (98% HPLC) were purchased from Sigma-Aldrich (St Louis, MO). The flavonoids were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to 15 mg/ml, shielded from light and stored at −20°C. In all experiments, cells were treated with genistein (0.5, 2.5, 15 μg/ml) or quercetin (0.5, 2.5, 5, 15 μg/ml) in complete medium for 72 h. The medium was replaced every 24 h during this treatment period. Treatment controls consisted of complete medium only and vehicle control [complete medium supplemented with 0.1% (v/v) DMSO].

Cell culture

The non-tumorigenic human breast cell line MSTV1-7 was used as a positive control for Cx43 expression and GJIC. MSTV1-7 cells were also used to determine whether genistein and/or quercetin treatments were toxic to non-tumorigenic breast cells. Hs578T cells are a human breast tumor cell line, which express abundant Cx43 and readily communicate through gap junctions. Hs578T cells were used as a positive control for Cx43 immunoblotting and GJIC. Experiments were performed on a metastatic human breast tumor cell line, MDA-MB-231. All cell lines were obtained from American Type Culture Collection (Manassas, VA).

MSTV1-7 cells were grown in Dulbecco's modified Eagle's Nutrient Mixture F-12 Ham medium (Sigma-Aldrich) supplemented with 5% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA), 1% (v/v) L-glutamine (Invitrogen), 0.1% (v/v) penicillin–streptomycin (Invitrogen) and 0.1% (v/v) insulin (Invitrogen). MDA-MB-231 cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 0.1% (v/v) penicillin–streptomycin. Hs578T cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum, 2% (v/v) L-glutamine and 0.1% (v/v) penicillin–streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% carbon dioxide.

Cx43 immunocytochemistry

Cells were grown to confluence on 12 mm coverslips (Fischer Scientific; Hampton, NH) in 35 mm plates. Following treatment, the cells were fixed with 80% methanol at −20°C for 20 min. Non-specific antibody binding was blocked with 10% (v/v) normal goat serum and 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The cells were then incubated with goat anti-rabbit Cx43 antibody (1:400 dilution; Sigma-Aldrich) in PBS with 1% (w/v) BSA for 1 h at room temperature. Following washes with PBS, cells were incubated with Alexa-Fluor 568 goat anti-rabbit IgG secondary antibody (1:500 dilution; Molecular Probes, Eugene, OR) in PBS with 1% (w/v) BSA for 1 h at room temperature. Cells were then washed with PBS, quickly rinsed with distilled, deionized water and then mounted with Vectashield medium (Vector Laboratories, Burlingame, CA).

Controls for immunocytochemistry consisted of PBS incubation in place of Cx43 primary and secondary antibody, affinity purified rabbit IgG in place of the Cx43 primary antibody and PBS in place of secondary antibody. Fluorescent immunoreaction was visualized with a Zeiss Axiophot photomicroscope (Carl Zeiss, Thornwood, NY) equipped with filters for fluorescein isothiocyanate (FITC) (excitation 470 nm/emission 525 nm) and Alexa 568 (excitation 546 nm/emission 590 nm). Differential interference contrast (DIC) and fluorescent images were viewed using the ×40 objective (oil). Fluorescent images were captured at an exposure of 490 ms using Zeiss Axioplan 4 software (Carl Zeiss).

Cx43, Ki67, DAPI immunocytochemistry

Ki67 (a proliferation marker), Cx43 and 4′-6-diamidine-2-phenylindole (DAPI) were combined in immunocytochemistry to test for the growth suppressive effects of genistein and quercetin and to quantify the proliferative activity in Cx43-expressing cells. The same procedure as mentioned above for Cx43 immunocytochemistry was performed with the following alterations. Primary antibody incubation consisted of mouse anti-human Ki67 antibody (1:500 dilution; Sigma-Aldrich) combined with rabbit anti-human Cx43 antibody (1:400 dilution; Sigma-Aldrich). Secondary antibody incubation consisted of Alexa-Fluor 568 goat anti-rabbit IgG (1:500; Molecular Probes) for Cx43 and Alexa-Fluor 488 goat anti-mouse IgG (1:500; Molecular Probes) for Ki67. Cells were incubated for 5 min with DAPI (1:34 000; Molecular Probes) before mounting the coverslips. The same immunocytochemistry controls were used as for Cx43 immunocytochemistry except that mouse IgG was used to control for the primary Ki67 antibody.

Ki67 staining was scored as positive or negative (31). All samples were scored by randomly selecting 20 fields of view and without knowledge of the treatment conditions. Chi-squared analysis was performed to determine whether there was a significant difference in Ki67 staining between treatment conditions. Immunocytochemistry was repeated on seven independent MDA-MB-231 cultures.

Protein isolation and western blot analysis of Cx43

Cells were rinsed once with PBS and then scraped off with lysis buffer [10% (v/v) glycerol (Fischer Scientific), 1% (v/v) Nonidet P-40 (Sigma-Aldrich), 0.1% (v/v) sodium dodecyl sulfate (SDS) (Fischer Scientific; Hampton, NH), 0.5 M NaCl, 5 mM Tris (pH 8.0) and protease inhibitor cocktail tablets (Complete, Mini; Roche, Indianapolis, IN)]. DNA in the lysate was sheared using 22- and 27-gauge needles (Beckton Dickinson, Franklin Lakes, NJ). Total protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce-BioLynx, Rockford, IL). Western blot analysis was carried out in triplicate as we have described previously (32).

Measurement of gap junctional dye coupling

Using established protocols, the presence of functional gap junctional coupling was determined (33). Cells were grown to confluence on 60 mm plates (two plates for each treatment condition; one donor plate and one recipient plate). Following aspiration of treatment medium, donor cells were preloaded with dye solution [5 uM calcein-AM (Molecular Probes) and 10 μM DiI (Sigma-Aldrich) in an isotonic (0.3 M) glucose solution] for 20 min in a humidified incubator (37°C, 5% carbon dioxide/95% air). Subsequently, the dye solution was aspirated from the donor plate, and the cells rinsed twice with glucose solution, trypsinized [0.25% trypsin (Invitrogen)] and suspended in 5 ml complete medium. The donor cells were then seeded onto the recipient (unlabeled) cells at a 1:500 ratio. Cells were maintained in the humidified incubator for 6 h and then examined using the aforementioned photomicroscope, capture software, and FITC and rhodamine filters (excitation 570 nm/emission 590 nm). In addition, dye coupling was also assessed using the scrape loading method as described by Ozog *et al*. (32).

Cell count

A total of 2.75×10^5 MDA-MB-231 cells were seeded in triplicate at 10% confluence for each treatment condition (genistein or quercetin, complete medium only, or 0.1% (v/v) DMSO vehicle control). Following 72 h treatment, the cells were trypsinized [trypsin–EDTA, Invitrogen] and suspended in 5 ml medium, and counted using the Beckman Coulter Counter (Beckman Coulter).

Data were analyzed using one-way analysis of variance (ANOVA). The treatment conditions were compared against the 0.1% (v/v) DMSO vehicle control using the Dunnett multiple comparisons test.

Cytotoxicity test

Lactate dehydrogenase (LDH) release was used as a measure of cytotoxicity. Released LDH in culture supernatants was measured with a 30 min coupled enzymatic assay using the CytoTox-96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). The ratio of treatment-induced LDH to total cellular LDH was determined for each day of the 3 day treatment period. Results were normalized to the 0.1% (v/v) DMSO vehicle condition. Data were analyzed using one-way ANOVA. The treatment conditions were compared against the 0.1% (v/v) DMSO vehicle using the Dunnett multiple comparisons test. This cytotoxicity procedure was repeated on extracts from three different cultures for both MDA-MB-231 and MSTV1-7 cells.

Results

Effect of genistein and quercetin on Cx43 protein levels

MDA-MB-231 cells are a metastatic human breast tumor line, which express a very low level of Cx43 protein, and do not form functional gap junctions (17). Western blot analysis was used to determine the relative amount of Cx43 produced following flavonoid treatment. Total protein loaded into each well was determined by probing with an antibody against GAPDH.

Compared with the 0.1% (v/v) DMSO vehicle and complete medium only, Cx43 protein levels increased following genistein and quercetin treatment (Figure 1A and B). Interestingly, as seen in Figure 1A, treatment with physiological levels of genistein (0.5 μg/ml) increased the level of Cx43. The amount of Cx43 increased further following 2.5 μg/ml genistein treatment. However, higher genistein concentrations (up to 20 μg/ml) did not raise Cx43 levels beyond that observed at 2.5 μg/ml. Quercetin treatment, at concentrations of 2.5 and 5 μg/ml, consistently increased Cx43 protein compared with the 0.1% (v/v) DMSO vehicle and complete medium only (Figure 1B). Interestingly, treatment with 15 μg/ml quercetin did not upregulate Cx43 protein compared with the 0.1% DMSO vehicle and complete medium only. This was probably due to cytotoxic effects (see below).

Effects of genistein and quercetin on Cx43 localization

Immunocytochemistry was performed to determine intracellular localization of Cx43. Cx43 was localized to the plasma membrane in non-tumorigenic human breast cells (MSTV1-7) (Figure 2A). Compared with the DMSO treated cells (Figure 2B), treatment with 2.5 and 15 μg/ml genistein resulted in an apparent increase in Cx43 protein (Figure 2D and E). Furthermore, some of the Cx43 appeared to localize as punctuate staining at the plasma membrane following genistein treatment. Quercetin treatment (0.5, 2.5, 5 μg/ml) also produced more Cx43 protein compared with the untreated cells (Figure 2F). Interestingly, Cx43 protein did not appear localized to the plasma membrane following quercetin treatment. Instead, cells treated with quercetin appeared to retain Cx43 in the perinuclear region. Immunocytochemistry revealed that MDA-MB-231 cells are heterogeneous with respect to Cx43 expression after treatment (Figure 2D–F).

Effect of genistein and quercetin on GJIC

The effect of genistein and quercetin on GJIC was studied using both the scrape loading and preloading dye transfer techniques. HS578T cells are shown as a positive control for GJIC as measured by the preloading technique (Figure 3A–C). In these cells, the gap junction-permeable dye, calcein, passed from the preloaded cells to many surrounding cells. Untreated MDA-MB-231 cells did not exhibit GJIC (data not shown). Genistein and quercetin treatment failed to increase GJIC in MDA-MB-231 cells (Figure 3D–F). Extra care was taken to observe preloaded cells in contact with surrounding cells because cell density was reduced following 72 h treatment with 15 μg/ml genistein and 5 μg/ml quercetin.

To test for a false negative result and to account for the observed heterogeneity in Cx43 expression in treated cells, GJIC was also measured by the scrape loading technique. The scrape loading results (Figure 3 G–J) confirmed the results obtained by the preloading technique.

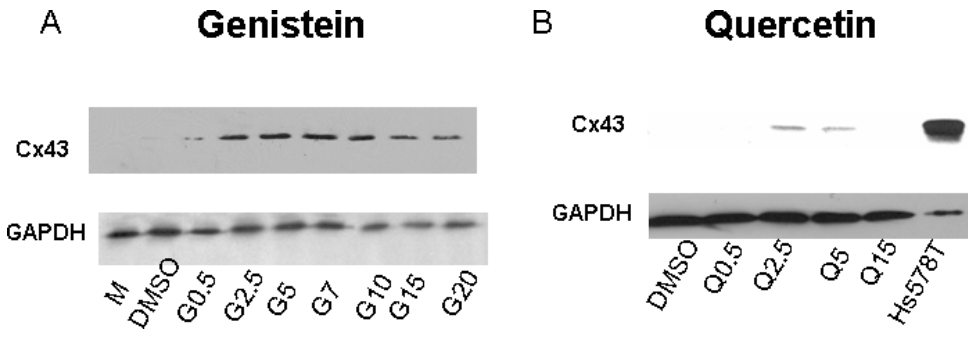

Fig. 1. Western blot analysis showing relative amount of Cx43 in MDA-MB-231 cells following genistein and quercetin treatment. Cells were treated for 72 h, replacing the treatment medium every 24 h. Control cells were grown in complete medium (M) and 0.1% (v/v) DMSO. DMSO was used to maintain genistein (G) and quercetin (Q) in solution. All treatment concentrations were in μg/ml. For example, G0.5 = 0.5 μg/ml genistein. All samples were loaded onto the gel at 200 μg/lane, except for Hs578T cells, which were loaded at 25 μg/lane. Hs578T cells express abundant Cx43, and serve as a positive control. All treatment concentrations of genistein (0.5, 2.5, 15 μg/ml) increased Cx43 compared with the 0.1% (v/v) DMSO vehicle and complete medium control conditions (**A**). Quercetin (2.5 and 5 μg/ml) increased Cx43 protein compared with the 0.1% (v/v) DMSO vehicle and complete medium controls (**B**). An aliquot of 15 μg/ml quercetin did not increase Cx43 protein by western blot analysis.

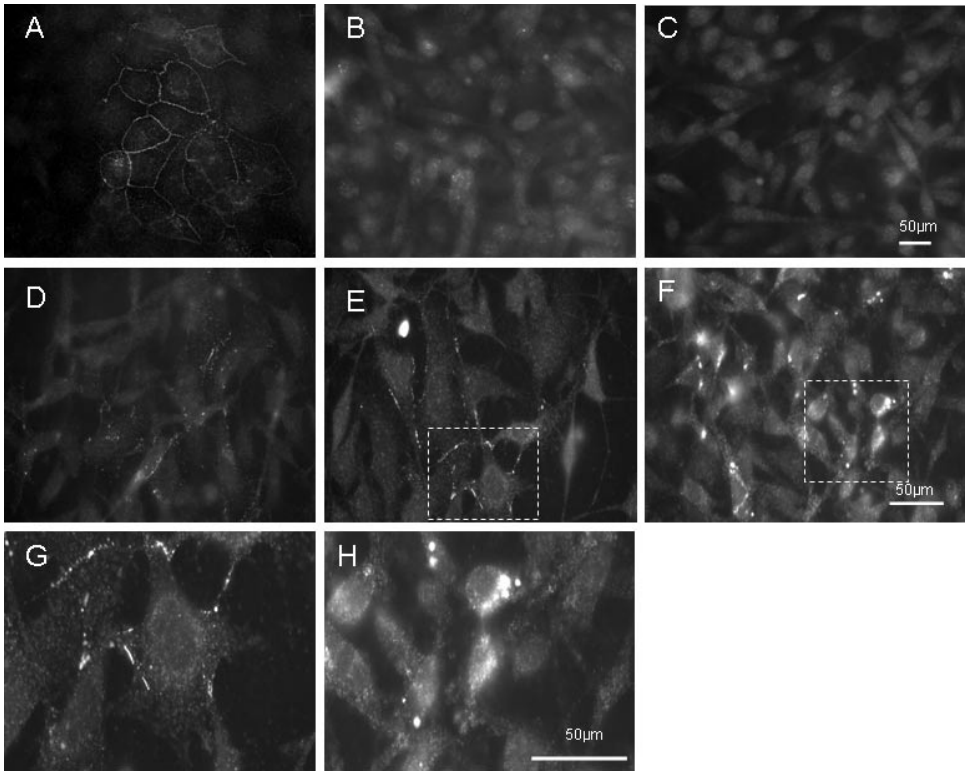

Fig. 2. Immunocytochemical analysis showing Cx43 localization following genistein and quercetin treatment. Cells were treated every 24 h for 72 h with 0.1% (v/v) DMSO vehicle, complete medium, genistein (0.5, 2.5, 15 μg/ml) or quercetin (0.5, 2.5, 5 and 15μg/ml). Non-tumorigenic human breast cells (MSTV1-7) show Cx43 localized at the plasma membrane (**A**). Following genistein treatment (2.5 and 15 μg/ml), punctuate staining for Cx43 is evident in many of the cells (**D** and **E**). Cx43 appeared to be retained in the perinuclear region following quercetin treatment (5 μg/ml) (**F**). Cx43 staining was not apparent in every cell, suggesting a heterogeneous cell population with respect to Cx43 expression and/or genistein or quercetin response. Higher magnifications of E and F are shown in **G** and **H**, respectively. DMSO treated MDA-MB-231 cells show no immunofluorescence above background (**B**), as did cells in which the primary Cx43 antibody was omitted (**C**). Magnification bar in C also applies to A and B; the bar in F also applies to D and E.

Effect of genistein and quercetin on cell proliferation

Cell counts were performed to determine whether genistein and quercetin treatment suppressed growth of MDA-MB-231 cells (Figure 4). There was no significant difference in cell number between the complete medium only and the 0.1% (v/v) DMSO vehicle. Comparing flavonoid treated cells with the 0.1% (v/v) DMSO vehicle, 2.5 μg/ml ($P < 0.05$) and 15 μg/ml ($P < 0.01$) genistein, and 2.5 μg/ml ($P < 0.05$), 5 μg/ml ($P < 0.01$) and 15 μg/ml ($P < 0.01$) quercetin significantly reduced cell number.

Cell proliferation was also measured by immunocytochemistry (Figure 5). Ki67 expression in MDA-MB-231 cells was examined following 72 h treatment with 15 μg/ml genistein, 5 μg/ml quercetin or 0.1% (v/v) DMSO vehicle. All cells which received 0.1% (v/v) DMSO vehicle treatment expressed some level of Ki67, and therefore were dividing (Figure 5B; Table I). In contrast, only 44.4% of cells were immunoreactive for Ki67 following 5 μg/ml quercetin treatment (Figure 5A; Table I). Approximately 73.8% of cells treated with 15 μg/ml genistein were immunoreactive for Ki67 (Table I). These results support the cell count results: namely, genistein ($P < 0.0001$) and quercetin ($P < 0.0001$) reduce proliferation of MDA-MB-231 cells compared with control conditions.

Cx43 staining was observed in conjunction with Ki67 and DAPI staining to determine whether genistein and quercetin-induced Cx43 contributed to reduced cell proliferation (Figure 5C and D). MDA-MB-231 cells, which expressed Cx43 following either 15 μg/ml genistein or 5 μg/ml quercetin treatment, did not show a difference in Ki67 immunoreactivity compared with cells that did not produce Cx43.

Cytotoxicity of genistein and quercetin

In order to determine whether the observed effects of proliferation might be due to cytotoxic effects of genistein and quercetin, we assessed LDH release during the time course of treatment. During the first 24 h of treatment, a significantly greater amount of LDH was released from MDA-MB-231 and MSTV1-7 cells treated with 15 μg/ml quercetin compared with the 0.1% (v/v) DMSO vehicle, consistent with cytotoxicity at this higher dose (data not shown). No other treatment concentrations were toxic to MDA-MB-231 or MSTV1-7 cells during any period of the 72 h treatment duration (Figure 6). Therefore, the significant reduction in cell number cannot be attributed to genistein or quercetin toxicity.

Discussion

Breast cancer is the second most frequent cause of death among North American women (34). There are effective treatments for this disease. Many breast cancer therapies focus on inhibiting the ER in an attempt to control proliferation. However, unfortunately, one-third of breast cancer patients lack ERs, and these patients respond very

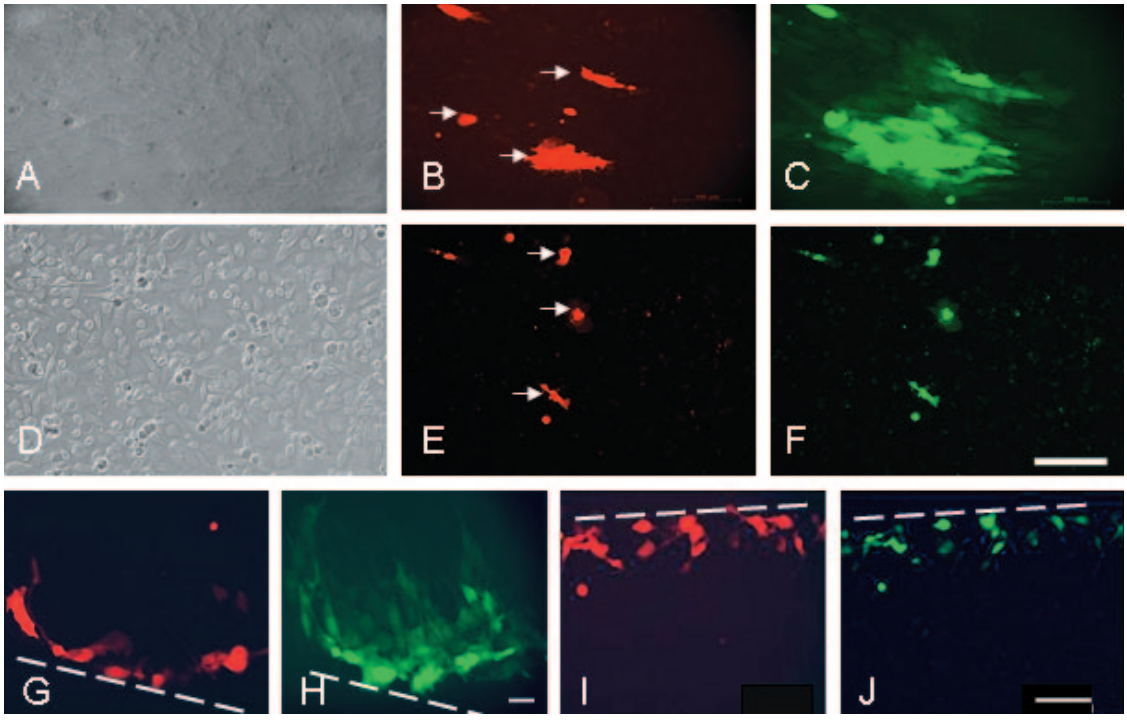

Fig. 3. Effect of genistein and quercetin on gap junctional dye coupling. Hs578T cells, which express Cx43, grown in complete medium act as positive controls for dye transfer (**A–C**). MDA-MB-231 cells are shown in **D–F**. Donor cells (some of which are indicated by white arrows in B and E) were labeled with calcein (green; gap junction permeable) and DiI (red; lipophilic membrane dye) and then seeded onto unlabeled recipient cells. Both donor and recipient cells received the same treatment. DIC images (**A** and **D**) demonstrate the confluent monolayer of recipient cells. Calcein passed from the donor cell to surrounding recipient cells in the Hs578T cells (A–C). Seventy-two hours of genistein (2.5 μg/ml) treatment (D–F) did not increase GJIC compared with the 0.1% (v/v) DMSO vehicle condition. Similar results were obtained using the scrape loading method (**G–J**). Hs578T cells were extensively coupled (G and H) while no coupling could be seen for genistein treatment of MDA-MB-231 cells (I and J). The hatched lines in G–J indicated the scrape line. All other treatment concentrations of genistein and quercetin failed to alter dye coupling.

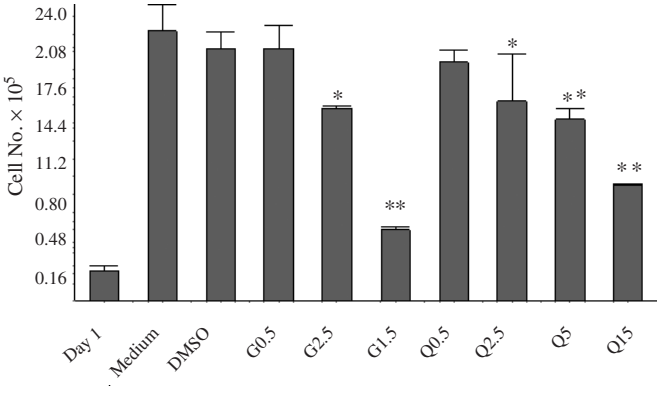

Fig. 4. Growth suppressive effect of genistein and quercetin. Flavonoid treatment reduced the cell number compared with complete medium and 0.1% (v/v) DMSO vehicle conditions. Cells were seeded at low density (Day 1) and treated every 24 h for 72 h with 0.5, 2.5, 15 μg/ml genistein or 0.5, 2.5, 5, 15 μg/ml quercetin. Cell number was significantly reduced following 2.5 μg/ml (*$P < 0.05$) and 15 μg/ml (**$P < 0.01$) genistein, and 2.5 μg/ml (*$P < 0.05$), 5 μg/ml (**$P < 0.01$) and 15 μg/ml (**$P < 0.01$) quercetin compared with the 0.1% (v/v) DMSO vehicle condition.

poorly to chemotherapy and endocrine therapy (35). Furthermore, many cancer therapies are burdened by significant physiological and psychological side effects. For example, tamoxifen, an effective adjuvant therapy for ER-positive breast cancer patients, has been associated with increased risk of endometrial cancer and thromboembolic disorders (2). Chemotherapy is associated with physical side effects including immunosuppression, hair loss, nausea, fatigue, diarrhea, weight gain, constipation and mucositis as well as cognitive deficits (2). Therefore, more effective preventative and therapeutic strategies are needed to manage this disease.

Increased connexin expression and improved GJIC may represent a new, effective therapy for breast cancer. GJIC is important for growth regulation. Dysfunctional GJIC has been associated with metastatic breast cancer (36), cell heterogeneity and is one of the earliest alterations associated with malignant transformation (8). Additionally, experimental research shows that connexin proteins have antitumor functions independent of GJIC. Connexin upregulation has been associated with improved response to chemotherapy agents such as etoposide, paditaxel and doxorubicin (37). Furthermore, human breast cancer cells transfected with Cx26 and Cx43 showed suppressed growth independent of GJIC. It was hypothesized that the connexin proteins disrupted the interaction between fibroblast growth factor and its receptor, reducing mitogenic activity (17).

Genistein and quercetin have been reported to induce apoptosis and halt the cell cycle in several types of tumors including breast, prostate, colon and lung (38). This study aimed to determine whether genistein and quercetin reduce proliferation of a metastatic breast tumor cell line (MDA-MB-231) through gap junctional mechanisms.

Cx43 is the most common connexin protein in breast tissue (7). An inverse relationship has been reported between the amount of Cx43 protein and tumor severity. Most high grade

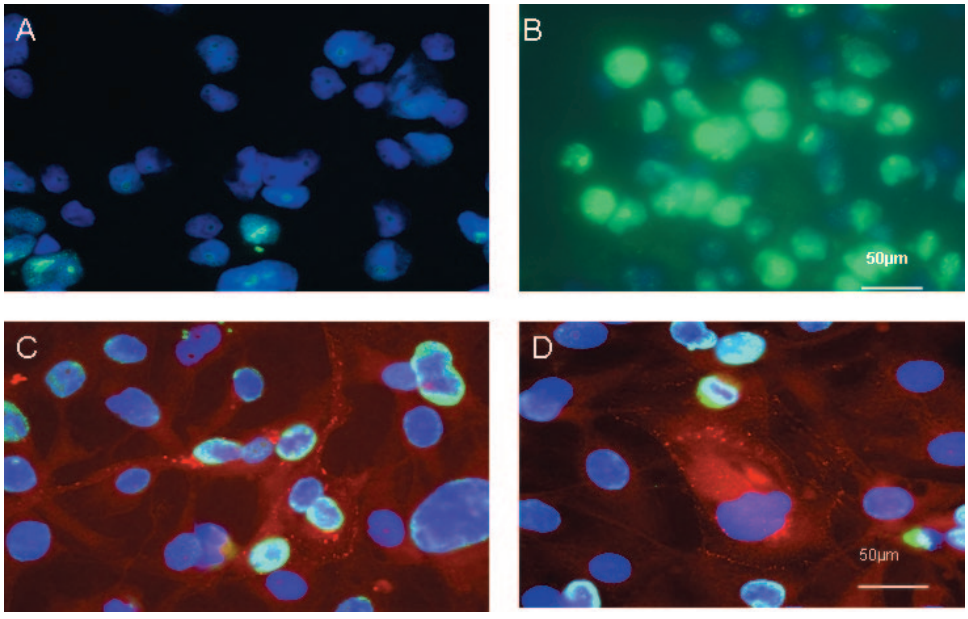

Fig. 5. Effect of genistein and quercetin on Cx43 and MDA-MB-231 cell proliferation. Cells, treated for 72 h with 5 μg/ml quercetin (**A**), or 0.1% DMSO vehicle (**B**), were double-labeled with Ki67 (green) and DAPI (blue). The fraction of cells expressing Ki67 was counted to determine the effect of flavonoids on proliferation. The results are summarized in Table I: quercetin and genistein treatment significantly reduced cell proliferation ($P < 0.0001$). (**C** and **D**) Ki67 (green), Cx43 (red) and DAPI (blue) triple labeling was analyzed to determine whether there was any association between Cx43 expression and proliferation. There was no apparent difference in Ki67 staining between cells which produced Cx43 and cells which did not produce Cx43 following flavonoid treatment. For example, some cells expressing Cx43 following 15 μg/ml genistein treatment show a high level of Ki67 staining (C). Some Cx43-expressing cells were negative for Ki67 (D, 15 μg/ml genistein).

Table I. Ki67 staining after genistein and quercetin treatment

Ki67 Staining	15 μg/ml genistein (number and % of cells)	5 μg/ml quercetin (number and % of cells)	1% (v/v) DMSO (number and % of cells)
Negative	54 (26.2)	100 (55.6)	0 (0)
Positive	152 (73.8)	80 (44.4)	388 (100)

and metastatically aggressive tumors have relatively low levels of Cx43 (8,36).

MDA-MB-231 cells are a highly metastatic human breast tumor cell line, which express very low levels of Cx43 protein. Our study showed that Cx43 expression was maximally stimulated following treatment with 2.5 μg/ml genistein. Quercetin maximally stimulated Cx43 protein at 5 μg/ml. Importantly, 0.5 μg/ml genistein (0.26 μM) increased Cx43 protein. This result is significant because it represents the average genistein plasma concentration (0.28 μM) reported in Japanese men (39).

The breast cancer incidence among Asian females is 4–6 times lower than among North American females. Asians consume a diet rich in genistein (20) and migratory studies demonstrate that breast cancer incidence among Asians dramatically increases when they move to North America (19). Therefore, it is possible that the high consumption of genistein among Asians is in part responsible for their lower breast cancer incidence (18). Given the results of this study, it would be interesting to measure the Cx43 protein levels in this population.

The mechanism by which genistein and quercetin elicit Cx43 upregulation requires further study. Both flavonoids can bind to ER-α and ER-β, translocate to the nucleus and affect gene transcription through estrogen response elements (40). There is evidence that flavonoids can increase Cx26 and Cx43 expression by this mechanism (41). However, MDA-MB-231 cells are ER-negative. Therefore, genistein and quercetin must be able to affect Cx43 expression by a mechanism independent of the ER. Genistein and quercetin are lipophilic molecules and are therefore able to diffuse through the plasma and nuclear membrane. It is possible that once inside the cell, genistein and quercetin may activate proteins involved in Cx43 transcription or directly stimulate Cx43 promoter regions.

Gap junction formation requires that connexin proteins be located at the plasma membrane. Few studies have investigated Cx43 localization following flavonoid treatment. Ale-Agha *et al*. (12) demonstrated that 75 μg/ml genistein counteracted the tumor promoter-induced internalization of Cx43 in rat liver epithelial cells. Chaumontet *et al*. (14) also used rat liver epithelial cells to show that Cx43 localized to cell–cell contact points following treatment with 7 μg/ml apigenin and 10 μg/ml tangeretin. In our study, there was no evidence of plasma membrane-associated Cx43 in untreated MDA-MB-231 cells. However, following treatments with 2.5 and 15 μg/ml genistein, punctuate Cx43 staining appeared at cell borders. In contrast, Cx43 appeared localized in the perinuclear region following quercetin treatment. While these treatments did result in the appearance of Cx43 protein, this was not observed throughout the entire culture, highlighting the heterogeneity of the response of these cells to genistein and quercetin. The absence of any increase in gap junctional coupling following flavonoid treatment is not surprising given the lack of Cx43 at the cell membrane.

Tumor promoting agents such as TPA and DDT inhibit GJIC contributing to alterations in cell proliferation and

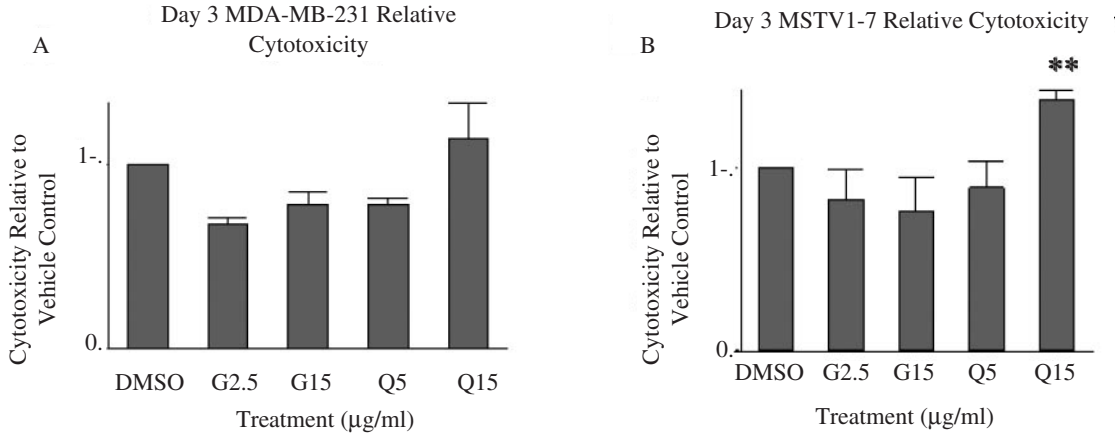

Fig. 6. Lack of cytotoxicity of genistein and quercetin on MDA-MB-231 and MSTV1-7 cells. Cells were treated with genistein and quercetin every 24 h for 72 h. LDH release was determined as outlined in the text. Only quercetin at 15 μg/ml significantly increased LDH release above control levels for MSTV1-7 cells (**$P < 0.01$).

transformation (11). Studies have shown the ability of flavonoids to counteract tumor promoter-induced reduction in GJIC. (−)Epicatechin, a flavonoid found in cocoa and green tea, is able to maintain GJIC in the presence of TPA in rat liver epithelial cells (12). Sai *et al*. (13) demonstrated that green tea protects against pentachlorophenol-induced inhibition of GJIC in mice. Without using a tumor promoter in the current study, it was found that neither genistein nor quercetin enhanced GJIC in MDA-MB-231 cells. The finding that Cx43 was localized to the plasma membrane following genistein treatment suggests that there must be factors other than aberrant Cx43 localization, which impede GJIC in MDA-MB-231 cells.

Cells must be held in close proximity to form functional gap junction pores. E-cadherin is an important intercellular adhesion molecule and is considered to be the most promising candidate for cell–cell interaction during gap junction formation (42). Interestingly, MDA-MB-231 cells do not express E-cadherin. This may represent one explanation for the lack of GJIC in this cell line.

Studies have reported the ability of genistein and quercetin to halt the cell cycle, induce apoptosis, and thus regulate the cell population (24–27). In this study, cell proliferation analysis showed that genistein and quercetin treatment reduced MDA-MB-231 cell number compared to control conditions. An aliquot of 15 μg/ml genistein and 5 μg/ml quercetin produced the greatest reductions in cell number independent of cytotoxicity. The same concentrations of genistein and quercetin also reduced cell number in a metastatic, 'sarcoma-like' human breast cancer cell line called Hs578T (data not shown).

Staining MDA-MB-231 cells with DAPI and the proliferative marker Ki67 showed that genistein and quercetin significantly reduced breast tumor cell proliferation. To observe whether or not flavonoid-induced Cx43 had an effect on proliferation, Cx43 was co-localized with DAPI and Ki67. We observed Cx43 staining in cells which were both positive or negative for Ki67, suggesting the lack of any correlation between the presence of these two proteins.

Cytotoxicity assays were performed on flavonoid treated MDA-MB-231 cells and MSTV1-7 cells. It was found that quercetin was more cytotoxic than genistein. Over a 3 day treatment period, genistein (0.5, 2.5, 15 μg/ml) was not toxic to either the non-tumorigenic MSTV1-7 cells or metastatic MDA-MB-231 cells. However, 15 μg/ml quercetin consistently produced a toxic reaction in both these cell lines. Interestingly, 15 μg/ml quercetin did not enhance Cx43 expression in MDA-MB-231 cells, suggesting that the toxic effects interfered with the transcription or translation of Cx43. Because of the estrogenic effect of genistein and quercetin, there is some concern regarding hormonal side effects. Chromosome aberrations have been reported following treatment with very high concentrations of genistein (25 μM) (43). However, *in vivo* studies have shown no short-term or long-term reproductive problems in humans (44). Although more *in vivo* studies are needed, genistein and quercetin suppressed cell growth at concentrations not toxic to non-tumorigenic breast cells, suggesting that these two flavonoids could be used therapeutically.

The growth suppressive effects of genistein and quercetin have been previously established. However, the antitumor effects of genistein and quercetin on ER-negative metastatic breast cells through gap junctional mechanisms has not previously been investigated. This study provides evidence that genistein and quercetin suppressed proliferation of MDA-MB-231 cells at concentrations which were not toxic to non-tumorigenic and metastatic human breast cells. The suppressed proliferation was independent of GJIC as demonstrated by dye transfer. Furthermore, based on the limited changes in Cx43 immunoreactivity observed in MDA-MB-231 cells following flavonoid treatment, the major effects on proliferation are likely not due to changes in Cx43 expression.

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