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ORIGINAL ARTICLE

Influence of soy isoflavones in breast cancer angiogenesis: a multiplex glass ELISA approach

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Summary

Purpose: *The aim of this study was to evaluate the anti-angiogenic properties of soy isoflavones using two breast cancer cell lines, by measuring the concentration of 30 cytokines involved in angiogenesis using a multiplex glass slide ELISA-based array.*

Methods: *Estrogen-dependent MCF-7 cells and estrogen-independent MDA-MB-231 cells were exposed to genistein (Gen), daidzein (Dai) and a soy seed extract (Ext) for 72 hrs, at selected concentration levels. The conditioned medium was analyzed using a glass slide, multiplex sandwich ELISA-based platform with fluorescent detection which allowed the identification and the quantification of 30 angiogenesis-related cytokines.*

Results: *In MCF-7 cells, low, stimulatory concentrations of test compounds determined the increase of CXCL16 and VEGF-A level. Gen induced the greatest effect, with 1.5-fold change compared to control. When MDA-MB-231 cells were exposed to inhibitory concentrations, all test compounds determined a reduction of CXCL16 and VEGF-A level with approximately 30%.*

Conclusions: *Soluble CXCL16 and VEGF-A are two promoters of angiogenesis and metastasis in breast cancer. The stimulation of these two angiogenesis-related cytokines could represent one of the mechanisms explaining the proliferative effects of low isoflavone doses in estrogen-dependent cells. In estrogen-independent cells, soy isoflavones inhibited their secretion, demonstrating promising anti-angiogenic properties.*

Key words: *angiogenesis, breast cancer cells, CXCL16, ELISA, isoflavones, VEGF-A*

Introduction

In the USA, breast, lung and colorectal cancers account for 50% of all cancer cases expected to occur in women in 2018. Of this percentage, breast cancer alone accounts for 30%, which embody 266,120 new diagnosed cases [1]. Breast cancer rates are generally higher in Northern America, Australia/New Zealand, Western Europe and low in most of Africa and Asia [2].

Several studies have related the low incidence rates of breast cancer in Asian countries with the local dietary patterns, showing that soy consumption could lower the risk of breast cancer for both pre- and post-menopausal women in Asian countries [3,4]. Later, these epidemiological observations were strengthened by *in vitro* data, soy isoflavones and especially genistein, showing antiproliferative

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effects by sustaining apoptosis, antioxidant defense and DNA repair and, not least, by inhibiting the development of tumor angiogenesis and metastasis [5].

Soy isoflavones have been explored as promising anti-angiogenetic agents as they appear to inhibit multiple angiogenic mechanisms, such as regulation of vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), epidermal growth factor receptor (EGFR) expressions and NF-κB, PI3-K/Akt or ERK1/2 signaling pathways [5,6]. However, despite the intensive research, the anti-angiogenic potential of soy isoflavones in breast cancer remains controversial, mainly due to their twofold effect [7].

Several *in vitro* assays have been developed to assess the angiogenic properties of exogenous agents. Most models focus on proliferation, migration, and differentiation of endothelial cells [8]. While these tests determine the effect or the outcome of drugs on blood vessel formation, more high-throughput tests have been developed in order to identify which particular angiogenic molecules or mechanisms are targeted by the test compounds. Such are the glass slide ELISA-based quantitative systems, extensively used for the rapid profiling of cytokine expression.

The main advantage of glass slides over the single-targeted 96-well plate ELISAs or Western blots is the possibility of performing simultaneous identification and quantification of multiple cytokines, growth factors, proteases, soluble receptors and other angiogenesis-associated proteins in a single experiment. Furthermore, they are highly specific and reproducible, require low sample volumes and are well-suited for high throughput assays [9].

To our knowledge, no ELISA-based quantitative array on breast cancer cells exposed to isoflavones has been performed so far. The identification and quantification of the key molecules involved in angiogenesis will provide a further understanding of isoflavones' anti-angiogenic properties. The aim of this study was to evaluate the anti-angiogenic properties of genistein (Gen), daidzein (Dai) and a soy seed extract (Ext) using two breast cancer cell lines, MCF-7 and MDA-MB-231, by measuring the concentration of 30 cytokines involved in angiogenesis using a multiplex glass slide ELISA-based array.

Methods

Chemical and standards

All chemicals and standards were purchased from Sigma-Aldrich (Taufkirchen, Germany), unless otherwise stated.

The soy extract was purchased from Hunan Goldliloo Pharmaceutical Co., Ltd. (Changsha, China). According to manufacturer's specifications, the extract was obtained from soy seeds (*Glycine max*), using an aqueous ethanolic solution followed by spray-drying. The extract contains 40% isoflavones, of which daidzein represents only 1.50%, glycitein 0.12%, and genistein 0.02%. The isoflavone distribution was confirmed in our laboratory by a validated HPLC-UV method [10].

Stock solutions of standard Gen, Dai, and Ext were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C.

Cell culture and culture conditions

The MCF-7 and MDA-MB-231 breast adenocarcinoma cell lines were obtained from CLS Cell Lines Service (Eppelheim, Germany) and routinely cultured as previously described [11]. All cells used in experiments were between passage number 5 and 20.

Cell treatment and sampling

The test concentrations of Gen, Dai, and Ext were established based on a MTT test, as previously described [11]. Briefly, in MCF-7 estrogen-dependent cells, all compounds induced a twofold effect, stimulating cell growth at relatively low concentrations and causing inhibition at higher concentrations. Therefore, we selected two concentration levels for each test compound: the concentrations that stimulated cell proliferation by 20% compared to control (SC_{20}) and the concentrations that inhibited cell growth by 20% compared to control (IC_{20}). The SC_{20} concentrations for Gen, Dai, and Ext were 5.62 µM, 19.01 µM, and 22.59 µg/mL respectively, while the IC_{20} concentrations were 22.44 µM, 52.24 µM, and 166.34 µg/mL respectively. For MDA-MB-231 estrogen-independent cells, only a dose dependent inhibitory effect was observed and, therefore, only the IC_{20} concentrations were selected. Precisely, these IC_{20} concentrations were 11.04 µM for Gen, 36.39 µM for Dai, and 26.36 µg/mL for Ext [11].

For cell treatment, 2.4×10^6 MCF-7 cells or 1.2×10^6 MDA-MB-231 cells were seeded in 150 mm cell culture dishes (Sarstedt, Germany) in 15 mL RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 1% non-essential amino acids and 1% penicillin-streptomycin.

The dishes were shaken for 1 min to ensure the homogeneous distribution of cells. Next, all plates were incubated for 24 hrs to allow cell attachment. After 24 hrs, the medium was replaced with 28 mL fresh medium containing the selected concentrations of Gen, Dai, Ext, or DMSO as solvent control. In all cases, the final concentration of DMSO did not exceed 0.01%. The incubation time was 72 hrs.

For sampling, 1.5 mL conditioned medium were centrifuged at 2000 rpm, at 4°C for 10 min. The supernatant was immediately frozen at -80°C until measurement.

Antibody array analysis of angiogenesis related cytokines

For the quantification of angiogenesis-associated cytokines, we used Quantibody Human Angiogenesis Array 3 (#QAH-ANG-3, RayBiotech, Norcross, Georgia, USA). This is a glass slide, multiplex sandwich ELISA-based platform which allows the identification and quantification of 30 cytokines, chemokines, growth factors, and other molecules involved in angiogenesis. The 30 spotted targets were angiogenin-1, angiostatin, C-X-C motif chemokine ligand 16 (CXCL16), epidermal growth factor, fibroblast growth factor 4, follistatin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, I-309, interleukin-1 beta, interleukin-4, interleukin-10, interleukin-12 subunit p40, interleukin-12 subunit p70, interferon-inducible T-cell alpha chemoattractant, monocyte chemotactic protein 2, monocyte chemotactic protein 3, monocyte chemotactic protein 4, matrix metalloproteinase-1, matrix metalloproteinase-9, platelet endothelial cell adhesion molecule-1, transforming growth factor alpha, transforming growth factor beta-3, tyrosine-protein kinase receptor Tie-1, tyrosine-protein kinase receptor Tie-2, urokinase plasminogen activator surface receptor, vascular endothelial growth factor-A (VEGF-A), vascular endothelial growth factor receptor 2, vascular endothelial growth factor receptor 3 and vascular endothelial growth factor D. Each antibody, together with two positive controls and a negative control, is printed in four identical spots, so each cytokine is measured four times per sample.

The assay was conducted according to manufacturer recommended protocol [9]. Briefly, the glass slides were first allowed to equilibrate and dry at room temperature for 2 hrs. In the blocking step, 100 µL sample diluent was added into each well and the slides were incubated for 30 min at room temperature. Next, the sample diluent was discarded and 100 µL calibration standard cytokines or conditioned medium were added into each well. The glass chamber was covered with adhesive film and incubated overnight, at 4°C, on a plate shaker (Titramax 101, Heidolph Instruments, Schwabach, Germany) at 200 rpm.

On the next day, the supernatant was discarded and each well was washed five times with Wash Buffer I and two times with Wash Buffer II. Subsequently, the biotinylated antibody cocktail was reconstituted and 80 µL were added per well. After 2 hrs, the antibody cocktail was removed, the wells were washed again with the two washing buffers and 80 µL of Cy3 Equivalent Dye-Streptavidin were added per well. The slides were incubated in the dark, at room temperature for 1 hr. After other washing steps, the slides were carefully removed from the gasket and allowed to dry at room temperature.

For fluorescence detection, a DNA Microarray Scanner (G2505C, Agilent Technologies, USA) with a scan resolution of 10 µm was used.

Data analysis

For background subtraction and densitometry measurement, the scanned images were analyzed using Image Studio Lite (v.2.5.2.). For each spot, the defined area for signal capture was a circle with a 158-micron diameter. The median intensity of a three-pixel border around the defined circle was used for local background subtraction.

As our cells were cultivated in serum-containing medium, which might contain various types of cytokines, the median signal intensity of each cytokine of the complete medium array was subtracted from the signal intensity of the corresponding cytokine from each other array.

Next, data normalization was carried out by accounting for the differences in signal intensities of the positive control spots across all arrays. The positive control spots represent standardized amounts of biotinylated antibody and the signal of these spots is dependent on the amount of streptavidin-fluor bound to that antibody. This bounding capacity will proportionally affect the signal intensity of every spot on the array. Therefore, the differences in the positive control signals between arrays will accurately reflect the differences between other spots on those arrays. The reference array was the solvent control (medium with DMSO only) corresponding to each cell line. The normalized values were calculated using equation 1 [9]:

$$nX(Y)=X(Y)\times P\ /\ P(Y)$$

nX(Y)= the normalized value for cytokine "X" of sample "Y", X(Y)= the signal density of the spots for cytokine "X" of sample "Y", P= the average signal density of the positive control spots on the reference array, P(Y)= the average signal density of the positive control spots of sample "Y"

Statistics and visualization

The calibration curves and the statistical analysis were executed using Prism (v.6.01, GraphPad Software). Each treatment was compared to the corresponding solvent control according to two-way ANOVA with Sidak's

Table 1. The concentration and the fold change of significantly altered cytokines ($p<0.05$, two-way ANOVA with Sidak's correction for multiple comparisons)

	pg/mL	*Fold change*
CXCL16 concentration		
In MCF-7 cells		
Control	1140.91	
Gen SC_{20}	1775.65	1.55
Dai SC_{20}	1691.43	1.48
Dai IC_{20}	547.37	-2.08
In MDA-MB-231 cells		
Control	1574.22	
Gen IC_{20}	1232.29	-1.28
Ext IC_{20}	1137.21	-1.38
VEGF-A concentration		
In MCF-7 cells		
Control	1309.22	
Gen SC_{20}	1982.84	-1.51
Ext SC_{20}	1808.55	-1.38
In MDA-MB-231 cells		
Control	1521.43	
Gen IC_{20}	1192.17	-1.27
Dai IC_{20}	1130.65	-1.34
Ext IC_{20}	950.75	-1.60

correction for multiple comparisons. Differences with p values less than 0.05 were considered as statistically significant.

Results

Exposure of both breast cancer cell lines to Gen, Dai, and Ext induced significant changes, especially in the signal intensity of two cytokines, CXCL16 and VEGF-A. In MCF-7 cells, SC_{20} of test compounds caused an increase in CXCL16 and VEGF-A signal intensity, while treatment of cells with IC_{20} concentrations led to a reduction of CXCL16 level. For MDA-MB-231 cells, inhibitory concentrations of test compounds triggered a decrease in the VEGF-A and CXCL16 signal intensity (Figure 1).

Next, the mean intensity of the significantly changed cytokines was plotted on the corresponding calibration curve (Figure 2). Using these curves, the absolute cytokine concentration was then calculated (Table 1).

Discussion

Soy isoflavones are known as promising anti-angiogenic agents, acting on multiple pathways, such as ERK1/2 signaling pathway, regulation of

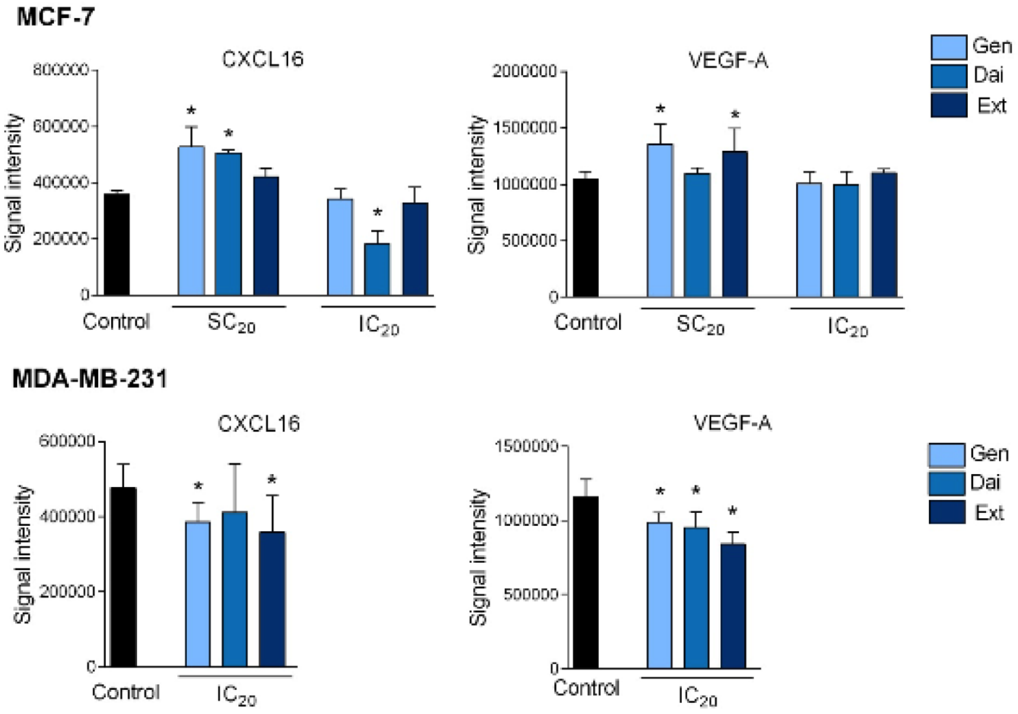

Figure 1. The signal intensity of CXCL16 and VEGF-A after MCF-7 and MDA-MB-231 cells were exposed to genistein (Gen), daidzein (Dai), and soy extract (Ext) at test concentrations. Asterisks indicate statistically significant differences ($p<0.05$) between solvent control and treated samples.

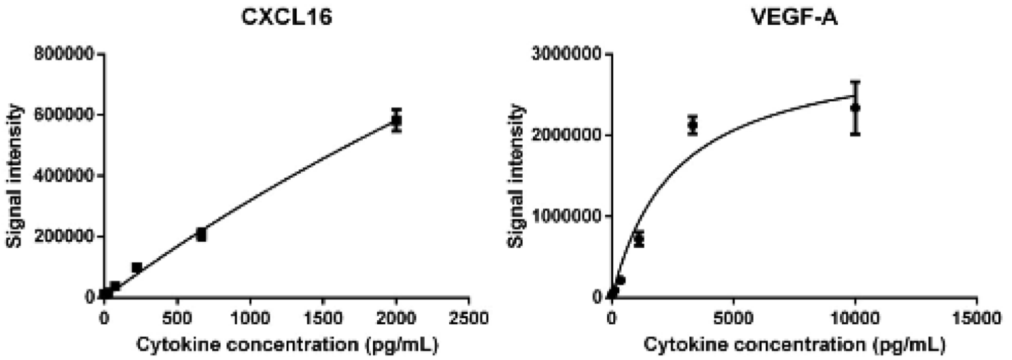

Figure 2. The calibration curves for CXCL16 and VEGF-A obtained by plotting their mean intensities against the predetermined concentrations. The curves were generated using a non-linear regression fit model (R^2=0.9974 for CXCL16 and R^2=0.9584 for VEGF-A).

VEGF or MMPs expression [5,6]. However, there is limited data regarding the influence of these natural compounds on CXCL16 expression.

CXCL16, along with CXCL12 (C-X-C motif chemokine ligand 12), belong to the superfamily of chemotactic cytokines, which govern the immune cell trafficking between or within tissues. Through coordinated interaction with its specific receptor, CXCR6 (C-X-C motif chemokine receptor 6), CXCL16 also plays a crucial role in tumor growth, invasion, angiogenesis, and metastasis in various types of cancers such as breast adenocarcinoma [12-14], lung cancer [15] or prostate cancer [16]. Moreover, for prostate and breast cancers, a positive correlation between CXCR6/CXCL16 expression and cancer aggressiveness was found [17,18], higher CXCR6 expression in nest site and metastatic lymph node being responsible for breast cancer progression [12].

So far, *in vitro* studies have shown that soy isoflavones can regulate other angiogenic chemokines, such as CXCL12. In MCF-7 estrogen-dependent cells, low doses of Gen or Dai (1–10 μM) induced a significant increase in CXCL12 level [19-21], triggering cell proliferation and invasion. When the same cell line was exposed to higher Gen concentrations (>25 μM), the CXCL12 mRNA level was significantly downregulated. This downregulation resulted in a subsequent inhibition of migration and invasion. In MDA-MB-231 cells, CXCR4 (C-X-C motif chemokine receptor 4), the cognate receptor of CXCL12, was downregulated by Gen in a dose-dependent manner [19].

To our knowledge, no study has assessed the effect of soy isoflavones on CXCL16 chemokine so far.

Our results show that isoflavone treatment triggers similar changes for soluble CXCL16 expression, as for CXCL12. Low doses of isoflavones (SC_{20}) significantly stimulated CXCL16 secretion in MCF-7 cells, Gen causing the highest CXCL16 increase. When MCF-7 were exposed to higher, inhibitory doses of isoflavones (IC_{20}), only Dai caused a significant decrease. The CXCL16 decrease caused by Dai could be due to the anti-inflammatory properties of Dai, which was shown to suppress the transcription of pro-inflammatory chemokines, such as CXCL2, by depressing PARP-1 activity [22].

However, the IC_{20} of Gen used in this study (22.44 μM for MCF-7 cells) was lower than the concentrations used in other studies [19,23]. Therefore, it is not excluded that higher Gen concentrations, most likely >50 μM, could decrease the CXCL16 secretion. In MDA-MB-231 cells, all test compounds generated a decrease in the CXCL16 level.

One of the mechanisms proposed for explaining the proliferative effects of CXCR6/CXCL16 breast cancer cells involves the activation of downstream signaling paths, such as ERK1/2 signaling pathway [12]. Apparently, stimulation of ERK1/2 pathway activates RhoA, a member of the RhoGTPase family. The effect leads to inhibition of cofilin activity, responsible for the regeneration of actin filaments. In response to cofilin inhibition, F-actin stability enhances, favoring breast cancer invasiveness and metastasis [12]. In fact, Gen can also act as direct modulator of ERK1/2 pathway, promoting MCF-7 cell growth through delayed and prolonged phosphorylation of ERK1/2 [24].

An alternative explanation could rely on the estrogenic effects of low isoflavones doses. Similar to estrogen, which upregulates CXCL12 and CXCR4 expression in breast cancer cells [25], isoflavones could upregulate CXCL16 expression and secretion acting through the same molecular mechanisms.

Isoflavone treatment also determined significant changes in the VEGF-A level. Compared to CXCL16, VEFG-A is a much more known player in the process of tumor angiogenesis and the most intensively studied member of VEGF family. Activation of the VEGF-receptor pathway triggers a network of signaling processes that promote cell growth, migration, and survival from pre-existing vasculature. The concentrations of the VEGF protein and VEGF receptors in the serum of breast cancer patients showed positive correlations with estrogen receptor status and the clinical stage of disease [26].

Soy isoflavones, and particularly Gen, have been intensively examined for their potential to modulate VEGF-A secretion, especially using cultured human umbilical vein endothelial cells [6,27]. In breast cancer, low concentrations (10^{-12}-10^{-6} M) of Gen have similar effects as estrogen in estrogen receptor (ER) positive cells like MCF-7 (ER positive), MELN (derived from MCF-7 cells) and MELP (derived from MDA-MB-231 cells and transfected with ER) inducing VEGF-A expression significantly. The same effect was not observed in MDA-MB-231 cells, suggesting that ER is necessary for VEGF stimulation [28]. On the other hand, when MDA-MB-453 cells were exposed to high Gen concentration, the VEGF mRNA expression decreased significantly [6] pointing to a second receptor and signaling pathway for Gen.

Our results are in line with the existing data, that low, stimulatory concentrations of Gen or Ext increase VEGF-A secretion in MCF-7 cells. Apparently, low isoflavone doses seem to mimic again the estrogen action, stimulating the secretion of VEGF-A. As VEGF-A promotes cell proliferation, upregulation of VEGF-A secretion could be one of the mechanisms explaining the proliferative effects of isoflavones.

Notably, concentrations of Gen below 5 μM correspond to a blood plasma concentration attainable in a soy-rich diet [29]. As CXCL16 and VEGF-A secretion were both stimulated at low isoflavone concentrations, special attention should be paid to the daily phytoestrogen intake by patients with estrogen responsive breast cancer subtype to avoid any pro-angiogenic effects.

In estrogen-independent MDA-MB-231 cells, IC_{20} of all test compounds triggered VEGF-A decrease. Inhibition of VEGF-A expression is a potential strategy especially in the triple negative breast cancer, the cancer subtype that lacks any targeted therapy and with the worst prognosis among all breast cancer subtypes. As isoflavones are capable of inhibiting VEGF-A secretion in MDA-MB-231 cells, they could represent promising anti-angiogenic agents.

Conclusion

Our study investigated the potential of soy isoflavones to modulate the main molecules involved in angiogenesis, using a quantitative glass slide ELISA-based array. The results showed that isoflavones exert dose dependent effects in both cell lines: in MCF-7 cells, low isoflavone doses stimulated the secretion of CXCL16 and VEGF-A, two promoters of angiogenesis and metastasis, while higher concentrations inhibited CXCL16 and VEGF-A secretion in MDA-MB-231 cells. The anti-angiogenic properties of isoflavones could be further exploited as an effective strategy, especially in triple negative breast cancers.

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Conflict of interests

The authors declare no conflict of interests.

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