Ethanol-based garlic extract prevents malignant evolution of non-invasive breast tumor cells induced by moderate hypoxia

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A B S T R A C T

Background: In breast cancer, low oxygen availability is associated with a more aggressive phenotype and with malignant evolution of non-invasive cells. Natural compounds have long attracted attention in cancer treatment, and in recent years garlic (Allium sativum) organosulfur derivatives have been shown to negatively affect growth and invasion of tumor cells.

Methods: Homemade ethanol-based garlic extract (GE) was administered to MCF7 and MCF10DCIS breast tumor cell lines grown under moderate hypoxia. Cell cycle, epithelial-to-mesenchymal transition and cancer stem cell markers were evaluated.

Results: We revealed that, in the non-invasive MCF10DCIS cells but not in the post-EMT MCF7 cells, low oxygen availability induced the decrease of E-cadherin and the increase of vimentin and motility, that were prevented by GE administration. In both cell lines, treatment with GE counteracted the up-modulation of CD133 positive cells induced by hypoxia.

Conclusions: Overall, our data firstly revealed anti-cancer properties of garlic in non-invasive breast cancer cells. In particular, they demonstrated a protective role of this natural product against the hypoxia-induced increase of molecules that play crucial roles in tumor evolution, suggesting that garlic derivatives can be considered in new approaches for preventing progression of breast tumors from non-invasive to infiltrating lesions.

1. Introduction

Breast tumors are the most frequently diagnosed and the leading cause of cancer death worldwide in women [1]. Alterations in the tumor microenvironment have been considered as crucial events in the development and progression of breast cancer [2,3], and hypoxia, which generates a hostile system in which tumor cells need to adapt to survive, has a key role in tumor malignancy [4]. In breast cancer, the adaptation of tumor cells is a crucial step in the progression towards a more malignant tumor [5-7], and epithelial-to-mesenchymal transition (EMT), a process whereby epithelial cells acquire the mesenchymal phenotype, improves the adaptive capabilities of cancer cells and is considered critical in tumor progression [4]. In accordance with the role of hypoxia in increasing the malignant potential of breast cancer, we found that, in breast tumor-derived cells, low oxygen availability down-modulated the EMT marker E-cadherin and up-regulated the cancer stem cell marker CD133 [8].

Ductal carcinoma in situ (DCIS), a heterogeneous non-invasive tumor with variable clinical, histopathological and molecular characteristics [9], represents, even if a non-obligate precursor, a crucial step in progression to invasive ductal carcinoma [10]. In the MCF10DCIS cell model of DCIS, we have previously demonstrated a clear correlation between low oxygen availability and malignant progression. We have found that hypoxia induces a loss of the epithelial-like shape in favor of a spindle-like phenotype, the acquisition of migratory capabilities, and the increased expression of the cancer stem cells marker CD133 [11], suggesting that treatments counteracting hypoxia-related events may prevent malignant progression of non-invasive breast lesions due to low...
oxygen availability.

An increasing number of studies report that garlic (Allium sativum) shows anti-tumor properties in several tumors acting at different stages of carcinogenesis [12,13]. In addition, clinical studies suggest that high garlic consumption is protective against various solid tumors, including breast cancer [14].

The effects of garlic compounds, and mainly of its organosulfur derivatives, were extensively studied in breast tumor, due to the high heterogeneity of this neoplasia and to the need of new and multidisciplinary approaches for personalized therapies [14]. Studies on animal tumor models and human invasive breast cancer cell lines showed that bioactive garlic compounds have a significant anti-proliferative role by inducing cell cycle arrest and apoptosis [15-18]. In human triple-negative breast cancer cells, garlic derivatives inhibit invasion and metastatic potential through inactivation of the β-catenin signaling pathway [19] and by down-modulation of EMT markers [20-22]. Finally, in vitro studies demonstrated that garlic compounds prevent tumor angiogenesis by inhibiting both the expression and secretion of VEGF-A protein [21].

Based on this evidence, the aim of this study was to assess the effect of a hydro-alcoholic garlic extract on non-invasive breast cancer cells grown under low oxygen availability, to establish an unidentified role for garlic in protection from breast cancer progression.

2. Materials and methods

All reagents were from Sigma (St Louis, MO, USA) unless otherwise indicated.

2.1. Cells and treatments

MCF7 cells were from the American Type Culture Collection (Rockville, MD, USA) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Laboratories) and 1% penicillin-streptomycin solution (Gibco Laboratories). The breast cancer-derived cell line MCF10DCIS, kindly provided and characterized by Dr. Macpherson (Beatson Institute for Cancer Research, Glasgow, UK), was cultured in Advanced DMEM/F12 medium (Gibco Laboratories), 1% L-Glutamine, 5% horse serum (HS, Gibco Laboratories) and 1% penicillin-streptomycin solution (Gibco Laboratories). Both cell lines were grown at 37 °C in a humidified atmosphere of 5% CO2 in air and tested monthly for mycoplasma and other contaminations.

Homemade hydro-alcoholic garlic extract (GE) was prepared essentially following the procedure described by Petrovic and colleagues [23]. In brief, 350 g of fresh garlic cloves were provided from “Consorzio Produttori Aglio di Voghiera DOP” (Ferrara, I), crushed in 250 ml 40% ethanol, and subjected to centrifugation at 2500g for 10 min at 4 °C. Supernatant, corresponding to GE, was stored at −20 °C until use. GE was treated with diethyl ether (1:1, vol/vol) for 24 h, and then subjected to analysis of organosulfur compounds by using a GC/MS system consisting of a Varian Saturn 2100 MS/MS ion trap mass spectrometer coupled to a Varian 3900 gas chromatograph (Varian, Palo Alto, CA, USA).

The mass spectrometer operated in scan mode (40–650 m/z) and the collected data were evaluated using NIST MS library for tentative identification of sulfur compounds. Both chromatogram and the list of the identified peaks were shown in Supplementary Fig. S1.

Increasing dilutions of GE (1:200, 1:400, 1:800, and 1:1600 in culture medium) were administered to MCF7 and MCF10DCIS cells growing under normoxia or hypoxia for 3 days. As extract contained ~22% ethanol [23], corresponding dilutions of 22% ethanol (vehicle) were used in untreated conditions. Exposure of cell cultures to moderate hypoxia (1% O2) was performed in a Forma™ Series II Water Jacketed CO2 Incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA), as previously reported [8,11].

Cells in all experimental conditions were subjected to evaluation of viability using the Trypan Blue Exclusion Test, in which cells were suspended in PBS containing trypan blue and then examined with an inverted phase-contrast microscope (Diaphot, Nikon, Melville, NY, USA).

2.2. Cell cycle analysis

The number of cells in each phase of the cell cycle was evaluated by means of flow cytometry after propidium iodide (PI) staining of ethanol-fixed cells. Briefly, 5 × 10^6 cells were fixed and incubated in the dark at room temperature for 30 min with 100 μg/ml RNase and 20 μg/ml PI. The PI fluorescence of individual nuclei was measured using a FACS Calibur flow cytometer (BD Biosciences, San José, CA, USA). The proportions of cells in the G0/G1, S and G2/M phases of the cell cycle were calculated by the CellQuest Pro 6.0 software (BD Biosciences), as previously described [24].

2.3. Immunohistochemical analysis

Total cell lysates were separated on 7.5% polyacrylamide denaturing gels and blotted to nitrocellulose membranes (GE Healthcare Life Science, Little Chalfont, UK), that were reacted with antibodies directed against vimentin, E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-tubulin, as previously reported [8]. The immunocomplexes were detected by chemiluminescence using the ECL system (Perkin-Elmer, Boston, MA, USA), according to the manufacturer’s instructions. The chemiluminescence derived bands were acquired with an ImageQuantTM LAS 4000 biomolecular imager (GE Healthcare Life Science) and the densitometrical analysis was performed by means of Image Quant TL software (GE Healthcare Life Science).

2.4. Real-time assays of cell migration

Cells were subjected to migration assays under normoxia and hypoxia by means of the xCELLigence RTCA system (Real-Time Cell Analyzer System, Acea Biosciences Inc., San Diego, CA, USA), developed to monitor cell events in real time, without incorporation of dyes, by measuring electrical impedance. In particular, 4 × 10^5 cells/well were seeded onto the top chambers of CIM-16 plates and the bottom chambers were filled with medium containing 5% FBS as chemoinhibitor. Each condition was performed in quadruplicate and the signal detection was programmed every 15 min for a total of 24 h. To evaluate migration under hypoxia, the RTCA station was allocated inside the incubator in which the oxygen concentration was 1% for the entire duration of the experiment, as previously reported [11].

2.5. Flow cytometric evaluation of CD133 expression

CD133 surface expression was measured by direct staining of cells with a phycoerythrin (PE)-conjugated anti-CD133/2 monoclonal antibody (293C3, Miltenyi Biotec, Bologna, I), following a previously reported procedure [6,11]. All samples were analyzed by a FACS Calibur flow cytometer (BD Biosciences) with CellQuest Pro 6.0 software (BD Biosciences). Data collected from 10,000 cells are shown as a percentage of positive cells.

2.6. Statistical analysis

The results were expressed as means ± standard deviations of three independent experiments. Statistical analysis was performed by using the 2-tailed Student’s t-test for unpaired data or the one-way ANOVA followed by Tukey’s multiple comparison test for more than two groups, using GraphPad Prism 6.0 statistical package (GraphPad Software, San Diego, CA, USA). P values < 0.05 were considered statistically significant.
3. Results

3.1. Garlic extracts reduces growth of non-invasive breast tumor cells

The effect of our handmade hydro-alcoholic garlic extract (GE) on cell viability and growth was firstly assessed in the invasive MCF7 cells, that were treated for 72 h with decreasing doses of unfractionated GE. As shown in Fig. 1A, a strong reduction of cell viability was revealed only at the lower tested dilution (1:200), that paralleled the increase of cells in the sub-G1 cell cycle phase (Fig. 1B), indicative of a predominant cytotoxic effect. A low decrease of the number of viable cells was induced by the 1:800 GE dilution (Fig. 1A), without significant effect on the number of death/apoptotic cells (Fig. 1B). Only 1:800 diluted GE also prompted the decrease of MCF7 cells in G0/G1 and their accumulation in the G2/M phases of cell cycle.

Because no data were available on the effects of garlic compounds in non-invasive breast tumors cells, GE was administered to the MCF10DCIS cell line, that was then evaluated for viability and cell cycle distribution. On the basis of the results obtained with MCF7, this investigation excluded the 1:200 diluted GE, as our intent was to identify an efficient GE dilution without evident toxic effects. At variance with MCF7, both 1:400 and 1:800 GE dilutions induced a low but significant decrease of the number of viable cells, albeit not accompanied by increase on the number of death/apoptotic cells (Fig. 1C and D). Both 1:400 and 1:800 GE dilutions reduced the number of cells in the G0/G1 and promoted their accumulation in the G2/M phases, with 1:800 GE capable of inducing the greatest effects in parallel with the lowest reduction of cell viability (Fig. 1C and D).

3.2. GE prevents the hypoxia induced EMT and motility in non-invasive breast tumor cells

To investigate the role of our homemade garlic extract in counteracting the effects of hypoxia on malignant progression of breast tumor, both the post-EMT MCF7 and the non-invasive MCF10DCIS cells were cultured at moderate hypoxia (1% oxygen) for 72 h in the presence of 1:800 diluted GE and evaluated for their malignant properties. We revealed that hypoxia failed to affect the expression of E-cadherin and vimentin in MCF7 cells, while these EMT markers were significantly modified by low oxygen in MCF10DCIS cells (Fig. 2A and B). In the latter cell line, the administration of GE completely prevented the decrease of E-cadherin and the increase of vimentin induced by hypoxia (Fig. 2A and B), revealing for the first time a role for garlic in preventing the EMT process induced by low oxygen availability in non-invasive breast tumor cells.

In order to assess if, in breast cancer as in other solid tumors, the hypoxia-induced mesenchymal phenotype is accompanied by enhanced cell motility, Real-Time assays of cell migration was performed in both MCF7 and MCF10DCIS cells grown under low oxygen. As shown in Fig. 2C, hypoxia induced an increase of the migration rate in both MCF7 and MCF10DCIS cells. No effects of GE were observed on migration of both cell lines grown under normoxia (Fig. 2C). On the other hand, GE administration prevented the increase of the migratory capability induced by low oxygen availability only in the non-invasive MCF10DCIS cells (Fig. 2C).

3.3. GE prevents the hypoxia induced stem cell marker CD133 in both invasive and non-invasive breast tumor cells

The possible role of garlic extract in affecting the CD133 surface marker was investigated in both MCF7 and MCF10DCIS breast tumor cells. No effects of GE on CD133 expression were revealed in both cell lines cell grown at normoxia, while cytofluorometric analysis of MCF7 and MCF10DCIS cells growing in under low oxygen availability confirmed our previous results showing the important increase of CD133 induced by hypoxia (Fig. 3A). The administration of GE during growth

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Fig. 1. Effect of garlic extracts on growth of MCF7 and MCF10DCIS cells. In (A) number of viable MCF7 cells after 72 h of culture in control conditions (Vehicle) or in the presence of increasing dilution of garlic extracts (GE). In (B) cell cycle analysis of MCF7 cells under the same experimental conditions. In (C) number of viable MCF10DCIS cells grown for 72 h in control conditions or in the presence of increasing dilution of GE. In (D) cell cycle analysis of MCF10DCIS cells under the above indicated experimental conditions. All the data are the mean of three separate experiments ± SD. *P < 0.05 with respect to Vehicle.
three separate experiments

1:800 garlic extract (GE) for 72 h. Fold changes of Cell Index (CI) relative to cells grown in control conditions (N)

proteins. In (C) xCELLigence-driven dynamic monitoring of migration of MCF7 and MCF10DCIS cells cultured at normoxia or hypoxia in the presence or absence of anticancer drugs and, in recent years, an increasing number of natural

4. Discussion

Botanical medicines have long attracted attention in the search for anticancer drugs and, in recent years, an increasing number of natural substances have shown potential in inducing the inhibition of breast cancer development and progression, without having the disadvantages of synthetic drugs [14,25]. Allium sativum, commonly known as garlic, has a long history as a bioactive plant with nutraceutical properties in the traditional medicine of different countries [26]. Even if there is no definitive evidence for the relationship between garlic and a reduced risk of cancer, some epidemiological studies demonstrated that garlic consumption is inversely associated with incidence of various tumors, including breast cancer [27,28]. Although the molecular mechanism at the basis of garlic effects is not completely understood, its phytochemical components, particularly oil- and water-soluble organosulfur compounds (OSCs), represent potential active molecules for cancer treatment [12,29]. It was reported that allicin, one of the most active OSCs in garlic, is the major component responsible for the anticancer properties of garlic through its bioactive oil-soluble derivatives, such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and ajene and some water-soluble organosulfur compounds such as S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC) [29].

Garlic derivatives may affect different stages of carcinogenesis, including the activation of carcinogens and oxidative enzymes, the proliferation of clonal cells, and growth and invasion of tumor cells [13, 29]. OSCs have been shown to inhibit cell cycle progression of cancer cells, with a specific role for DADS in suppressing progression of cells from the G2 phase into M phase and of DAT in increasing the number of cells arrested at G2/M phase [30].

As no previous data demonstrated a role for garlic in the progression of breast tumors induced by the tumor microenvironment, our work focused on the possible role of this functional food to counteract cell modifications that characterize malignant evolution of non-invasive breast tumor derived cells induced by low oxygen availability. To optimize the role of garlic, we used an unfractionated hydro-alcoholic garlic extract (GE), prepared as described by Petrovic et al. [23], which, as it contains a large number of active OSCs, has been reported to inhibit growth of cancer cells in vitro and in vivo more efficiently than GE fractions containing allicin or other OSCs, including DADS and its isomers [23].

The first part of the work was aimed to assess if our garlic extract, containing a number of bioactive oil-soluble allicin derivaties (DAS, DADS, DATS), was effective on breast tumor cells grown at normoxia. GE was then tested on MCF7 cells, a low invasive breast tumor cell line in which the effects of garlic on cell cycle and apoptosis has been previously demonstrated [17], and in the non-invasive MCF10DCIS cell line, that in nude mice gives rise to lesions that are predominantly high-grade comedo ductal carcinoma in situ [31]. Our results confirmed literature data indicating that, in MCF7, garlic derived OSCs induce a cell-cycle arrest [15,16] and demonstrated, for the first time, that garlic decreases growth of breast tumor cells with a non-invasive phenotype by blocking their progression through the cell cycle.

Based on substantial data on invasive breast cancer highlighting the ability of garlic to promote an epithelial phenotype [16,19,21,22], the further step of our work was to assess if our GE may revert the EMT, which represents a crucial step in malignant progression of solid tumors [32]. We found that administration of a non-lethal amount of GE induced a significant increase of E-cadherin in both the post-EMT MCF7 and the non-invasive MCF10DCIS cells, in the last of which also a strong reduction of the mesenchymal marker vimentin was revealed, demonstrating the ability of garlic derivatives to promote an epithelial phenotype also in non-invasive breast tumor cells.

In invasive breast cancer, it is well known that EMT is induced by low oxygen availability, which also supports the invasiveness of tumor cells, and regulates their stem cell properties [8,12]. We have previously demonstrated that hypoxia increased the malignant potential of DCIS-derived cells [11], promoting EMT and motility, and inducing the expression of the stem cell marker CD133. As in vitro and in vivo studies demonstrated that garlic derivatives, including DAS and DAT, reduced the effects of hypoxia in invasive breast cancer, we investigated the role of GE in counteracting the effects of hypoxia on malignant progression of breast tumors.
specific role of GE on events related to the EMT process and suggesting capability of both cell lines, we found that GE administration prevented this event only in the non-invasive MCF10DCIS cells, confirming the non-invasive breast tumor cells. Despite hypoxia enhanced migratory counteracting the EMT process induced by low oxygen availability in breast tumor cells and plays a phenotype and reducing cell growth. Notably, unfraccionated hydro-alcoholic extract enriched in organosulfur compounds may exert a protective role against hypoxia-induced transition from epithelial to mesenchymal phenotype, and expression of cancer stem cell markers that play crucial roles in tumor evolution. Even though further substantial studies are needed to identify the most suitable method to ensure its management as well its administration to patients, our results clearly indicate that hydro-alcoholic garlic extract can be considered in new approach for preventing progression of non-invasive to infiltrating breast tumors.

**CRediT authorship contribution statement**

**Federica Brugnoli**: Conceptualization, Methodology, Investigation, Writing – original draft. **Paola Tedeschi**: Methodology, Investigation. **Silvia Grassilli**: Investigation, Software. **Annalisa Maietti**: Investigation. **Vincenzo Brandolini**: Writing - review & editing. **Valeria Bertagnolo**: Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

**Conflict of interest statement**

The authors declare no conflict of interest.

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112052.

**References**


**Fig. 3.** Effect of GE on the CD133 stem cell marker in MCF7 and MCF10DCIS cells grown in hypoxia. In (A) flow cytometric analysis of CD133 expression in MCF7 and MCF10DCIS cells cultured for 72 h under normoxia (N) or hypoxia (H) in the presence or absence of 1:800 garlic extract (GE). A phycoerythrin (PE)-conjugated anti-CD133 antibody was used and surface antigen expression is reported as percentage of positive cells. All the data are the mean of three separate experiments ± SD. *P < 0.05 with respect to normoxia (N + Vehicle); **P < 0.05 between bars. In (B), the surface expression of CD133 in MCF7 and MCF10DCIS cells treated with GE and grown under hypoxia is shown on a bi-parametric dot plot in which a gate based on the isotype control was fixed to select high expressing cells. The percentage of cells showing high cell surface levels of CD133 is indicated at the upper right of each panel, together with their mean fluorescence intensity (MFI). Both MCF7 and MCF10DCIS cells confirmed that hypoxia induced the EMT shift, affecting E-cadherin and vimentin, in MCF10DCIS but not in the post-EMT MCF7 cells [8], and we demonstrated that this event can be prevented by GE, revealing for the first time a role for garlic in the post-EMT MCF7 cells [8], and we demonstrated that this event can be prevented by GE, revealing for the first time a role for garlic in the post-EMT MCF7 cells [8].

It is well known that hypoxia also influences stem cell development and maintenance [12], and we have previously demonstrated an increase of the sub-population expressing the cancer stem cell marker CD133 in both MCF7 and MCF10DCIS cells cultured in low oxygen [8,11,33]. Since OSCs, including allicin, were reported to affect cancer stem cells proliferation and CD133 expression in other solid tumors [34, 35], the possible role of garlic extract in affecting this surface marker was investigated in breast tumor cells growing under hypoxia, demonstrating, for the first time, that administration of GE strongly counteracted the up-modulation of CD133 positive cells induced by low-oxygen in both MCF7 and MCF10DCIS cell lines.

**4.1. Conclusions**

Overall, our data first demonstrated that garlic shows anti-cancer properties in non-invasive breast tumor cells, promoting an epithelial phenotype and reducing cell growth. Notably, unfraccionated hydro-alcoholic extract enriched in organosulfur compounds may exert a protective role against hypoxia-induced transition from epithelial to mesenchymal phenotype, and expression of cancer stem cell markers that play crucial roles in tumor evolution. Even though further substantial studies are needed to identify the most suitable method to ensure its management as well its administration to patients, our results clearly indicate that hydro-alcoholic garlic extract can be considered in new approach for preventing progression of non-invasive to infiltrating breast tumors.
In this study, we investigated the effects of garlic and its derivatives on breast cancer cells. We found that garlic and garlic-derived compounds can significantly inhibit cell proliferation and invasion, as well as promote apoptosis. Furthermore, our results suggest that garlic may be a promising candidate for cancer therapy.

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