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Original Research Article

G-protein-coupled estrogen receptor agonist G-1 inhibits the proliferation of breast cancer cells through induction of apoptosis and cycle arrest

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Abstract

Purpose: *To determine the effect of G-1, a G-protein-linked estrogen receptor (GPER) agonist on apoptosis, cell cycle, and proliferative potential of mammary tumor cells, and the associated mechanisms of action.*
Methods: *Three groups of human breast cancer cell line MDA-MB-231 were used: control group, estradiol (E2) group and G-1 group. Control group was not treated. The effects of treatment (10 □M G-1) on cell proliferation were determined and compared amongst the groups. Cell cycle distribution and apoptosis were determined while expression levels of proteins related to pi3k/AKT/MAPK were assessed by western blotting.*
Results: *Apoptosis was significantly reduced in E2 group relative to control, but was enhanced in G-1 group, when compared to the other 2 groups ($p < 0.05$). There were marked down-regulations in protein levels of cylinb1, p21, caspase 6, p53, p-ERK in E2 group, relative to the corresponding expression levels in the control group.*
Conclusion: *GPER agonist G-1 suppresses the proliferation of mammary tumor cells and induces apoptotic changes and cycle blockage in the cells via inhibition of pi3k/AKT pathway and activation of MAPKs pathway. Thus, GPER is a potential target in breast tumor treatment, and G-1 is a potential new anti-tumor drug.*

Keywords: *GPER agonist G-1, Breast cancer, Apoptosis, Cycle block, Cell proliferation*

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INTRODUCTION

Breast tumor is a frequently-occurring malignancy, mostly in middle-aged women aged 40 to 55 years [1]. It majorly accounts for cancer-associated death in Chinese ladies. Indeed, new cases of breast cancer and breast cancer-associated deaths in China account for 12.20 and 9.60 %, respectively, thereby seriously endangering the life and health of Chinese women [2]. With recent changes in style of living and increase in social pressure, the incidence of breast cancer in China has continued to increase annually, and it has continued to attract the

attention of Chinese medical researchers [3]. The mammary gland is an organ regulated by estrogens. It has been suggested that the occurrence of breast cancer is closely associated with changes in estrogen receptors [4].

Studies have shown that GPER is a novel estrogen receptor which activation is involved in the pathogenesis of vertebral column injury, trauma-induced brain lesions, cardiorespiratory illness, kidney disease, diabetic condition, and other diseases [5]. Studies have shown that GPER significantly inhibited the growth of tumor cells and promoted their death [6].

Estradiol (E2) is a steroid hormone which performs important physiological roles by binding to the estrogen receptor, thereby mediating in a variety of diseases. In addition, E2 is implicated in pathogenesis of breast cancer and tumors of the female reproductive organs [7]. Moreover, it has been revealed that only cell-permeable E2 derivatives rapidly activate GPER [8].

The present study was carried out to investigate the influence of GPER agonist G-1 on apoptosis, cell cycle, and proliferation of human breast cancer cell line MDA-MB-231. Moreover, the associated mechanisms were investigated.

EXPERIMENTAL

Cell line

Human breast cancer cell line MDA-MB-231 was provided by Shanghai Cell Bank, Chinese Academy of Sciences.

Cell grouping and treatments

A cell suspension was prepared at a density of 5×10^4 cells/mL, and 100 µL of the cell suspension was inoculated into 96-well plates. The supernatant was discarded after 24 h of adherent growth, and 3 groups of cells were set up: control, E2 and G-1 groups, with 5 repeated wells in each group. The control group was not treated.

Assessment of cell growth

MDA-MB-231 cells in the three groups were harvested at 0, 24, 48 and 72 h post-treatment. The cells in each group were enumerated thrice using blood counting plate. Each experimental group was set up in triplicate, and the results were calculated as mean of triplicate determinations.

Determination of protein expression level with western blot assay

Cells in each group were rinsed twice in PBS buffer, and total protein extraction was done by lysing with RIPA cell lysis buffer for 15 min. The total lysate protein was quantitated using bicinchoninic acid method. Then, 10 µg protein from each well was subjected to separation using SDS-polyacrylamide gel electrophoresis, followed by electro-transfer to PVDF membrane and membrane sealing at room temperature for 1 h. The membrane was rinsed thrice with TBST buffer (each rinse for 5 min). Thereafter, the membrane was incubated overnight at 4 ℃ with primary antibodies against p-AKT, p-ERK and GAPDH, after which the membrane was rinsed thrice with TBST buffer, followed by incubation with horse radish peroxide-conjugated 2º antibody at laboratory temperature for 60 min. Thereafter, the membrane was rinsed thrice with TBST buffer. Image development was done with ECL in line with ECL kit manufacturer's protocols. The gray values of protein bands were analyzed using ImageJ analysis, and the protein expression levels were normalized to that of GAPDH which served as the house keeping gene.

Determination of cell cycle

The cells were inoculated in 6-well plates (1×10^5 cells per well), followed by addition of 1 mL of serum medium. After 12 h, the starved cells were rinsed thrice using PBS buffer, and complete medium was added. After 48 h, the cells were subjected to tryptic digestion, after which they were rinsed with PBS buffer, treated with alcohol, and subjected to 12-h incubation at 4 ℃. Thereafter, the cells were stained using propidium iodide (PI) cell cycle dye for 30 min, followed by flow cytometric analysis of the cell cycle.

Flow cytometry

Logarithmic growth stage cells were subjected to tryptic digestion. Then, the cells were inoculated into 6-well plates at a density of 1×10^5 cells per well, and collected in Eppendorf (EP) tube after 48 h. Thereafter, the cells were rinsed in a mixture of PBS buffer and binding solution, after which cells in each well were stained with 5 µL of Annexin V-PE staining solution for 10 min in the dark. This was followed by addition of 5 µL of 7-ADD staining solution to each well. The staining was done for 5 min. Normal cells, early apoptotic cells and necrotic cells were identified via flow cytometry, and apoptosis was calculated.

Table 1: Comparison of cell growth amongst the three groups

Group	0 h	24 h	48 h	72 h
Control	7.24±0.25	14.12±0.38	18.46±0.52	21.36±0.62
E2	7.21±0.25	16.38±0.41[a]	21.49±0.58[a]	25.64±0.69[a]
G-1	7.19±0.26	10.26±0.34[ab]	13.06±0.46[ab]	16.98±0.58[ab]
F	0.202	1586.901	1336.832	939.911
P-value	0.821	<0.001	<0.001	<0.001

[a, b, c, d, e]$P < 0.05$: [a] vs the control; [b] vs E2; [c] vs the same group at initial time, [d] vs the same group at 24 h; [e] vs the same group at 48 h

Statistical analysis

SPSS 20.0 software package was used for statistical analysis of data. Measurement data consistent with normal distribution are expressed as mean ± SD. Comparison amongst multiple groups was performed using one-way ANOVA, while SNK-q test was used for paired comparison. Statistical significance of difference was assumed at $p < 0.05$.

RESULTS

Cell growth in each group of cells

As shown in Table 1, there were marked and time-dependent increases in cell population in the three groups. At each time point after 0 h, the number of cells was markedly raised in E2 group, relative to the number in controls, while the population of G-1 group was significantly reduced, when compared to the other 2 groups.

Cell cycle phase

The population of M/G2 phase cells was markedly reduced in E2 group, relative to controls, but it was markedly increased in control and E2 groups ($p < 0.05$). The results are shown in Table 2.

Table 2: Comparison of cell cycle in each group of cells

Group	G0/G1	S	M/G2
Control	62.15±0.56	19.58±0.26	18.27±0.27
E2	67.42±0.26[a]	19.74±0.28	12.84±0.18[a]
G-1	29.05±0.21[ab]	30.79±0.40[ab]	40.16±0.24[ab]
F	61030.800	8097.780	77043.550
P-value	<0.001	<0.001	<0.001

Expression levels of cell cycle-associated factors

There were significantly down-regulated levels of cylinB1 and P21 in E2 group than in controls, while PCNA protein level was up-regulated in E2, relative to control level ($p < 0.05$). In contrast, cylin B1 and P21 protein levels were elevated in G-1 group, relative to the corresponding levels in the control and E2 groups, while the protein level of PCNA was significantly lower in G-1 than in control and E2 groups ($p < 0.05$). These data are shown in Table 3.

Table 3: Expression levels of cell cycle-related proteins in each group of cells

Group	Cylin B1	P21	PCNA
Control	1.01±0.11	0.99±0.09	1.02±0.08
E2	0.67±0.06[a]	0.58±0.05[a]	1.36±0.13[a]
G-1	1.64±0.16[ab]	1.49±0.14[ab]	0.51±0.07[ab]
F	351.911	412.652	389.431
P-value	<0.001	<0.001	<0.001

[a]$P < 0.05$, vs control; [b] $p < 0.05$, vs E2

Apoptosis

Table 4 shows that apoptosis was less in E2 group than in control group, but markedly increased in G-1 group, relative to control and E2 groups ($p < 0.05$; Table 4).

Table 4: Comparison of apoptosis amongst the groups

Group	Apoptosis (%)
Control	18.64±1.46
E2	12.46±1.01[a]
G-1	46.38±2.16[ab]
F	2505.043
P	< 0.001

[a,b]$P < 0.05$: [a]vs the control; [b]vs E2

Levels of apoptosis-associated proteins

Table 5 shows that the protein expression levels of caspase-6 and P53 were markedly lower in E2 group than in control group, while MCL-1 protein was markedly up-regulated, relative to control value. The relative amounts of caspase-6 and P53 proteins were markedly higher in G-1 than in E2 and control groups, while MCL-1 protein was markedly down-regulated in control and E2 groups.

Levels of PI3K/AKT and MAPKs pathway-linked factors

In G-1 group, p-ERK protein was markedly up-regulated, relative to the corresponding control and E2 levels, while p-AKT protein level was

markedly down-regulated in E2 and control groups (Table 6).

Table 5: Apoptosis-related protein levels in the 3 group of cells

Group	Caspase-6	MCL-1	P53
Control	1.01±0.09	1.01±0.08	1.02±0.09
E2	0.59±0.06[a]	1.41±0.16[a]	0.64±0.04[a]
G-1	1.42±0.14[ab]	0.81±0.05[ab]	1.34±0.15[ab]
F	330.164	162.323	228.826
P-value	< 0.001	< 0.001	< 0.001

[a]*P* < 0.05, vs control; [b]*p* < 0.05, vs E2

Table 6: Relative expression levels of PI3K/AKT and MAPKs pathway proteins in the 3 groups

Group	p-AKT	p-ERK
Control	1.01±0.09	0.99±0.08
E2	1.35±0.14[a]	0.79±0.04[a]
G-1	0.61±0.04[ab]	1.37±0.16[ab]
F	280.961	155.002
P-value	< 0.001	< 0.001

[a,b]*P* < 0.05: [a]vs control; [b]vs E2

DISCUSSION

Various tissues and cells of the female reproductive system, central hippocampal tissue and cardiovascular system contain GPER which participates in several processes in cells [9]. In recent years, GPER has been involved in research on cervical carcinoma, lung tumor, thyroid cancer and other cancer cells. It has been reported that changes in the expression of GPER regulated the invasion and metastatic capacities of endometrial cancer [10]. In another study, E2 derivatives induced GPER expression, thereby increasing the proliferation of undifferentiated thyroid carcinoma [11]. It has been reported that GPFR level was significantly abnormal in mammary carcinoma cells [12]. In the present study, analysis of the survival curves of breast cancer cells in each group revealed that the number of cells in the three groups was increased over time, and at the same time, the number of cells in E2 group was markedly raised, but cell number in the G-1 group was markedly reduced. These results suggest that the GPER agonist G-1 inhibited the growth of mammary tumor cells. This finding is similar to that reported in an earlier study [13].

The cell cycle is easily affected by environmental conditions. Changes in the cell cycle are of great significance in cell proliferation and tumor growth. Cylin B1 is a crucial cell cycle regulatory factor. Increased levels of cylin B1 promote cell proliferation. It is known that P21 controls changes in the cell cycle [14]. Moreover, PCNA is a cell cycle marker expressed only at the stage of cell proliferation. It has been revealed that PCNA is involved in cell cycle regulation in laryngeal carcinoma, duodenal adenocarcinoma and vascular smooth muscle cells [15]. In this study, the population of M/G2 phase cells in E2 group was markedly reduced, while it was markedly raised in G-1 group. There were marked decreases in cylin B1 and P21 in E2 group, while PCNA protein amounts were significantly increased. The protein levels of cylinB1 and P21 in G-1 group were markedly up-regulated, while PCNA protein levels were significantly decreased. These results suggest that G-1 activated cell cycle blockage at the M/G2 stage by regulating cyclin, thereby inhibiting cell proliferation.

Apoptosis is crucial for the removal of unwanted cells in multicellular organisms. Caspase-6 is a newly discovered apoptosis regulator. Activated caspase-6 acts on cytoplasmic components and induces apoptosis. It has been revealed that MCL-1, a member of the Bcl-2 family, inhibits cell apoptosis [16]. Indeed, an increase in MCL-1 level is indicative of occurrence of tumors [17]. The tumor suppressor gene, P53 enhances cell apoptosis, and it is often used as an indicator of cell apoptosis and cell survival status in clinical practice. In this study, the percentage apoptosis in E2 group was markedly reduced, but apoptosis in G-1 group was markedly increased. Moreover, caspase-6 and P53 levels in E2 group were markedly decreased, while the MCL-1 protein levels were markedly up-regulated. In contrast, caspase-6 and P53 levels in G-1 group were markedly increased, while MCL-1 protein expression levels were significantly decreased. These results indicate that the GPER agonist G-1 induced apoptosis via modulation of expressions of apoptosis-associated factors.

The PI3K/AKT pathway and MAPKs pathway control the growth, apoptotic changes and invasiveness of tumor cells, as well as regulation of the pathogenesis of tumors [18]. In this study, p-ERK protein level in the E2 group was markedly decreased, while p-AKT protein level was markedly increased. The protein level of p-ERK in the G-1 group was up-regulated, while the p-AKT protein level was significantly down-regulated. These results suggest that the GPER agonist G-1 inhibited cell proliferation by regulating the PI3K/AKT and MAPKs pathways, thereby delaying the occurrence of breast cancer.

CONCLUSION

The GPER agonist G-1 suppressed the proliferation of breast cancer cells via induction of apoptosis and cycle arrest through inhibition of

the PI3K/AKT pathway and activation of MAPKs pathway. Thus, GPER is a likely target in the treatment of breast tumor. Moreover, G-1 has promising potential as a novel anti-tumor drug.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lihui Ma designed the study, supervised the data collection, and analyzed the data. Hancheng Liu interpreted the data and prepared the manuscript for publication. Minghui Wang, Chunyu Tian, Jie Zhang and Hongxu Zhang supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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