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ORIGINAL ARTICLE

Glutathione-*S*-transferase enhances proliferation-migration and protects against shikonin-induced cell death in breast cancer cells

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Abstract Glutathione-S-transferase (GST) is a cytoplasmic protein responsible for detoxification, but the effect of the enzyme on cell biological events, including proliferation and migration, has never been reported. Thus, we evaluated the detoxification effect of *in vitro* —applied GST on cancer cell proliferation and migration. Assays for proliferation and migration of human breast cancer cells in the presence of GST were carried out. Binding of GST on the surface of the cancer cells was studied by flow cytometry. Detoxification through GST pathway was studied in the presence of shikonin. The effective dosage of GST in enhancement of cell proliferation was 10—50 nM, and the cell migration could be significantly enhanced after 6 hours in the presence of 2—50 nM GST. Therefore, overall cell proliferation and migration could be enhanced in the presence of 10 nM or greater concentration of GST, and 15 μM shikonin-induced toxification of the cancer cells could be neutralized by 1.0 μM GST. Flow cytometry showed that GST directly bound to the surface of the cancer cells, and this was confirmed by fluorescence confocal microscopic observation. It is concluded that human class π-GST enhances proliferation and migration of human breast cancer cells by means of direct binding to the cell surface and maintaining cell viability by detoxification.

Introduction

Glutathione-S-transferase (GST) is a dimer that shares homology in almost all eukaryotes and plays a role in internal cell detoxification. Recently, it was found that GST might neutralize toxic effects caused by drugs, food additives, environmental chemicals, and carcinogens [1—3]. A few copies of *gst* genes are located in human chromosomes

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6p encoding for α-GST, 1p for μ-GST, 11q for class π-GST (π-GST), and 22q for θ-GST [4—7]. The natural substrate of GST is glutathione, a water-soluble molecule widely present as both oxidized and reduced (GSH^{Red}) forms in eukaryotes. Binding of GSH^{Red} with GST causes oxidation of the substrate and, thus, maintains the balance of redox states in cells [8].

Internal detoxification by means of GST occurs in conjugation pathway (Phase II), in which GST binds with substrates forming conjugates, such as GSH S-conjugate. The conjugate is then catalyzed to cysteine S-conjugate by γ-glutamyl transpeptidase and is finally released in feces and urine [9,10].

It is well known that free radicals, including hydrogen peroxide, superoxide anion, and hydroxyl radicals, are toxic substances generated in electron transfer pathways in mitochondria, especially under the stimulation of UV light. Excess amounts of these radicals cause damage to the cells, including large-molecule nucleic acids, proteins, and lipids [11]. More severe radicals, such as alkoxy radical and α,β-unsaturated aldehydes, may be generated readily when lipid is attacked by hydroxyl radical and singlet oxygen, causing high oxygen pressure in cells [12]. GST plays an important role in neutralizing these radicals, decreasing accumulation of fatty acid hydroperoxides and phospholipid hydroperoxides, and preventing the production of α,β-unsaturated aldehydes in the cells [13,14].

Antiapoptic effects of GST had been reported in leukemia K562 cell line, which expressed large amounts of human α-GST. The enzyme inhibited the activation of stress-activated protein kinase/c-jun N-terminal kinase and caspase-3. Thus, GST prevents apoptosis probably by decreasing superoxide anion under high oxygen pressure [15].

Shikonin is a natural substance of *Radix arnebiae*, a herbal drug used very often in the treatment of immune-mediated diseases, including systemic lupus erythematosis and cancers. This herbal drug was first listed as a middle-grade drug in *Shen Nong's Herbal Classics*, the earliest extant monograph on material medica in China, which appeared about 2020 years ago (during Qin and Han Dynasties). Recently, shikonin was found to inhibit bacterial growth [16,17], suppress replication of HIV [18] and inflammation [19], and inhibit platelet aggregation [20,21]. Shikonin was also found to enhance apoptosis of cancer cells [22]. Our preliminary results also showed that shikonin was toxic to human breast cancer cells under culture conditions.

Our previous experimental results showed that exogenous application of *Schistosomiasis japanicum* GST to human breast cancer cell line (MDA-MB-435s) could enhance proliferation and migration by stimulating over-expression of matrix metalloproteinases 2 and 9 [23]. To reveal GST the detoxifying mechanism of action in cancer cell proliferation and migration against used shikonin as cellular toxic substance, we set up the current experiments applying recombinant human π-GST to incubate with MDA-MB-435S cells and used flow cytometry and confocal microscope to detect the binding of GST with MDA-MB-435s cells. Our present results provide some molecular insights of GST on proliferation, migration, and extracellular detoxification in human breast cancer cells.

Methods

Reagents

Recombinant *Taq* DNA polymerase with proofreading activity, polymerase chain reaction buffer, deoxyribonucleotide triphosphate mix, and restriction enzymes were purchased from TaKaRa (Tokyo, Japan). The intermediate polymerase chain reaction cloning kit (pGEM-T easy vector system) was obtained from Promega (Madison, WI, USA), and expression vector (pAcGFP-N1) was purchased from Invitrogen (San Diego, CA, USA). General chemicals were purchased from Sigma (Shanghai, China).

Purification of GST

Expression plasmid was constructed with prokaryotic cell expression vector pKK233-2 containing π-GST gene. Recombinant π-GST (rGST) was purified from π-plasmid GST-transformed *Escherichia coli* strain JM109. Briefly, 100 mL of fresh Luria Broth medium containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 80 μg/mL ampicillin was inoculated with π-plasmid GST-transformed *Escherichia coli* strain [BL21 (DE3)] and was grown overnight at 37°C with vigorous shaking. The stock cells were then transferred to 900 mL of fresh Luria Broth medium containing the same concentration of antibiotic and continued to grow for 2 hours before IPTG was added to the final concentration of 0.3 mM to induce gene expression. The growth was maintained for another 4 hours, cells are harvested, sonicated, in solubilization [50 mM Tris-HCl (pH 8.0)] was achieved by centrifugation. The supernatant containing rGST was dialyzed against 100-fold volume of phosphate-bufferred saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4); centrifuged to remove insoluble materials; filtered through 0.45-μm membrane; and then loaded onto glutathione-agarose column equilibrated with PBS. The column was washed in 5—10 bed volumes of PBS or until zero absorbance at 280 nm. The recombinant protein was eluted in elution buffer (10-mM GSH^{Red} in Tris-HCl, pH 8.0), and protein concentration was estimated spectrophotometrically [24], used immediately or stored at −80°C until use.

Cell viability assays

The measurement of viability was based on the ability to cleave tetrazolium salts by dehydrogenases [25]. Augmentation in the amount of developed color directly correlated with the number of metabolically active cells. Assays were performed according to manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a density of 5×10^4 cells/well in culture medium for 4 hours to allow attachment. The cell viability—detecting reagent 4-[3-(4-Iodophenyl)-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10-μL pure solution) was added to each sample after every treatment with various concentrations of shikonin, and the cells were incubated for another 2 hours in a humidified atmosphere (37°C). The absorbance of the samples (A_{450}) was determined using a scanning multiwell spectrophotometer. Absolute optical density was normalized to the absorbance of