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Energy metabolism analysis reveals the mechanism of inhibition of breast cancer cell metastasis by PEG-modified graphene oxide nanosheets

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ABSTRACT

Recent advances in nanomedicine provide promising alternatives for cancer treatment that may improve the survival of patients with metastatic disease. The goal of the present study was to evaluate graphene oxide (GO) as a potential anti-metastatic agent. For this purpose, GO was modified with polyethylene glycol (PEG) to form PEG-modified GO (PEG-GO), which improves its aqueous stability and biocompatibility. We show here that PEG-GO exhibited no apparent effects on the viability of breast cancer cells (MDA-MB-231, MDA-MB-436, and SK-BR-3) or non-cancerous cells (MCF-10A), but inhibited cancer cell migration *in vitro* and *in vivo*. Analysis of cellular energy metabolism revealed that PEG-GO significantly impaired mitochondrial oxidative phosphorylation (OXPHOS) in breast cancer cells; however, PEG-GO showed no effect on OXPHOS in non-cancerous cells. To explore the underlying mechanisms, a SILAC (Stable Isotope Labeling by Amino acids in Cell culture) labeling strategy was used to quantify protein expression in PEG-GO-exposed breast cancer *versus* non-cancerous cells. The results indicated that PEG-GO selectively down-regulated PGC-1α in breast cancer cells and thus modified the expression of diverse energy generation-related proteins, which accounts for the inhibition of OXPHOS. The inhibition of OXPHOS by PEG-GO significantly reduced ATP production and impaired assembly of the F-actin cytoskeleton in breast cancer cells, which is required for the migratory and invasive phenotype of cancer cells. Taken together, these effects of PEG-GO on cancer cell metastasis may allow the development of a new approach to treat metastatic breast cancer.

1. Introduction

Cancer deaths are caused more frequently by metastasis to distant sites than by the primary tumor [1–3]. Despite significant advances, the efficacy of conventional cancer chemotherapy is limited by cytotoxicity to normal cells, the inability to reach the primary tumor or metastatic site(s), and by selection of multidrug-resistant tumor cells [4]. The use of nanotechnology has increased our ability to improve detection and treatment of cancer. Because nanomaterials selectively gain access to cancer cells by passive or active targeting, they show great promise for cancer therapy [5,6]. For example, in a phase III clinical trial, patients with metastatic breast cancer were treated with albumin-bound paclitaxel nanoparticles (ABI-007), and this formulation was both more efficacious and safer than standard paclitaxel [7]. In a phase I trial, a polyethylene glycol (PEG)-modified liposomal formulation of the semisynthetic camptothecin analog S-CKD602 showed increased antitumor activity as well as prolonged circulation time with low toxicity [8].

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Numerous nanomaterials such as liposomes, polymers/dendrimers, metal nanostructures and carbon nanostructures are used to selectively target drugs to tumors [9]. Nanomaterials fabricated using graphene have drawn considerable attention for use in biomedical applications because of their unique physical and chemical properties [10–13]. Graphene oxide (GO), a one-atom-thick sheet of sp^2-bonded carbon atoms with carboxyl, hydroxyl, and epoxide functional groups on its basal planes and edges, has been characterized in detail. Functionalized GO with PEG (PEG-GO) exhibits increased stability in aqueous solution as well as improved biocompatibility [14,15], suggesting that it could be used in drug delivery and photothermal therapy [16,17]. PEG-GO shows superior efficiencies for transfecting cells with nucleic acids (plasmid DNA and small interfering RNA) and for administering conventional drug treatment [18]. Moreover, transfection efficiency can be enhanced using irradiation with a low-power near-infrared laser [19]. PEG-GO coupled to hydrophobic aromatic molecules such as the camptothecin analog SN38 is highly soluble in aqueous solvents while remaining cytotoxic to cancer cells [20]. Furthermore, silver-embedded nanoparticles deposited onto doxorubicin-coupled GO exhibit very high drug-loading efficiency and impart excellent chemo-photothermal therapeutic efficacy, tumor targeting, and laser-controlled drug release; they also aid in enhanced X-ray imaging [21].

Nanoparticles are effective drug carriers; however, some nanoparticles show potential inhibitory effects against tumor growth and metastasis on their own, without any added drug [22]. We recently reported that graphene attenuates mitochondrial respiration on its own by inhibiting the activity of electron transport chain (ETC) complexes [23]. However, the effects of PEG-GO on tumor metastasis *in vivo* and on the metabolism of cancer cells are unknown. Here, we investigated the effects of PEG-GO on MDA-MB-231, MDA-MB-436, and SK-BR-3 breast cancer cells, all of which exhibit metastatic phenotypes. Normal mammary epithelial cells (MCF-10A) and primary macrophages were used as non-cancerous cell controls to test whether effects of PEG-GO were specific to cancer cells. The assembly of the F-actin cytoskeleton, cellular ATP generation, mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis were measured in PEG-GO-exposed cells. In addition, stable isotope labeling with amino acids in cell culture (SILAC)-based quantitative proteomics was used to understand the molecular mechanism of the inhibition of cancer cell OXPHOS by PEG-GO. Finally, the *in vivo* effect of PEG-GO on the migration of breast cancer cells was assessed using animal models.

2. Materials and methods

2.1. Materials

Culture medium and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Alexa Fluor 488-labeled phalloidin was purchased from Life Technologies (Grand Island, NY, USA). Anti-cortactin antibody was from Cell Signaling Technology (Danvers, MA, USA). Antibodies against SDHB (Complex II) and UQCRC2 (Complex III) were from MitoSciences (Eugene, OR, USA). Antibodies against CDC42, Rac1 and RhoA were from Proteintech (Chicago, IL, USA). Antibodies against PGC-1α, β-actin and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Matrigel was purchased from BD Biosciences (San Jose, CA, USA). Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA). Cell culture dishes and Transwell plates were purchased from Corning (Tewksbury, MA, USA). Fluorescent-labeled latex beads (amine-modified polystyrene, 1 μm mean particle size), peroxidase-labeled second antibodies, TRITC-labeled second antibodies and other reagents were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation and characterization of PEG-modified GO

GO was prepared from graphite flakes using a modified Hummer's method [24]. Briefly, graphite was oxidized using H_2SO_4 and $KMnO_4$, stirred vigorously (37 °C, 3 h), and treated with H_2O_2 to remove residual $KMnO_4$ and MnO_2. The sample was washed with 5% HCl and deionized H_2O and then dialyzed to remove residual metal ions and acids. Graphite oxide was exfoliated for 2 h using a sonicator set to 325 W and then centrifuged at 5000× *g* for 20 min to remove graphite oxide that was not exfoliated.

The GO suspension was diluted to ~2 mg/ml with deionized water. PEG solution (5 wt%) was added dropwise into the suspension with sonication and then sonicated in a water bath for an additional 1 h. After centrifugation (12,000× *g*) for 1 h, the supernatant was collected as the final product. The shapes and thicknesses of the PEG-modified GO were assessed using a Nanoscope IIIA atomic force microscope. Their size distribution was analyzed using a Beckman Coulter Delsa Nano C particle analyzer.

2.3. Cell culture

The MDA-MB-231, MDA-MB-436, and SK-BR-3 human breast cancer cell lines, the MCF-10A human mammary epithelial cell line, and the NIH-3T3 mouse embryonic fibroblast cell line were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured under conditions recommended by the ATCC. Primary peritoneal macrophages were harvested from female BALB/c mice (Vital River Experimental Animal Center, Beijing, China) and cultured as described previously [25].

2.4. Cell viability assays

Cell viability was assessed using an Inno-Alliance IC1000 automated cell counter. Cells were seeded in 60-mm culture dishes and then incubated in medium containing 0, 5, 10, 20, 40, or 80 μg/ml of PEG-GO at 37 °C for 24 h. Trypan blue was added to each culture and unstained (viable) cells were counted.

2.5. Transwell migration and invasion assays

Matrigel invasion assays were performed at 37 °C using 24-well Transwell inserts coated with 30 μg of Matrigel [26,27]. After incubation with 40 or 80 μg/ml PEG-GO for 24 h, cells (50,000) were suspended in 200 μl of serum-free medium and added to the upper chamber, and the migration inducer (NIH-3T3 cell-conditioned medium) was added to the lower chamber. Transwell cell migration assays were performed similarly but without Matrigel. Cells that migrated and invaded through the membrane were stained with 0.005% crystal violet and quantified, and the data were normalized to those of the control cells.

2.6. Assessment of F-actin cytoskeletal assembly

The F-actin cytoskeletal assembly was stained with phalloidin. Briefly, cells were cultured on cover slips, incubated with 40 or 80 μg/ml PEG-GO for 24 h, treated with NIH-3T3 cell-conditioned medium for 5 h, and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized using 0.1% Triton X-100, and 1% BSA and 10% horse serum were added to reduce the nonspecific binding of antibodies. Cells were then incubated with Alexa Fluor 488-labeled phalloidin, cortactin antibody, and DAPI. Cells were viewed with an Olympus FV1000 laser scanning confocal microscope. Lamellipodia, the flattened F-actin-rich leading edge of migrating cells, were outlined and measured using Image-Pro Plus software. The summed lengths of the lamellipodia were expressed as a percentage of total cell circumferences. The images represent at least 50 cells.

2.7. Endocytosis assay

Endocytosis was assessed by measuring the ability of cells to ingest fluorescent-labeled latex beads (1 μm mean particle size). Both MDA-MB-231 cells and primary murine macrophages were incubated with 40 or 80 μg/ml PEG-GO for 24 h and then exposed to 1-μm latex beads at a bead-to-cell ratio of 10:1 for 4 h. The percentages of cells with internalized beads were analyzed by a BD FACSCalibur flow cytometer as described previously [25].

2.8. Analysis of intracellular ATP levels

Intracellular ATP levels were quantified using an ATP Assay Kit (Beyotime, Haimen, China). Briefly, cells were seeded in six-well cell-culture plates, incubated with or without 40 μg/ml PEG-GO, lysed with 400 μl ice-cold ATP-releasing buffer, and centrifuged at 12,000× *g* for 10 min. The supernatant was transferred to a new tube; then, 10 μl of supernatant was added to 100 μl of ATP detection buffer, and luminescence was measured using a Berthold Sirius luminometer. The ATP concentration was determined using a standard curve, and ATP levels were normalized to protein concentrations.

2.9. Mitochondrial OXPHOS and glycolysis assays

Mitochondrial OXPHOS and glycolysis were analyzed using a Seahorse XF24-3 extracellular flux analyzer by real-time measurements of the oxygen consumption rate (OCR, indicative of mitochondrial OXPHOS) and extracellular acidification rate (ECAR, indicative of glycolysis). For OCR analysis, cells (20,000 cells per well) were seeded in complete growth medium in the wells of 24-well plates designed for the XF24. The cells were treated the next day with 40 μg/ml PEG-GO for 24 h, incubated in unbuffered media, and then incubated in a CO_2-free incubator for 1 h prior to measurement. A program with a typical 8-min cycle of mix (3 min), dwell (2 min), and measurement (3 min) was used. During measurement, oligomycin, carbonyl