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Dimethyl sulfoxide-caused changes in pro- and anti-angiogenic factor levels could contribute to an anti-angiogenic response in HeLa cells

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ABSTRACT

Dimethyl sulfoxide (DMSO) is widely used in biological research as a general solvent. While it has been previously demonstrated that DMSO possesses a wide range of pharmacological effects, there is no published work regarding the effects of DMSO on pro-angiogenic factor levels. This study was designed to investigate the possible effects of DMSO on the levels of three pro-angiogenic factors released from HeLa cells in vitro. Cells were treated with two different and previously determined concentrations of DMSO. The cytotoxic effects of DMSO concentrations on HeLa cells were determined via MTT. Survival rates of DMSO-treated cells were determined by Invitrogen live/dead viability/cytotoxicity kit and trypan blue exclusion assay.

Changes in the pro-angiogenic levels in media were evaluated by Cayman's Substance P Enzyme Immunoassay ELISA kit. Vascular endothelial growth factor ELISA kit and interferon gamma ELISA kit for substance P, VEGF and IFNγ respectively. Changes in substance P levels were corrected by standard western blotting. Changes in VEGF and IFNγ levels were corrected both by western blot and real time PCR.

Treatment with 1.4 μM DMSO caused a time-dependent inhibition of cell proliferation at 24, 48 and 72 h. 1.4 μM DMSO caused a significant reduction in VEGF levels at 72 h of incubation and sharp increases in IFNγ levels at both 48 and 72 h of incubation. According to real time PCR analyses, DMSO (1.4 μM) exhibited an inhibitory effect on VEGF but acted as an augmenter of IFNγ release on HeLa cells in vitro.

This is the first report showing that the general solvent DMSO suppressed HeLa cell proliferation, decreased the levels of two pro-angiogenic factors (substance P and VEGF) and increased the release of an anti-angiogenic factor IFNγ in vitro.

1. Introduction

A lignin derivative dimethyl sulfoxide (DMSO), accepted as a general solvent for many water-insoluble drugs, is often used to dissolve hydrophobic substances in biological research and medical applications (Santos et al., 2003). DMSO has a wide range of pharmacological effects including vasodilation, diuresis and drug permeability inside the cell. These effects depend on the DMSO concentration, type of DMSO treatment and the cell type (Fossum et al., 2008). Based on in vitro experiments, it is known that DMSO is able to pass through many biological membranes and barriers by altering the structure of bilayer and lipid packing. DMSO is also used to treat inflammatory diseases such as cerebral ischemia and interstitial cystitis because of its anti-inflammatory properties. In 1978, the U.S. Food and Drug Administration (FDA) approved the usage of a 50% aqueous solution of DMSO (RIMSO-50) for the treatment of interstitial cystitis (Shimizu et al., 1997). It has also been used for leukemia treatment due to its anti-proliferative effects (Wang et al., 2012).

Angiogenesis is a multistep process in cancer progression which is regulated both by pro- and anti-angiogenic factors (Mravec et al., 2008). The metastasis capacity of a tumor depends on the expression levels of angiogenic factors. Since the discovery of angiogenic inhibitors, anti-angiogenic therapy has become a novel strategy for cancer treatment. In recent years, it has been accepted that neuro-immune mechanisms (especially the substance P (SP)/NK1 receptor pathway) play a crucial role in local tumor development, carcinogenesis and distant metastasis (Simsek Oz et al., 2011; Grimsholm et al., 2008). It is known that SP plays a major role in neurogenic inflammation, inducing local inflammatory responses and angiogenesis (Simsek et al., 2014).

In our previous study, it was shown that 100 μM thalidomide (dissolved in 1.4 μM DMSO) showed a cytotoxic effect on 4T1 and 4THMpc mouse breast cancer cell lines. DMSO (1.4 μM) alone in a concentration equivalent to dissolved thalidomide was capable of exhibiting cytotoxic effects as well (Simsek Oz et al., 2012). Therefore,

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we speculated that the cytotoxic effect of thalidomide may partially be attributed to the solvent DMSO.

On the other hand, we showed that water-soluble thalidomide had no significant effect on SP levels in the same cell lines. In our previous study, we showed that a combination of thalidomide plus irradiation therapy and irradiation therapy alone could increase SP levels in the media (Simsek Oz et al., 2011).

Regarding our previous results, it should be pointed out that DMSO could have been responsible for the anti-angiogenic property of thalidomide by decreasing SP levels. The goal of the study was to test the possible effects of DMSO on vascular endothelial growth factor (VEGF, the major angiogenic factor in angiogenesis) and on interferon gamma (IFNγ, one of the main anti-angiogenic factor related with inflammation) levels in vitro.

2. Material and methods

2.1. Cell culture conditions

HeLa cells (ATCC®CCL-2™) were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 10 μg/ml gentamicin and 5% sodium pyruvate. The cells were incubated in 5% CO_2 with 95% humidity at 37 °C.

2.2. Cell proliferation

Cell proliferation was estimated using a CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega, Madison, WI, USA), which is based on the cleavage of 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) into a formazan product which is soluble in tissue culture medium. Briefly, cells were seeded at 1×10^4 cells per well in 200 μl complete medium onto 96-well plates and allowed to attach for 24 h. After the cells reached 80–90% confluency, the media was removed and cells were washed with phosphate-buffered saline (PBS). Subsequently, cells were treated with two concentrations of DMSO (1.4 μM and 0.14 μM) prepared in 1% FBS containing complete medium. Each treatment was performed in eight well replicates. The cells were grown at 37 °C for 24 h, 48 h and 72 h. The medium was gently aspirated to terminate the experiment, 180 μl serum-free complete medium and 20 μl of MTT were added to each well and cells were incubated for 4 h. The absorbances at 490 nm were measured in a microplate reader (Thermo Labsystems Multiskan Spectrum, Thermolabsystem, Chantilly, VA, USA) using wells without cells as background. Sample readings were calculated by subtracting the average of background absorbances. All experiments were performed at least three times.

2.3. Cell viability

The live/dead viability/cytotoxicity kit for mammalian cells (Invitrogen, Eugene, OR, USA) was used to determine cell viability. According to the manufacturer's protocol, cells were dually stained with two probes (calcein AM for live cells, EthD-1 for dead cells) that enable the simultaneous determination of live and dead cells in a sample. Fluorescence intensity was measured on a LS55 Luminescence Plate Reader (PerkinElmer Inc., Waltham, MA, USA) at 485–530 nm excitation and 630–645 nm emission wavelengths, respective to each reagent dye. The experimental data was pooled and subjected to statistical comparisons using Student's t-test.

2.4. Trypan blue exclusion assay

To determine the number of dead cells and total cell numbers, trypan blue (4% trypan blue in Hank's Balanced Salt Solution (HBSS)) exclusion assay was used. Detection was a double-blind trial. Photographs were captured under a phase contrast microscope. The percentage of decrease in cell survival was calculated according to the results of three independent experiments. The following formula was used; 1 − [no. of live cells in treated (DMSO) / no. of live cells in the control group] × 100.

2.5. Determination of SP levels

Cells (200,000 cells per well) were seeded in 6-well plates and incubated for 24 h. Then, 1.4 μg/ml DMSO (in 1% serum containing medium) was applied to all wells except controls. In control wells, media was replaced with fresh medium containing only 1% serum. Conditioned medium was collected 24 h after treatment and the SP concentration was measured in duplicate using a sensitive (20 pg/ml detection limit) competitive EIA kit according to the manufacturer's instructions (Substance P Enzyme Immunoassay kit; cat. no. 583751; Cayman, Ann Arbor, MI, USA). Absorbances were read at 420 nm with a microplate reader (Model 450; Bio-Rad, Richmond, CA, USA).

2.6. Determination of VEGF and IFNγ levels by ELISA

In order to determine the possible effects of DMSO on VEGF levels in HeLa cells, Quantikine Human VEGF ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) was used in accordance with the manufacturer's protocols. Briefly, 5×10^3 cells were plated in 96-well plates, cells were treated with two different concentrations of DMSO (0.14 and 1.4 μg/ml) and incubated for 48 h (Simsek Oz et al., 2012). Human VEGF specific polyclonal antibody coated wells were used. Serial dilutions of purified human VEGF (as standards) and cell media (200 μl) were added into each well. The procedure was started with the incubation of a primary antibody and a biotinylated secondary antibody. Then, streptavidin-peroxidase enzyme and the relative substrate solution were added and the absorbances were determined at 450 nm in an ELISA plate reader.

IFNγ levels in DMSO-treated cell media were assayed by Human IFNγ sandwich ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, standards and samples were pipetted into human IFNγ specific monoclonal antibody coated wells. Biotinylated polyclonal detector antibody was added to all wells. After washing, streptavidin-horseradish peroxidase (HRP) was added to the wells. After an incubation period, substrate solution (TMB) was added for color development. The optical densities of samples were determined on a standard ELISA reader. All measurements were performed three times and average results were compared to non-treated cells.

2.7. Western blotting

To ascertain whether alterations in the SP, VEGF and IFNγ levels of HeLa cells were due to changes in protein content, western blotting was performed as previously described (Simsek Oz et al., 2011). Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL, USA).

Briefly, 25 μg of homogenate proteins were separated on a 12.5% acrylamide gel by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia Biotech, Piscataway, NJ) with a semi-dry transfer apparatus. The membranes were blocked with TBS-5% milk and then probed with human anti-SP, anti- VEGF and anti-IFNγ (sc-2710281, sc-30345 and sc-166800; 1:200, 1:100 and 1.100, respectively). The primary antibodies were detected with horseradish peroxidase-conjugated anti-human secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, sc-2453 diluted 1:5000, 1:2000 and 1:2000 for SP, VEGF and IFNγ, respectively).

2.8. Real time PCR for VEGF and IFNγ

2.8.1. RNA isolation

5×10^5 HeLa cells were plated in 6-well plates and allowed to attach for 24 h. After the incubation period, cells were treated with 1.4 μM