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Superoxide-hydrogen peroxide imbalance interferes with colorectal cancer cells viability, proliferation and oxaliplatin response

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ABSTRACT

The role of superoxide dismutase manganese dependent enzyme (SOD2) in colorectal cancer is presently insufficiently understood. Some studies suggest that high SOD2 levels found in cancer tissues are associated with cancer progression. However, thus far, the role of colorectal cancer superoxide-hydrogen peroxide imbalance has not yet been studied. Thus, in order to address this gap in extant literature, we performed an in vitro analysis using HT-29 colorectal cell line exposed to paraquat, which generates high superoxide levels, and porphyrin, a SOD2 mimic molecule. The effect of these drugs on colorectal cancer cell response to oxaliplatin was evaluated. At 0.1 μM concentration, both drugs exhibited cytotoxic and antiproliferative effect on colorectal cancer cells. However, this effect was more pronounced in cells exposed to paraquat. Paraquat also augmented the oxaliplatin cytotoxic and antiproliferative effects by increasing the number of apoptosis events, thus causing the cell cycle arrest in the S and M/G2 phases. The treatments were also able to differentially modulate genes related to apoptosis, cell proliferation and antioxidant enzyme system. However, the effects were highly variable and the results obtained were inconclusive. Nonetheless, our findings support the hypothesis that imbalance caused by increased hydrogen peroxide levels could be beneficial to cancer cell biology. Therefore, the use of therapeutic strategies to decrease hydrogen peroxide levels mainly during oxaliplatin chemotherapy could be clinically important to the outcomes of colorectal cancer treatment.

1. Introduction

Biological role of superoxide dismutase manganese dependent enzyme (MnSOD/SOD2) in healthy cell metabolism is undisputable since, under oxidative stress conditions, this enzyme is considered as the first line of mitochondrial defense against oxidative damages by removing excessive superoxide anion (Kim, 2009; Montagner et al., 2010). However, the role of SOD2 cancer cells is not completely understood, and several conflicting results have been published in the literature including genetic investigations of some polymorphism such as Val16Ala-SOD2 (Oberley, 2005; Bresciani et al., 2015). In the first studies involving SOD2 and cancer performed during the 70s, Oberley and Buettner proposed that SOD2 could to be a tumoral suppressor molecule (Oberley et al., 1980). However, contrary evidence reinforced the dual nature of SOD2 in tumor development and progression, depending on cancer type and stage. In some cases, such as breast cancer, high levels of SOD2 genetically determined an increased risk of cancer development, whereas low SOD2 levels were found to stimulate the metastasis occurrence (Bica et al., 2009, 2010).

In colorectal cancer in particular, controversial results related to SOD2 enzyme have been reported. An investigation conducted by Janssen and colleagues showed that colorectal tumor tissues present up to four times more SOD2 than normal mucosa (Janssen et al., 1999). A more recent study conducted by Nozoe et al. revealed that high SOD2 content was associated with colorectal lymph node metastasis and with poor 5-year overall survival (Nozoe et al., 2003). Supporting these findings, some causal mechanisms associated with high SOD2 levels and colorectal cancer development and metastasis have been reported since. Authors that examined casual mechanisms suggested that SOD2 could induce the colorectal cancer surveillance by apoptosis inhibition from down-regulation of molecules, such as factor-related apoptosis-inducing ligant (TRAIL) (Mohr et al., 2008). In addition, SOD2 overexpression caused by down-regulation of microRNA-212 has been related to colorectal tumor metastasis (Meng

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et al., 2013). On the other hand, a study performed by Zhang and colleagues pointed toward a strong antitumoral effect in cells with SOD2-TRAIL genes obtained from a replication-competent recombinant adenovirus with E1B 55-kDa gene deletion (ZD55). According to the authors, the overexpression of SOD2-TRAIL genes promoted tumor suppression, as production of hydrogen peroxide derived from SOD2 activated caspase-8 triggering apoptosis events (Zhang et al., 2006).

These results make understanding of the role of SOD2 in colorectal cancer biology very difficult. As SOD2 continually dismutates superoxide anion in hydrogen peroxide, it is likely that the imbalance of this reaction could be the main factor in colorectal cancer regulation, and not just SOD2 enzyme regulation. This assumption is corroborate by a recent study that suggest that glutathione peroxide 1 gene (GPX1) may play a critical role in the development of colorectal cancer (Bus and Gibson, 1984; Deferme et al., 2015). In this case, evaluation of the superoxide-hydrogen imbalance effects on colorectal cancer using pharmacological drugs can be considered relevant. Therefore, we performed an in vitro analysis using HT-29 colorectal cell line exposed to two drugs (paraquat and porphyrin) that induce superoxide-hydrogen peroxide imbalance. Paraquat is an organic compound that interferes with electron transfer through the reduction in the number of donor electrons, such as NADPH in the mitochondria, increasing the superoxide anion levels. On the other hand, porphyrin is a SOD2 mimic molecule that increases the rate conversion of superoxide anion in hydrogen peroxide (Zhang et al., 2006; Bus and Gibson, 1984). We also tested the effect of this imbalance on the colorectal cell response to oxaliplatin chemotherapeutic drug in order to determine whether superoxide-hydrogen peroxide imbalance plays a role in cancer drug resistance.

2. Materials and methods

An in vitro investigation using human cancer colorectal HT-29 cell line (ATCC HTB38 — Rio de Janeiro Cell Bank, RJCB 0111, Brazil) was performed in controlled conditions. Cells were exposed to paraquat and porphyrin in order to generate a superoxide and hydrogen peroxide imbalance. Its effect on viability, apoptosis induction, cell proliferation, cell cycle modulation and expression of antioxidant and apoptotic genes was subsequently evaluated. The influence of this imbalance on oxaliplatin effect on HT-29 cells was concomitantly analyzed in our protocols.

2.1. Cell culture conditions

The cell cancer line was cultured in Dulbecco's Modified Eagle Media (DMEM) with 10% fetal bovine serum (FBS), supplemented with 1% penicillin/streptomycin and amphotericin B. The cells were cultured at 37 °C with 5% CO2 and were expanded by obtaining the optimal amount for the experiments. Cell suspension was placed in each of 96-well plates (2.5×10^5 cells/well). After cell attachment, the cultures were treated with paraquat, porphyrin and oxaliplatin. Further, the cells were incubated at 37 °C in humidified atmosphere with 5% CO2 for 24 and 72 h in order to produce the assays.

2.2. Viability and cell proliferation assay

The HT-29 viability and cellular proliferation were initially evaluated by MTT Assay. The supernatant of the treatments was removed and the cells were resuspended in phosphate buffer (PBS, 0.01 M; pH 7.4). The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolic bromide) reagent (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a 5 mg/mL PBS added into a 96-well plate containing the sample treatments and was incubated for 1 h at 37 °C. Next, the supernatant was removed from the wells and the cells were resuspended in 200 μL of DMSO (dimethyl sulfoxide). The absorbance at 560 nm was read in the fluorimeter (Barbisan et al., 2014).

2.3. Apoptosis and cell cycle assays by flow cytometry

Complementary analyses of flow cytometry were performed to confirm the treatment cytotoxicity using Annexin-V-FITC and Propidium Iodide (PI) staining kit (BD Pharmingen™). Annexin-V-FITC can detect early apoptotic process, whereas PI allows identifying late or necrotic apoptotic cells. The detection is realized through the bond the cell receptors form with these fluorescents antibodies, Annexin-V and PI. The analysis was performed following the manufacturer's instructions. Briefly, cells were seeded in 6-well plates at 1×10^6 cells per well in 2 mL of different treatments in DMEM and were incubated for 24 h. Following incubation, the cells were trypsinized in order to ensure that they are detached and were washed twice with cold PBS before being resuspended in 1X Binding Buffer at a concentration of 1×106 cells/mL. After transferring the cells into 100 μL of the solution (1×10^5 cells), the contents were placed into a 5 mL culture tube. The resuspended cells were subjected to gentle vortex and were stained with 5 μL of Annexin-V-FITC and 5 μL of PI. Following a short incubation (15 min) in the dark at room temperature, 400 μL of 1X Binding Buffer was added to each tube, and the cell florescence was analyzed by flow cytometry according to the manufacturer's specifications (Zhao et al., 2014).

The cell cycle analysis (William-Faltaos et al., 2006) was also performed using flow cytometry after 24 h and 72 h of HT-29 prior to being treated with paraquat, porphyrin and oxaliplatin. The PI reagent binds to the DNA by intercalating between the bases with little or no sequence preference. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. In our work, cells were seeded in 6-well plates at 5×10^4 cells per well in 2 mL of different treatments in DMEM and were incubated for 24 and 72 h. Following incubation, the cells were trypsinized, washed with PBS and resuspended in 70% ethanol. Further cells were stored at −20 °C overnight. Prior to conducting analyses, the cells were centrifuged and washed once with PBS, before being resuspended in 500 μL PI-solution in PBS: 50 μg/mL PI from 50× stock solution (2.5 mg/mL) 0.1 mg/mL RNase A 0.05% Triton X-100 and incubated for 40 min at 37 °C. Finally, 3 mL of PBS was added for washing and was resuspended in 500 μL PBS for flow analysis.

2.4. Quantitative real time RT-PCR (qRT-PCR) analysis

Total RNA was extracted using Trizol, following manufacturer's instructions (Ludwing-Biotec, Brazil). The extracted RNA was measured by Thermo Scientific NanoDrop™ 1000 Spectrophotometer at the 532 nm wavelength. For realizing the reverse transcription, RNA was used, whereby the samples of RNA (1 μg/mL) were added to 0.2 μL of DNAase (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37 °C for 5 min, followed by heating at 65 °C for 10 min. The cDNA was generated with 1 μL of Iscript cDNA and 4 μL of Mix Iscript (Bio-Rad Laboratories, Hercules, CA-USA). The reaction consisted of the following steps: heating at 25 °C for 5 min, at 42 °C for 30 min, and at 85 °C for 5 min, followed by incubation at 5 °C for 60 min. qRT-PCR was realized in the Passo One Plus (Applied Biosystems, Foster City, CA, EUA) with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) at 95 °C for 3 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The reactions of each sample were made in triplicate, using 0.5 μM of each primer and 2X Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) for the final volume of 15 μL. The Beta-acting gene played the housekeeping role (Pfaffl, 2001). The characteristics of the primers used are presented in the (Table 1).

2.5. Statistical analysis

The results were expressed as mean ± standard deviation (SD). All experiments were replicated tree times. To perform the statistical analysis we used mean ± standard error of each independent experiment.