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Original Article

Silencing peroxiredoxin-2 sensitizes human colorectal cancer cells to ionizing radiation and oxaliplatin

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ABSTRACT

Colorectal cancer (CRC) remains one of the leading causes of cancer-related death worldwide. Antioxidant enzymes decrease the generation of ionizing radiation (IR)-induced free radicals and therefore are associate to radioresistance. The main goal of this work is to study the involvement of peroxiredoxin-2 (Prx2) in the radio and chemoradiotherapy response in CRC cells *in vitro* and *in vivo*. We found that Prx2 oxidation state is associated to differential response to ionizing radiation in CRC cell lines. HCT116 radioresistant CRC cell line have lower ROS levels and a higher monomer/dimer Prx2 ratio, compared to halfway resistant Caco-2 and T84, and radiosensitive LoVo cell line. Constitutive and transient Prx2 silencing in CRC cells increase ROS levels, and most importantly, enhance *in vitro* radiation sensitivity. In addition, we showed that administration of IR plus oxaliplatin in down regulated Prx2 HCT116 cells has higher citotoxic effect than in control cells. Finally, radiosensitizing effect of Prx2 depletion was confirmed *in vivo*. These results suggest that Prx2 is an important component in tumoral radiation response, and their inhibition could improve radio and chemoradiotherapy protocols in patients with CRC.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females. Nowadays, despite significant advances in CRC treatment, it remains as one of the leading causes of cancer-related death worldwide [1]. Pre-surgical radiochemotherapy is commonly used to improve CRC treatments outcome; however, only a few percentage of patients have shown a complete response to this treatment [2,3]. Intrinsic cellular resistance is one of the most important constraints that yet remain to be solved and new therapeutic strategies should be explored to overcome this barrier. Oxidative stress generated by IR damages cells and induces the activation of antioxidant signal transduction pathways associated with radioresistant phenotypes [4]. Thus, antioxidant enzymes decrease the generation of IR-induced free radicals and therefore reduce radiation damage. This phenomenon has been established as one of the main mechanism of cancer radioresistance [5].

Peroxiredoxins (Prxs) enzymes constitute a family of antioxidants that consist of six isoforms (Prx1–Prx6) encoded by distinct genes in mammals, which are classified into three mechanistic subgroups (typical 2-Cys, atypical 2-Cys, and 1-Cys) [6]. All Prxs contain a conserved NH2-terminal cysteine residue that serves as the active site for catalysis and typical 2-Cys Prxs, (Prx1–Prx4) have an additional conserved C-terminal cysteine residue responsible for resolving the oxidized active site cysteine [7]. Prxs protect cells by removing constitutive levels of H_2O_2, hydroperoxides and peroxynitrite [8,9]. In this process, the conserved catalytic cysteine of typical 2-Cys, named peroxidatic cysteine (Cys–SPH) is oxidized to a cysteine sulfenic acid (Cys–SOH), which is attacked by the resolving cysteine (Cys–SRH) located in the C terminus of the other subunit [10]. This condensation reaction results in the formation of a stable inter-subunit disulfide bond [11]. This modification is completely reversible

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by the subsequent reduction that regenerates the active cysteine [12].

Prxs are up-regulated in many cancers, including cancers of the lung [13], thyroid [14], breast [15], and mesothelioma [16], suggesting a possible role of these enzymes in cancer cell maintenance. Particularly in CRC has been reported Prx2 overexpression in biopsies tumor tissue versus normal tissue [17]. So, here we studied the role of Prx2 in the intracellular ROS levels regulation and in the tumor response to IR and chemotherapeutic drugs as oxaliplatin (OXLP). We show that Prx2 knocking down through RNAi sensitizes CRC cells to IR and IR plus OXLP, in addition to the strong decrease of cell proliferation and tumorigenicity of this type of cells.

Materials and methods

Cell culture

Human CRC cell lines HCT116, Caco-2, T84 and LoVo were was kindly given by Dr Podhajcer (Fundación Instituto Leloir, Argentina). Cell lines were cultivated in complete Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Invitrogen Argentina SA) supplemented with 10% (vol/vol) fetal bovine serum (FBS, NatoCor, Argentina), 50 units/mL of penicillin G and 50 μg/mL of streptomycin sulfate, at 37 °C and at 5% (vol/vol) of CO_2 in humidified atmosphere. Subcultures were performed following standard procedures.

Prx2 small hairpin RNA (shRNA) transfection and isolation of clones expressing Prx2 shRNA

Prx2 specific shRNA (plasmid pRFP-shPrx2, TR310194) and the negative scrambled control (psh-control plasmid TR30015) were purchased from OriGene Technologies, Inc (USA). The targeted sequence of pRFP-shPrx2 was 5′-CTGACTTCAAGGCCACACGCGGTGGTTGAT-3′ and the control sequence does not share homology with any known sequences in the human genome.

HCT116 cells were seeded on 60-mm-culture plates and transfected either with 0.5 μg of pRFP-shPrx2 or psh-control plasmid at 24 h (70–80% confluence). Transfection was performed using DreamFectGold (Quiagen, USA), according to the manufacturer's guidelines. Cells were incubated for 48 h, and then were reseeded at low density (1/16 dilution) in complete medium, puromycin (1 μg/mL) was added as selection antibiotic. Prx2 knock-down was evaluated by Western Blot. The clone with the most decreased expression of Prx2 was selected and named as HCT116shPrx2. Control clone was named HCT116shc.

Prx2 small interfering RNA transfection

Prx2 small interfering RNA (Prx2 siRNA) was purchased from Ambion (USA). The sense strand nucleotide sequence for Prx2 siRNA was 5′-AGAUCAUCGCGUUCAGCAAtt-3′, and the anti-sense sequence was 5′-UUGCUGAACGCGAUGAUCUcg-3′. The negative control siRNA (Neg siRNA) sense sequence was 5′-CUUACGCUGAGUACUUCGAtt-3′, and the antisense sequence was 5′-UCGAAGUACUCAGCGUAAGtt-3'. The Neg siRNA does not share homology with any known sequences in the human genome. siRNA duplexes were transfected into HCT116 cells using DreamFectGold (Quiagen, USA) according to the manufacturer's guidelines and using magnetofection technique in Caco-2 and T84 cell lines as described elsewhere [18]. Prx2 knock-down was evaluated by Western blotting.

Clonogenic survival assay

Clonogenic survival assays were used to determine the radiosensitivity of cells transfected with Prx2 siRNA, Neg siRNA or without transfection. Exponentially growing cells were seeded at 800 cells per T25 culture flask, incubated for 12 h until were irradiated with γ-rays from a ^{137}Cs source (IBL-437C Irradiator; CIS BioInternational, CEBIRSA, Argentina) at a dose rate of 6 Gy/min. After 14 days, colonies were fixed with methanol/acetic acid solution 3:1 and stained with 1% of crystal violet in 25% methanol. The fraction of clonogenic cells was determined by scoring colonies containing ≥50 cells. Surviving fraction (SF) corresponding to each dose was determined as the number of colonies formed at the different doses divided the number of colonies formed at 0 Gy. Survival curves were fitted to the linear quadratic model $S = \exp - (\alpha D + \beta D^2)$. Clonogenic survival assays were used to determine the response of cells transfected with Prx2 or Neg siRNA to subsequent treatment with OXLP and IR. Before irradiation cells were incubated with the IC30 of OXLP (5 μM) for 2 h. Then the medium was changed and the irradiation was performed as described above.

Clonogenic survival assays were also used to determine the sensitivity of the clones HCT116shPrx2 and HCT116shc to H_2O_2. In this case, 12 h after cells seeded in 60-mm tissue culture plates cells were treated with 0–100 μM H_2O_2. Twenty-four hours following treatment, medium was change by fresh medium and the cells were maintained for 14 days. Colonies staining and surviving fraction determination was performed as described above.

Western blot analysis

Prx2 protein expression in CRC cells and their induction post irradiation with 2Gy was examined by Western blotting. Cells were grown to 70–80% confluence in 60-mm tissue culture plates, washed twice in phosphate buffered saline (PBS), lysed in RIPA buffer, stored 30 min on ice and then centrifuged for 10 min at 4 °C. Supernatants were collected and protein concentrations were determined using the Bio-Rad kit (Bio-Rad Laboratories). Cell lysates containing 20 μg proteins were mixed with sample buffer (250 mM Tris–HCl pH6.8, 8% SDS, 0.04% bromophenol blue, 40% glycerol and 20% β-mercaptoethanol) and heated for 5 min at 95 °C. They were then resolved by SDS-PAGE (10% polyacrylamide gels), and transferred to nitrocellulose membranes (Hybond ECL Membrane, Amersham Biosciences, GE Health Care) by electroblotting. The membranes were blotted with 5% nonfat milk, washed in Tris-Buffered saline Tween (TBS-T) and incubated with primary rabbit polyclonal antibodies anti-Prx-2 (1:5000) or mousse polyclonal anti-β-actin (1:2000) (Sigma–Aldrich, Argentina) overnight at 4 °C. The membranes were washed with TBS-T and then were incubated with anti-rabbit or anti-mousse horseradish peroxidase conjugated IgG (1:2000) for 60 min at RT. Fluorescence detection was conducted using the enhanced chemiluminescence detection system (ECL Western Blotting Substrate, Invitrogen) and the detector Bioespectrum® (UVP). Quantification was performed by densitometric analysis with NIH Image J software. To analyze the Prx2 thiol oxidative status in CRC cells, immunoblot assays were performed in the absence of β-mercaptoethanol reducing reagent.

Measurement of intracellular ROS

Intracellular ROS were detected using the cell-permeable probe DCFH-DA (Invitrogen, Argentina). Cells were incubated in 10 μM DCFH-DA for 20 min at 37 °C and were collected by trypsinization, washed three times with PBS and suspended in 2 ml of this buffer. Fluorescence was detected with a FACSCalibur (Becton Dickinson, San Diego, CA, USA).

Measurement of sulfhydryl groups

The sulfhydryl group reacts with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB; Ellman's reagent) and produces a yellow-pigmented 5-thio-2-nitrobenzoic acid (TNB). Measurement of the absorbance of TNB at 405 nm provides an accurate estimation of sulfhydryl groups in the sample. 50 μg protein sample of HCT116, HCT116shc and HCT116shPrx2 cells were placed in 96-well plates and brought to a final volume of 180 μl with reaction buffer (0.1 M sodium phosphate, 1 mM EDTA) and 20 μl of 10 mM DTNB (Thermo Scientific, Argentina). Plates were incubated for 15 min at RT and the absorbance was determined at 412 nm.

Measurement of cell growth and doubling time

Cell growth and proliferation were determined using a doubling time assay. Cells were seeded at 2×10^3 cells/well of a 96-wells plate, collected and counted every 24 h during 72 h. Doubling time was calculated as $dt = \log 2 \times t/(\log N - \log N_0)$, where dt = doubling time, t = time point of counting (h), N = number of cells counted, and N_0 = initial number of cells seeded.

Tumor xenograft experiments

Fifteen 5-week-old female athymic *nu/nu* BALB/c mice obtained from the animal facility of the National Atomic Energy Commission (Buenos Aires, Argentina) were divided into 3 groups as follows: control group (implanted with HCT116 cells), control shRNA group (implanted with HCT116shc cells), Prx2 shRNA group (implanted with HCT116shPrx2 cells). Tumor cells (1×10^7) were injected subcutaneously into each lateral flanks. Four days after implantation tumors reached approximately 4–5 mm in diameter and only right side tumors were irradiated. Animals were anesthetized with ketamine/xylazine (80–10 mg/kg and then 2Gy from a γ-rays source of 192Ir were applied (GammaMedplus, Varian Medical System, Instituto de Oncología Ángel H. Roffo). Tumor sizes were measured by an electronic caliper every three days and volumes were estimated as following equation tumor volume (mm3) = 0.5 × DM × (Dm) [2], where DM and Dm are the major and minor tumor diameters respectively. Forty days later, all mice were sacrificed. Animal care and experimental procedures were followed institutional guidelines approved by the National Institutes of Health.

Statistical analysis

GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Data are represented as means ± standard deviation (SD) for three independent experiments. Student's unpaired t test or two-way analysis of variance (ANOVA) was used to determine differences between groups, differences were considered significant at $p < 0.05$.