The effect on radioresistance of manganese superoxide dismutase in nasopharyngeal carcinoma

YAMING QU1, HUA ZHANG1, SUPING ZHAO1, JIDONG HONG2 and CANE TANG3

Departments of 1Otolaryngology Head and Neck Surgery and 2Radiation Oncology, and 3Central Laboratory of Medical Research, Xiang Ya Hospital, Central South University, Xiangya Road 87, 410008 Changsha, P.R. China

Received November 8, 2009; Accepted December 30, 2009

DOI: 10.3892/or_00000726

Abstract. Failure to control nasopharyngeal carcinomas (NPC) is mainly due to a portion of radioresistant phenotype. Identifying gene targets for radiosensitization is an important strategy in improving anticancer treatments. Exposure of cells to ionizing radiation leads to the formation of reactive oxygen species that are associated with radiation-induced cellular apoptosis and necrosis. The antioxidant enzyme manganese superoxide dismutase (SOD2) catalyzes the dismutation of the superoxide anions into hydrogen peroxide. We reasoned that SOD2 could contribute to the radioresistant phenotype in NPC cells. We compared CNE1 cells with CNE2 cells in radiation character and SOD2 protein. SOD2 gene silencing through the plasmid transfer using a microRNA interference optimized for transcription in NPC cell lines inhibited the radioresistance of human NPC cells. We compared radioresistant NPC with radiosensitive NPC in SOD2 expression. CNE1 cells and CNE2 cells demonstrated dose-modifying factors at 10% isosurvival of 1.529 and 1. CNE1 cells were 1.94-fold higher than CNE2 cells at SOD2 protein baseline, and CNE1 cells exposed to ionizing radiation demonstrated 1.15- to 1.39-fold increase in SOD2 immunoreactive protein. Radioresistance in CNE1 cells was reduced following expression of miRNA targeting SOD2. Radioresistant tumors (11/23) were SOD2-positive, and 2/46 radiosensitive tumors were SOD2-positive before commencement of radiotherapy. The results presented suggest that SOD2 expression can participate in radioresistance of NPC, being markers of a subset of tumors in which routine radiation treatment failure is likely. Combination of the SOD2 gene silencing therapy and conventional radiotherapy should be attempted to improved cancer therapy for NPC.

Introduction

The intrinsic radioresistance of the cells is among the possible reasons for the failure of radiation treatment to control the tumor. Improving the therapeutic ratio thus requires knowledge of the radiobiological factor that could influence tumor control by fractionated radiation treatment. Exposure of eukaryotic cells to ionizing radiation (IR) results in the formation of free radicals and in oxidative damage to biomolecules such as DNA, proteins and lipids that contribute to the biological effects of radiation (1-3). Manganese superoxide dismutase (MnSOD now known as SOD2) is a mitochondrial enzyme that catalyzes the dismutation of superoxide anion (O2•−) to form hydrogen peroxide (H2O2) and molecular oxygen (O2) (4). SOD2 works in concert with other antioxidant enzymes to defend cells against the damage associated with exposure to reactive oxygen species (ROS) (5) and is thought to be an important determinant of sensitivity to ROS-induced cytotoxicity. Scavenging the reactive species produced at the time of irradiation can mitigate some of the effects of IR-induced injury. Active SOD enzymes and SOD mimetic compounds can lead to inhibition of the deleterious effects induced by IR in a wide variety of in vitro and in vivo studies ranging from transformation assays, bystander effects, inflammatory responses and fibrosis (6-12). The protective effect of SOD2 against normal tissue damage caused by radiation is highlighted by in vivo experiments showing induction of SOD2 following radiation in the heart and gut (13,14) and the finding that overexpression of SOD2 in normal mouse epithelial tissues protected them from radiation injury (15).

Despite expression of endogenous SOD2 has been found to be reduced in many human cancer cells and transformed cell lines (16-18). Expression of SOD2 causes significant alterations in the malignant phenotype as well as inhibition of tumor growth in vivo (16,18,19). In many human tumor cells, SOD2 immunoreactive protein and activity are barely detectable in contrast to other AEs (CuZnSOD, CAT and GPx) (20). Therefore, increase of endogenous SOD2 could contribute to radioresistance seen in some tumor cells treated with radiation. A fraction of genes in the radiation-induced gene expression profiles in tumor cells represent genes that are regulated by SOD2 induction (8). Identifying SOD2 gene could therefore yield crucial insights into the genes in radioresistant phenotypes.
few studies have been undertaken to assess whether expression of SOD2 can be involved in the radioresistance of cancers and the results of these studies are contradictory (15,21-23). Yamaguchi et al found that endogenous SOD activity protects human leukemic and cancer cells from radiation. Veldwijk et al reported that the human superoxide-dismutase gene does not confer radioresistance on HeLa cervical carcinoma cells.

To determine in the present study if a causal relationship between SOD2 expression and radioresistance exists in human nasopharyngeal carcinoma (NPC) cells, gene expression of SOD2 in CNE1 cells was compared to that in CNE2 cells. Increased expression of SOD2 protein was detected in radioresistant CNE1 cells, relative to that in CNE2. The involvement of SOD2 in the IR-induced alterations in gene expression was confirmed by a lack of radiation responsiveness in radiosensitive CNE2 cells. Moreover, expression of miRNA for SOD2 inhibited the up-regulation of SOD2 in CNE1 cells, as well as inhibiting the radiation-resistant phenotype. Finally, SOD2 expression using immunohistochemical staining on paraffin tissue sections was associated with radioresistant NPC. These results provide strong evidence that SOD2 expression induces the radioresistant phenotype of human nasopharyngeal carcinoma following radiation exposure.

Materials and methods

Cell cultures. Human nasopharyngeal carcinoma cell lines CNE1 and CNE2 were purchased from Xiang Ya Central Experiment Laboratory, Central South University and utilized because relatively characterized radioresistance among NPC cell lines was available in these cell lines. Cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Longgreen Co., China) and non-essential amino acids. Cells were maintained at 37°C and 5% CO2 during all experiments.

Generation of miRNA expression vectors. The pcDNA™6.2-GW/EmGFP-miR-SOD2 plasmids were constructed by inserting a double-stranded oligonucleotide (ds oligo) encoding a pre-miRNA for SOD2 into pcDNA™6.2-GW/EmGFP-miR-SOD2 expression vectors described above using the cationic lipid-based Lipofectamine™ 2000 (Invitrogen Co.). Two days after transfection, cells harboring an expressing integrant were viewed by fluorescence microscopy based on GFP. The cells were transfected for 72 h, trypsinized, and cultured in the selecting medium with 4 μg of blasticidin/ml for 14 days. The transfected cells were cultured for at least two passages in blasticidin-free medium before the experiments.

Clonogenic survival after irradiation. Radioresistance was measured by clonogenic survival following exposure to IR. Control and pcDNA™6.2-GW/EmGFP-miR-neg control plasmid and pcDNA™6.2-GW/EmGFP-miR-SOD2 expression construct infected cells were trypsinized and plated in triplicate at cell population of 100, 100, 200, 400, 103, 104 and 105 per 3 cm2 dish respectively, then were irradiated with 0-8 Gy using medical linear accelerator. Irradiated cells were trypsinized and cloned in a 37°C incubator with 5% CO2 for 14 days. Clones containing more than 50 cells were scored as survivors, and the data were normalized to the appropriate sham-irradiated control group. Dose-modifying factors (DMFs) were calculated using the following expression: DMF = (dose to reach the specified survival in resistant cells)/ (dose to reach the same survival in the control). A linear quadratic equation was fit to the radiation dose response data using the programme DRFIT (24). The parameters obtained from the fitted data were α (a measure of the initial slope), β (a measure of the final slope), MID (mean inactivation dose, the area under the curve in linear coordinates), and SF2 (surviving fraction at 2 Gy).

Immunoblotting techniques. Wild-type CNE1, CNE1+ miR-neg and CNE1+ miR-SOD2 cells were cultured in 10% FBS-RPMI-1640, and total protein extracts were prepared. Equal quantities of sample protein were mixed with 40 μl of loading buffer, heated at 95°C for 10 min, size separated in 12% acrylamide SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membrane (PVDF, Millipore Co. USA). The membranes were then blocked at room temperature for 2 h in 5% non-fat dry milk solution, washed with 0.01% Tween-phosphate-buffered saline, and incubated overnight at 4°C with either of the following primary antibodies: goat anti-SOD2 at a dilution of 1:500; anti-ß-actin at a dilution of 1:400 (Santa Cruz Biotechnology, Santa Cruz, USA). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:500. Protein bands were visualized using the DAB detection kit (Maixin Biotechnology, Fuzhou, China).
Reverse transcription-PCR (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen Co.). After confirmation of the RNA integrity on an agarose gel, total RNA was digested using RNase-free DNase I for 20 min, extracted with phenol-chloroform, then precipitated with 2.5 volumes of ethanol. The cDNA was synthesized from 2 μg of total RNA using RevertAid™ M-MuLV reverse transcriptase and oligo(dT) (MBI Fermentas Life Sciences). For PCR, 5 μl of cDNA products was mixed with PCR buffer (Promega, Madison, WI) and 0.1 μM concentrations of human SOD2 PCR primers synthesized according to the sequence in GenBank. The primers sequences utilized in this study are as follows: SOD2 forward, 5'-CGACCTGCCCTACGACTA-3'; SOD2 reverse, 5'-ACCTGAGCCTTGGACACC-3' and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) forward, 5'-CAAGGTCATCCATGACAACTTTG-3'; GAPDH reverse, 5'-CAAGGTCACTGACAACTTTG-3'. Gene expression levels were estimated by densitometry.

Patients and immunohistochemical staining. Sixty-nine patients were studied from Department of Radiation Oncology, Xiang Ya Hospital between January 2005 and October 2006. These patients were aged ≤78 years, had histologically confirmed poorly-differentiated squamous cell in nasopharynx which was UICC stage II or III, and had not been previously treated. All patients were treated with 70 Gy using medical linear accelerator and were followed up >6 months. Formalin-fixed, paraffin-embedded tumor tissue was analyzed from 23 patients (18 males and 5 females) with uncontrolled local or regional tumor and 46 patients (36 males and 10 females) with well-controlled tumor. Immunohistochemical staining of SOD2 was performed on paraffin
sections using the streptavidin-biotin peroxidase method. Briefly, 6-mm sections were dewaxed and endogenous peroxidase was blocked by 20 min pretreatment with 0.5% H2O2 in 70% methanol. Following rehydration, non-immune goat serum (1:30) was used to block non-specific staining. The primary antibody used for SOD2 detection was BMS122 (Bender Co., Austria; dilution 1:100). Tissues were then incubated with biotinylated anti-rabbit IgG and avidin-biotin peroxidase complex (Maixin Biotechnology) in sequence, with 0.05% diaminobenzidine as substrate. Sections were lightly counterstained with Harris haematoxylin. Controls were a known positive specimen from a nasopharyngeal mucous membrane expressing SOD2, and negative controls included omitting the primary antibody and reacting sections with diluent buffer only and the use of an irrelevant rabbit antibody (IgG isotype) with each staining set. Staining was evaluated using a semi-quantitative method as follows: -, negative; ±, equivocal; +, strongly positive. Pathological assessment and all immune reactions were evaluated in a blinded manner by independent researchers who had no knowledge of the outcome of therapy for individual patients.

Results

Radioresistance of CNE1 and CNE2. To determine the radioresistance in CNE1 and CNE2 cells, clonogenic survival was determined, and the DMFs at 10% isosurvival were found to be 1.529 and 1 for CNE1 and CNE2 cells (relative to CNE2, Fig. 1A). Survival curve data were analyzed using a modified linear quadratic model the programme DRFIT. CNE1 cells demonstrated increased radioresistance as shown by a significant decrease in α, increase in MID and increase in SF2, compare to CNE2 cells (Fig. 1A, right schematic diagram). There was no significant difference in the β value.
These results are in agreement with previous reports of cellular radiobiological characteristics human NPC cell lines (25).

**Increased SOD2 in relatively radioresistant CNE1 cells.** Fig. 1 shows that, compared to CNE2 cells (Fig. 1B, line 2), levels of SOD2 expression were increased in CNE1 cells (lane 1). The SOD2 protein levels were estimated by densitometry and normalized to ß-actin (Fig. 1B, lower panel). CNE1 cells were 1.94-fold higher than CNE2 cells at SOD2 protein base line and radioresistance correlated to SOD2 expression levels. Enzyme activity detected by water-soluble tetrazolium salt microplate assay further showed that endogenous SOD2 activity was increased in CNE1 cells.

**Endogenous SOD2 expression in CNE1 and CNE2 cells exposed to IR.** By using the SOD2/ß-actin ratio (Fig. 1C) series doses or time of radiation 1, 2, 4 Gy or 6, 12, 24 h induced 1.15-fold to 1.39-fold increase in the level of SOD2 transcript in CNE1 cells, but not in CNE2 cells that constitutively demonstrated a 2-fold-greater level of endogenous SOD2 (Fig. 1C). These results indicate that the induction of SOD2 by radiation is more pronounced in CNE1 cells. These results suggest that the expression of SOD2 is linked to radiosensitivity.

**Expression of miRNA for SOD2 sensitized CNE1 cells to IR.** To determine if a causal relationship between SOD2 expression and radiosensitivity exists, CNE1 cells were transfected with the miRNA expression construct for RNAi targeting SOD2 (Fig. 2A). CNE1 cells stably transfected with pcDNATM6.2-GW/EmGFP-miR-SOD2 down-regulated the SOD2 mRNA levels and exhibited decreases in SOD2 immunoreactive protein (Fig. 2B). Cells from the parental line and those transfected with pcDNATM6.2-GW/EmGFP-miR-neg or pcDNATM6.2-GW/EmGFP-miR-SOD2 were irradiated with dose ranging from 0-8 Gy, clonogenic survival was determined. The results (Fig. 2C) show that expression of miRNA for SOD2 inhibited resistance to radiation-induced cell kill, as shown by DMFs of 0.435 and 1.043 for CNE1+miR-SOD2 and CNE1+miR-neg cells, respectively, at 10% isosurvival (relative to CNE1). Survival curve data were analyzed using a modified linear quadratic model the programme DRFIT. CNE1 cells transduced with miR-SOD2 demonstrated decreased radiosensitization as shown by a significant increase in α, decrease in MID and decrease in SF2 (Fig. 2C, right schematic diagram). There was no significant difference in the β value. Fig. 2B shows reduced expression of SOD2 mRNA and protein in CNE1+miR-SOD2 cell line. These observations support the hypothesis that SOD2 expression regulates a portion of the radioresistant phenotype.

**Increased expression of SOD2 in radioresistant NPC tissues.** The following variables, staging of tumor, sex and age, and treatment-planning method were not significantly different in the radioresistant tumor group and the radiosensitive tumor group. SOD2 expression was cytoplasmic. Heterogeneity was observed both in the percentage of cells positive and in the level of expression of protein: 11/23 (47.8%) radioresistant tumors were SOD2-positive and 12/23 (52.2%) were SOD2-negative; 2/46 (4.5%) radiosensitive tumors were SOD2-positive and 44/46 (95.5%) were SOD2-negative before commencement of radiotherapy. Associations of expression of SOD2 protein with patient and tumor characteristics for radioresistance are shown in Table I.

**Discussion**

Carcinoma of the nasopharynx is prevalent in the South China region (26). The most effective means of treatment of NPC is generally radiation therapy (27,28). Patients with NPC often have to face deleterious treatment consequences as the price of cure (29); the therapeutic ratio of routine fractionated radiation, however, has not improved over several decades and there is plenty of room for improvement of the treatment of NPC in China (30). The 5-year survival rate of
nasopharyngeal carcinomas is about 50% overall (31) and failure to control the cancer is mainly due to a portion of radioreistant phenotype of NPC. Hence, therapeutic efficacy can be improved by devising an approach that will increase the response of the tumor relative to that of the surrounding normal tissues (32). The approach involves exploiting biological factors that result in differences in the response of tumors and normal tissues to radiation therapy. Thanks to the advancements in molecular medicine, specific treatment to the potential target using technologies such as immunotherapy and RNAi becomes a potential genetic marker of radiosensitivity discovery more meaningful for NPC management.

The mechanisms causing resistance to radiotherapy in NPC are poorly understood. However, identifying gene targets for radiosensitization is an important strategy in improving anticancer treatments (33-35). Exposure of cells to ionizing radiation leads to the formation of reactive oxygen species that are associated with radiation-induced cytotoxicity. The antioxidant enzyme manganese superoxide dismutase (SOD2) catalyzes the dismutation of the superoxide anions into hydrogen peroxide, to enhance the radioresistance of a mammalian cell. A number of studies in other body sites have shown cellular resistance to radiation-induced DNA-damaging in the overexpression of SOD2 (36). But the results of some studies are contradictory (7,15). SOD2 may alter radioresistance via gene regulation. However, SOD2-mediated induction of radioresistance may be species and cell line-dependent (8).

The present study provides the first evidence that SOD2 expression regulates a portion of the radioreistant phenotype in NPC cell lines. Indirect evidence in support of this conclusion includes the fact that CNE1 with relatively increased expression of SOD2 showed significant radiosresistance to CNE2 with relatively decreased SOD2 expression, and endogenous SOD2 was induced in irradiated CNE1 cells. Further support was obtained when expression of SOD2 protein induced in CNE1 cells was also found to be reduced in CNE2 cells with low SOD2 protein base line following irradiation. A causal relationship between SOD2 expression and increased radiation resistance in NPC cell line was further suggested by experiments showing that expression of miRNA for RNA interference with SOD2 down-regulated CNE1 cells. We have shown that enforced expression of miRNA for SOD2 in CNE1 cells significantly decreases radioresistance. SOD2 was found to be up-regulated in CNE1 cells, and the protein level was down-regulated following expression of miRNA for SOD2 in CNE1 cells during the same time that radiosensitization was occurring (Figs. 1 and 2). Therefore, we hypothesize that SOD2 may play a specific role in radioresistance relevant to nasopharyngeal carcinoma cell lines. Finally, in the study of 69 nasopharyngeal carcinomas, we have demonstrated that incidence of SOD2 immunostain in the radioresistant group was significantly higher than in the radiosensitive group. Expression of SOD2 protein may contribute to the radioresistance of some nasopharyngeal carcinomas. The present results provide evidence for SOD2 expression participating in radioresistance.

Although the mechanism by which SOD2 impacts resistance to ionizing radiation is currently unknown, SOD2 overexpression has been suggested by other studies to alter the intracellular redox environment via changes in mitochondrial hydroperoxide production (37), initiating downstream signaling cascades and transcription factors that control the stress-responsive signaling pathways (38). The stress-signaling proteins responsive to SOD2 expression have been correlated to the signaling pathways governing radiation response and radiosensitivity. Down-regulated cyclin B1 increases radiosensitization (39); 14-3-3 proteins are phosphoserine-binding molecules with a primary function of inhibiting apoptosis (40); c-Rel controled SOD2 was found to play a key role in antiapoptosis (41); E2F1 and c-Myc appear to potentiate apoptosis through inhibition of NF-κB activity that facilitates SOD2-mediated ROS elimination (42). A pathway activated by SOD2 to effector genes with antiapoptotic function is a contributor to radiation-induced adaptive responses (8) that results in enhanced tolerance to the subsequent cytotoxicity of ionizing radiation. In addition, modifying intracellular or extracellular SOD activity reduce the oxygen enhancement ratio leading to the effects of oxygen on radiosensitization (1). SOD2 with superoxide dismutation capability following radiation could result in radioprotection.

Our study suggests that the levels of SOD2 expression, which can be rapidly applied, are important markers of a subset of tumors (and thus of patients) in which fractionated radiation treatment failure is likely. Further work is clearly required for a complete understanding of what extent of SOD2 expression are responsible. In this regard, several authors have recently reported that overexpression of SOD2 may result in protecting normal tissues from radiation injury (6,14,18), and endogenous SOD activity is also involved in protecting human leukemic and cancer cells from radiation (21), confirming that detailed analysis of SOD2 gene expression and their biological functions should be considered (43). If future studies confirm that SOD2 expression are associated with a tumor residual or confer a radioresistance when a particular radiation treatment-planning method is used, then identifying patients with tumors that carry increased SOD2 may improve treatment selection. Furthermore, regulating the expression of SOD2 in vitro and in mouse model has improved the sensitization of cell lines and experimental tumors to radiation therapy, or the protection of the hematopoietic compartment and normal tissues from the effects of ionizing radiation, demonstrating the effectiveness of a combination of gene therapy and conventional radiotherapy (10,15,44-46). Accurate detection of SOD2 status prior to therapy will also allow SOD2 inhibitors or radiosensitizers to be used in clinical trials in NPC to reduce failure from radiotherapy. Our findings therefore have important therapeutic implications for the future in moving towards an improved cancer therapy for NPC.

Acknowledgements
We thank Dr Xinzhang Cai for insightful advice and Mr. Chenglong Wang for technical assistance.

References


