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Original Contribution

MnSOD drives neuroendocrine differentiation, androgen independence, and cell survival in prostate cancer cells

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ABSTRACT

An increase in neuroendocrine (NE) cell number has been associated with progression of prostate tumor, one of the most frequent cancers among Western males. We previously reported that mitochondrial manganese superoxide dismutase (MnSOD) increases during the NE differentiation process. The goal of this study was to find whether MnSOD up-regulation is enough to induce NE differentiation. Several human prostate cancer LNCaP cell clones stably overexpressing MnSOD were characterized and two were selected (MnSOD-S4 and MnSOD-S12). MnSOD overexpression induces NE morphological features as well as coexpression of the NE marker synaptophysin. Both MnSOD clones exhibit lower superoxide levels and higher H_2O_2 levels. MnSOD-overexpressing cells show higher proliferation rates in complete medium, but in steroid-free medium MnSOD-S12 cells are still capable of proliferation. MnSOD up-regulation decreases androgen receptor and prevents its nuclear translocation. MnSOD also induces up-regulation of Bcl-2 and prevents docetaxel-, etoposide-, or TNF-induced cell death. Finally, MnSOD-overexpressing cells enhance growth of androgen-independent PC-3 cells but reduce growth of androgen-dependent cells. These results indicate that redox modulation caused by MnSOD overexpression explains most NE-like features, including morphological changes, NE marker expression, androgen independence, inhibition of apoptosis, and enhancement of cell growth. Many of these events can be associated with the androgen dependent–independent transition during prostate cancer progression.

Mitochondria are the main source of reactive oxygen species (ROS) inside the cell. Mitochondrial superoxide dismutase (MnSOD/SOD2) is one of the most important antioxidant enzymes. This enzyme scavenges superoxide radicals normally produced through the respiratory electron transport chain. The rate of superoxide radical production inside mitochondria is the most important among all the naturally occurring free radical sources in the cell. MnSOD's subcellular location, allowing it to scavenge the main radical in the cell at the same place where it is produced, gives this enzyme an essential role in the living cell. A clear example is the early death of newborn mice of MnSOD knockout lineages due to multiorgan failure [1].

The role of oxidative stress in tumor initiation and promotion is well accepted [2]. Several aspects of MnSOD, such as genetic variations [3], expression, and activity, have been studied in vitro [4,5] and in vivo in several kinds of tumors [6,7]. One of these works showed a complex behavior of MnSOD protein in tumor initiation and progression, and an imbalance between MnSOD and catalase in lung tumor patient samples was reported [8]. Similarly, in pancreatic tumors, in which a decrease in antioxidant enzymes as shown by immunohistochemistry was described [9], it was also observed that metastatic sites and ascites showed significantly increased levels of MnSOD protein and activity in mouse models [6]. Additionally, in several reports, it MnSOD overexpression was implicated in the resistance to anti-cancer therapies [10], especially radiotherapy [11–13].

In prostate adenocarcinoma, one of the most common malignancies in the Western world, two clear phases appear during illness development. The first stage is characterized by an androgen-dependent state, which is frequently easy to treat with either surgical or androgen deprivation strategies. However, after a lapse of 1–5 years the tumor reappears in a second androgen-independent stage, showing high resistance to radio- and chemotherapy. Neuroendocrine (NE) differentiation (NEd) is a process observed during the progression of prostate and other tumor types. Usually, cells appear in the tumor showing features similar to NE cells from the normal plexus and they are called NE-like cells [14]. NE and NE-like cells display a characteristic neurite-containing morphology; they are positive for NE markers such as synaptophysin (Syn) or neuron-specific enolase (NSE) and they show a high resistance to apoptosis and androgen-independent behavior. Furthermore, they produce an array of several bioactive molecules (e.g., bombesin, serotonin) with mitogenic properties. Androgen deprivation

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promotes NEd in patient samples [15] and has been proposed as an intermediate step between the androgen-dependent and the androgen-independent stage [16]. Several strategies are able to induce NEd in prostate cancer cells in culture, including treatment with interleukin-6 [17], cyclic adenosine monophosphate (cAMP) [18], melatonin [19], or silibinin [20] or androgen deprivation [21], among others. In a previous report our group showed that an increase in MnSOD protein levels and activity was associated with the NE-like differentiation process in vitro using melatonin, androgen deprivation, or cAMP [22]. The aim of the present study was to clarify the molecular mechanisms by which MnSOD promotes NE differentiation.

Materials and methods

Reagents

Protease inhibitors were all obtained from Sigma (St. Louis, MO, USA), as well as Triton X-100, Hepes, and EDTA. Tumor necrosis factor α (TNF-α) was purchased from R&D Systems (Minneapolis, MN, USA). Etoposide, docetaxel (Fluka), and reagents for the enzymatic determination of glutathione (GSH) were all obtained from Sigma. Hoechst 33258 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for proliferation and viability assays were also purchased from Sigma.

Cell culture

LNCaP cells, a human prostate androgen-dependent epithelial adenocarcinoma cell line, were purchased from ECACC (Salisbury, UK) and were grown in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), ampicillin, kanamycin, and L-glutamine (Gibco, Invitrogen, Carlsbad, CA, USA). All experiments were performed between passages 10 and 20. PC-3 cells are a human androgen-independent adenocarcinoma cell line. PC-3 cells were purchased from ATCC (Manassas, VA, USA) and were grown in DMEM/F12 medium (Lonza) supplemented with 10% FBS, antibiotic–antimycotic cocktail, and L-glutamine (Gibco, Invitrogen). All experiments were performed between passages 15 and 30. Both cell lines were grown at 37 °C in a humidified 5% CO_2 environment.

MnSOD overexpression

Transfection was carried out on LNCaP cells from passages 5–12, when they were at a confluency of 60–70%. Lipofectamine 2000 transfection reagent (Invitrogen) was used according to the manufacturer's instructions for 4 h. Transfected cells were selected using 300 μg/ml G418 (Sigma) as the selective antibiotic for 7 passages. MnSOD expression was tested by immunoblotting. The plasmid expression vector pcDNA3 containing the human MnSOD cDNA sequence, as well as the corresponding empty vector, was kindly provided by Dr. Wenqing Sun (University of Iowa, Iowa City, IA, USA). All experiments were done on transfected cells between passages 8 and 15 (equivalent to 18 and 25 passages in LNCaP cells).

Fluorescence microscopy

For phalloidin staining, cells were grown on Thermanox coverslips, washed twice with phosphate-buffered saline (PBS), and then fixed in 3% paraformaldehyde for 24 h at 4 °C. For visualization, cells were incubated with phalloidin–TRITC conjugate (1 μg/ml) for 90 min at room temperature and counterstained with DAPI (5 ng/ml). Coverslips were finally mounted using Fluoromount G and observed with a Nikon Eclipse 80i using the appropriate fluorescence filters. Micrographs were taken with a Nikon DS-L1 digital camera.

Immunoblotting

After being treated, the cells were washed with ice-cold PBS and lysed in RIPA lysis buffer containing 1 mM dithiothreitol and the protease inhibitors 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin and 200 μM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride.

For cytoplasm and nuclear protein extraction, cells were collected by brief trypsinization and washed in PBS. After a first wash in buffer A (10 mM Hepes–KOH, 2 mM $MgCl_2$, 15 mM NaCl, 0.1 mM EDTA), cells were then lysed with buffer A containing protease inhibitors, including 0.2% Igepal CA 630 (Fluka, Sigma). Cytoplasm lysates were centrifuged at 15,000 *g* and then buffer B (420 mM NaCl, 20 mM Hepes–KOH, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol) and buffer C (50 mM KCl, 20 mM Hepes, 0.2 mM EDTA, and 20% glycerol) were added. Nuclear extracts were collected after centrifugation at 15,000 *g* and protein from both fractions was quantified using Bradford reagent.

Total protein lysates were clarified by centrifugation at 13,000 *g* for 15 min at 4 °C and subjected to SDS–PAGE and electrophoretic transfer to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The following antibodies were used: anti-MnSOD (Calbiochem, Merck Chemicals Ltd, Nottingham, UK), anti-CuZnSOD (Calbiochem), anti-NSE (Neomarkers, Thermo Scientific, Fremont, CA, USA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA), proliferation cell nuclear antigen (PCNA; Santa Cruz Biotechnology), survivin (Santa Cruz Biotechnology), Bcl-2 (Calbiochem, San Diego, CA), glutathione peroxidase (Gpx; Abcam, Cambridge, UK), catalase (CAT; Calbiochem), androgen receptor (AR; Abcam), prostate-specific antigen (PSA; Neomarkers), and histone deacetylase 1 (HDAC2; Santa Cruz Biotechnology). Primary antibodies were visualized by binding of horseradish peroxidase-conjugated anti-sheep (Calbiochem), anti-mouse (Calbiochem), or anti-rabbit (Cell Signaling, Danvers, MA, USA) secondary antibodies and detected with chemiluminescence substrate (Millipore). Blots were scanned (300 dpi gray scale) and densitometry was quantified using software downloaded from www.scioncorp.com and following the instructions provided by the company.

Morphological studies

Five micrographs of each experimental group were taken at a final magnification of 200×. At least 30 cells were observed in each field. Total neurites and number of neurites larger than twice the cell body length were counted. Total neurite number was compared to the total number of cells. The number of neurites larger than twice the length of the cell body was expressed as an average of each field (%).

Native gel SOD activity assay

Protein samples for SOD assay were obtained by lysing cells using freeze–thaw cycles in PBS. Protein extracts were cleaned by centrifugation (13,000 *g*) and quantified by Bradford assay. Briefly, equal amounts of total cellular protein extracts were separated by 12.5% native gel electrophoresis, and SOD activity was determined by incubating gels with nitroblue tetrazolium (2.43 mM) and riboflavin–Temed (2.8×10^{-5} M riboflavin and 28 mM Temed). A parallel set of samples was run using 5 mM potassium cyanide to specifically inhibit CuZnSOD activity to assay MnSOD activity exclusively in the gel.

Immunocytochemistry

LNCaP cells were seeded on cell-culture-treated glass coverslips (Thermanox provided by Nunc, Roskilde, Denmark), fixed in 1% formaldehyde–PBS, and permeabilized with 0.1% Triton X-100/PBS. Anti-AR antibody (Abcam) was employed at a final dilution 1:300 in TBS-T(Tris Buffer Saline-Tween 20). Anti-rabbit secondary antibody