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Role of superoxide anion on the proliferation and c-Ha-*ras* or p53 expression in prostate cancer cell line PC3

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Abstract The purpose of this study was to investigate the role of superoxide anion ($O_2^{-\cdot}$) in the regulation of p53 or c-Ha-ras expression and proliferation in the prostate cancer cell line PC3. Cell proliferation was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in the presence of $O_2^{-\cdot}$, basic fibroblast growth factor (bFGF) or their combination. p53 or C-Ha-*ras* expression in the cells treated with $O_2^{-\cdot}$ was assayed by fluorescence in situ hybridization (FISH). The proliferation was significantly inhibited by $O_2^{-\cdot}$ in a concentration-dependent manner ranging from 9 to 36 μmol/l nicotinamide adenine dinucleotid (NADH) combined with 2–8 μmol/l *N*-methylphenazonium methyl sulfate (PMS). Enhancement of proliferation by 2 ng/ml bFGF was significantly inhibited by $O_2^{-\cdot}$. Although $O_2^{-\cdot}$ was not able to alter c-Ha-*ras* gene expression, $O_2^{-\cdot}$ at the concentrations of 18 μmol/l NADH and 4 μmol/l PMS upregulated the expression of p53. $O_2^{-\cdot}$ may modulate proliferation and gene expression in PC3 cells.

Key words Superoxide anion · Proliferation · Gene expression · p53 · c-Ha-*ras* · Prostate cancer cell

Introduction

Reactive oxygen species (ROS) involved in the regulation of cellular proliferation and gene expression could act as a biological messenger rather than a mediator of cellular damage (23). The modulation of ROS on cells depends on relative concentrations of exogenous superoxide or hydrogen peroxide in relation to the numbers of cells exposed as well as different cell types [3]. The growth response to low concentrations of hydrogen peroxide are positive in BHK-21, 208F, Hela and mouse fibroblastic cells Baib 3T3 [19], but in certain situations they can be negative in mouse osteoblastic cells MC3t3-E1 [16]. Tyrosine phosphorylation of three protein bands were stimulated in the presence of a nitric oxide generating system and epidermal growth factor [17], indicating that ROS might modulate the action of cytokine.

The elucidation of the pathogenesis of prostate cancer, therefore, would contribute to the understanding of the mechanisms of development, although detailed mechanisms of prostate cancer are as yet unknown and the relationship of ROS and development of prostate cancer is not completely clear.

Oncogene expression and androgen are crucial in the growth of the prostate cancer. Basic FGF (bFGF) which may be involved in malignant prostate cellular proliferation [8] is one member of the FGF family [1]. ROS supplied exogenously can reverse the effect of bFGF [12]. In addition, transfection of transformed cells with wild-type p53 results in arrest in the G1 phase of the cell cycle or induction of apoptosis [7, 29]. Soini [20] saw immunohistochemically detectable p53 protein in only 6% of fixed radical prostatectomy specimens. Moreover, the *ras* family of proto-oncogenes is believed to play a role in the transduction and processing of growth factor signals and consists of three different genes (Ki-*ras*, N-*ras*, Ha-*ras*) that encode three highly related 21 000 MW peptides (designated *ras* p21) (4). Immunostaining for *ras* p21 in prostate tissues was specific for adenocarcinoma suggesting a correlation of p21 with prostate cancer (27). Whether ROS may modulate expression of p53 or p21 genes and proliferation induced by bFGF in prostate cancer cell is rarely reported.

This study was undertaken to investigate the role of superoxide anion ($O_2^{-\cdot}$) in the regulation of p53 or c-Ha-*ras* expression in the prostate cancer cell line PC3. In

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addition proliferation of the PC3 cell was also assayed in the presence of $O_2^{-\cdot}$, bFGF or their combination.

Materials and methods

Chemicals

Catalase (Cat) was purchased from Institute of Xiahe Bioproducts (China). *N*-methylphenazonium methyl sulfate (PMS) and nicotinamide adenine dinucleotid (NADH) were provided by Fluka (Swizerland). RPMI 1640 medium was obtained from GIBCO. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), proteinase K and L-glutamine were obtained from Sigma. Fetal calf serum (FCS), formamide, dextran sulfate, diethylenetriaminepentaacetic acid (DETAPA), sodium formate, probe (cDNA, xba^I; 1.8 Kb) of human wild p53 and probe (cDNA, $EcoR^I$, 0.8 Kb) of human wild p53 were purchased from Sino-American Biotechnology Company (China). Fluorescein isothiocyanata (FITC) labeling p53 or c-Ha-*ras* probes were kindly prepared by the Institute of Hematopathy at Beijing Medical University and the specificity was determined by negative control (no staining in rabbit lymphocytes) and an immunocytochemisty test which is consistent with fluorescence in situ hybridization (FISH) result using the probes.

Tumour cells

Human prostate cancer cell line PC3 was kindly provided by the Institute of Urology, Beijing Medical University. The cells were maintained in complete medium (CM) which consists of RPMI 1640, 100 U/ml penicillin, 100 U/ml streptomycin, 50 U/ml gentamycin, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and 15% heat-inactivated FCS at 37°C in 5% CO_2 air enviroment. Tumour cells were harvested by overlaying the monolayer with a solution of 0.05% trypsin (Sigma) and 0.53 mmol/l EDTA and then resuspended in CM. The cells were placed in 96-well plates at 3×10^3 cells per well in 150 µl CM for proliferation assay.

Generation of $O_2^{-\cdot}$

The $O_2^{-\cdot}$ was generated in the CM supplemented with 2–36 µmol/l NADH and 0.5–8 µmol/l PMS plus 426 units Cat, 0.1 mmol/l DETAPA and 0.4 mmol/l sodium formate in order to generate $O_2^{-\cdot}$ only.

Proliferation assays

The proliferation of the PC3 cell was determined by MTT assay [15]. The medium containing PC3 cells in 96-well plates for 48 h was replaced by 250 µl test medium which comprised CM supplemented with different concentrations of bFGF (0–4 ng/ml), $O_2^{-\cdot}$ (CM supplemented with 0–36 µmol/l NADH and 0–8 µmol/l PMS) and their combinations. After culture for 48 h, the medium was removed and the plates were washed with RPMI 1640 and 2% FCS. RPMI 1640 medium containing 0.5% MTT and 0.5% FCS was then added in each well. After incubation for 4 h, the medium was replaced by 100 µl DMSO (Sigma). The absorbance (A) at 570 nm in each well was determined by using a microplate autoreader (Nanjing, China). The proliferation assay was totally repeated four times, each for four wells.

Fluorescence in situ hybridization

The PC3 cells treated with $O_2^{-\cdot}$ (CM supplemented with 18 µmol/l NADH and 4 µmol/l PMS) for 48 h were harvested by overlaying the monolayer with a solution of 0.05% trypsin (Sigma) and 0.53 mmol/l EDTA and washed with Hanks solution three times. The cells were then resuspended in 70% ethanol and stored at −20°C until further use.

The cells were added to the slides treated with 0.1% gelatin and 0.01% chrome alum. The slides were incubated at 70°C for 1 h and then treated with a methanol/ethanol solution (3:1) at 0 C for 10 min, 70% acetic acid for 20 s, and washed with water and treated with degradation ethanol. After digestion with proteinase K solution (0.9 µg/ml proteinase K in 20 mmol/l TRIS-HCl, pH 7.5) for 15 min and washing with PBS three times, the slides were placed in 4% paraformaldehyde for 5 min followed by washing with PBS and 2 × SSC, incubation in denatured solution, which contained 70% formamide, 50% dextran sulfate and 500 ng/µl herring sperm DNA (HSDNA) in 2 × SSC at 80°C for 5 min, then in ice, and finally in degration ethanol. Hybridization was carried out in 50% formamide, 10% dextran sulfate, 500 ng/µl HSDNA, 10 ng/µl FITC labeling probe of human wild p53 or human probe of c-Ha-*ras* probes in 2 × SSC at 42°C overnight. Avidin D-FITC (20 µl) was added to the slides which were further incubated at 37°C for 30 min and then washed in PBS three times. 20 µl Biotin (20 µl) combined with antiavidin D antibody were added and the slides were incubated at 37°C for 30 min followed by washing with PBS, incubation with avidin D-FITC for 30 min and washing with PBS again. The slides were counterstained with 0.1 mg/l propidium iodide and observed under fluorescence microscopy (Olympus, bandpass filter BG-12, 435 nm) and positive cells, in which there were reddish orange granules, were counted (Fig. 1). The slides used sense probes or slides whose probes were omitted as negative controls. There were no false positive signals under these conditions. The test was performed four times.

Statistical analysis

Statistical analysis of cell numbers was performed by analysis of variance. The statistical analysis of gene expression was carried out by using Wilcoxon's U test.

Results

Effect of $O_2^{-\cdot}$ on proliferation of the prostate cancer cell line PC3

The proliferation of PC3 cell was significantly inhibited by $O_2^{-\cdot}$ in a concentration-dependent manner ranging from 9 to 36 µmol/l NADH and 2 to 8 µmol/l PMS (Fig. 2).

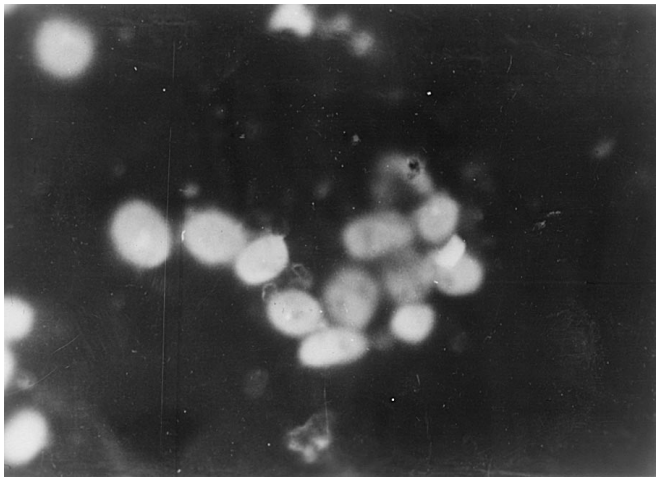

Fig. 1 p53 gene expression in PC3 cells treated with $O_2^{-\cdot}$ at the concentration of 18 µmol/l NADH and 4 µmol/l PMS. Fluorescence in situ hybridization technique, counterstained with propidium iodide and observed under fluorescence microscopy, original magnification ×400

The inhibition of $O_2^{-\cdot}$ on the proliferation of PC3 cells induced by bFGF

Enhancement of proliferation of PC3 cells treated with 2 ng/ml bFGF was observed (Fig. 3, $P < 0.05$). The cell proliferation induced by bFGF was significantly inhibited by $O_2^{-\cdot}$ in a concentration-dependent manner ranging from 9 to 36 μmol/l NADH and 2 to 8 μmol/l PMS (Fig. 4).

Expression of p53 and c-Ha-*ras* of PC3 cells treated with $O_2^{-\cdot}$

Although $O_2^{-\cdot}$ was not able to alter c-Ha-*ras* gene expression, $O_2^{-\cdot}$ at the concentrations of 18 μmol/l NADH and 4 μmol/l PMS upregulated the expression of p53 (Fig. 5).

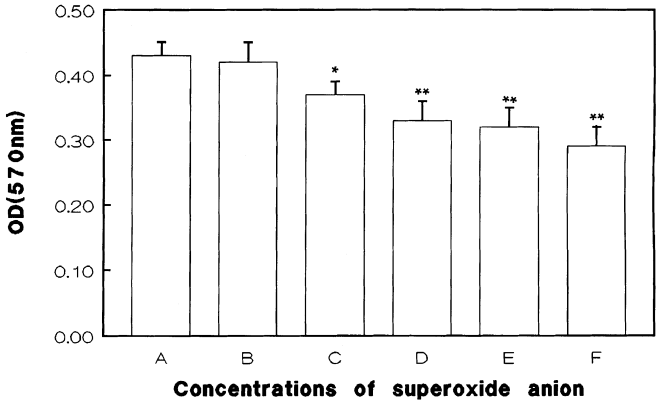

Fig. 2 The inhibition of proliferation of PC3 cell by $O_2^{-\cdot}$. PC3 cells were cultured in CM supplemented with 0 μmol/l NADH and 0 μmol/l PMS (*A*), 2 μmol/l NADH and 0.5 μmol/l PMS (*B*), 4 μmol/l NADH and 1 μmol/l PMS (*C*), 9 μmol/l NADH and 2 μmol/l PMS (*D*), 18 μmol/l NADH and 4 μmol/l PMS (*E*), 36 μmol/l NADH and 8 μmol/l PMS (*F*). Each group contained four wells and the experiment was repeated four times ($n = 4$). $*P < 0.05$, $**P < 0.01$ compared with *A*

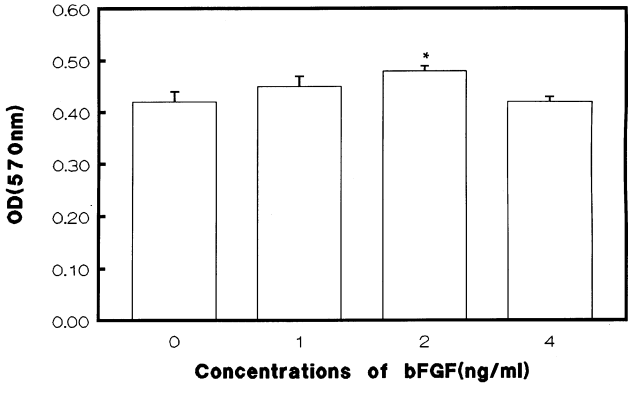

Fig. 3 Enhancement of proliferation of PC3 cells treated with bFGF. Each concentration group contained four wells and this experiment was repeated four times ($n = 4$). $*P < 0.05$ compared with control

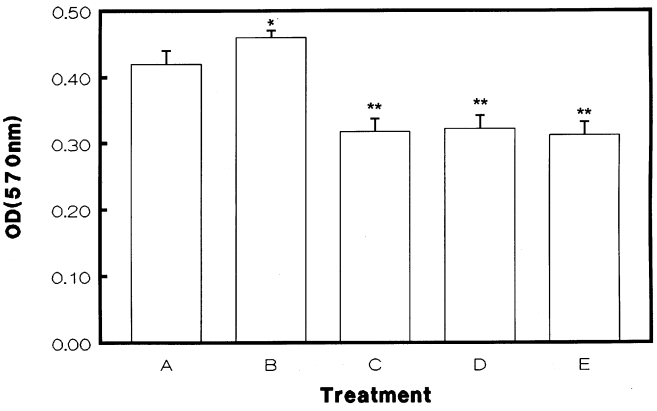

Fig. 4 Effect of superoxide anion on the proliferation of a PC3 cell line induced by bFGF. The PC3 cells were treated with CM (*A*), CM containing 2 ng/ml bFGF (*B*), CM containing 2 ng/ml bFGF plus 36 μmol/l NADH and 8 μmol/l PMS (*C*), CM containing 2 ng/ml bFGF plus 18 μmol/l NADH and 4 μmol/l PMS (*D*), CM containing 2 ng/ml bFGF plus 9 μmol/l NADH and 2 μmol/l PMS (*E*). Each concentration group contained four wells and the experiment was repeated four times ($n = 4$). $*P < 0.05$, $**P < 0.01$ compared with *A*

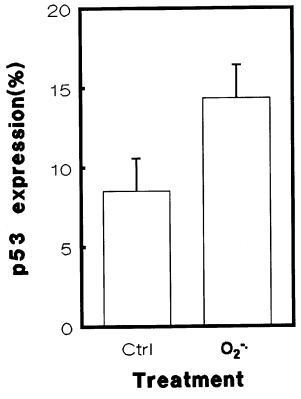

Fig. 5 Regulation of p53 expression in PC3 cells by superoxide anion. The PC3 cells treated with $O_2^{-\cdot}$ (CM supplemented with 18 μmol/l NADH and 4 μmol/l PMS) for 48 h. The percentage of p53 positive expression cells was assayed. The experiments were performed four times

Discussion

Superoxide or hydrogen peroxide are the main kinds of ROS, which derive from activated neutrophils and monocytes, and play an important role in immunocytes-mediated tumor cell damage [18, 28]. In addition, high levels of hydrogen peroxide are constitutively released from a wide range of human tumor cells [24]. The modulation of ROS on cells depends on relative concentrations of exogenous superoxide or hydrogen peroxide in relation to the numbers of cells exposed as well as different cell types, so it is important to assess how this modulation may be modified biologically in different cell types. In BHK-21 cells, symptoms of apoptotic cell death are evident when exposed to 10–100 μmol/l

hydrogen peroxide [3]. The study presented here also demonstrated that $O_2^{-\cdot}$ significantly inhibited proliferation of PC3 cell in a concentration-dependent manner. Nose et al. [16] suggested that hydrogen peroxide can act as a negative signal in mouse osteoblastic cells late in the G1 phase. In addition, there have been a number of observations that indicate an apparent inverse relationship between levels of cellular lipid peroxidation, resulting from ROS, and rates of cell proliferation [3]. Lipid peroxides can break down nonenzymatically to yield a variety of carbonyls such as 4-hydroxynonenal which may play a central role in the downregulation of cell proliferation [3, 5].

The expression of bFGF mRNA was detected in hyperplastic and cancerous tissues of the human prostate [14]. In rat prostate cancer FGF can stimulate growth even without androgens or EGF [21]. bFGF stimulated anchorage-independent cell proliferation of human prostate cancer cell line LNCap [8]. The study presented here demonstrated that bFGF at 2 ng/ml stimulated the growth of PC3 cells and the proliferation of PC3 cells induced by bFGF was significantly overcome by certain concentrations of $O_2^{-\cdot}$. This is in agreement with the results of Kather and Kreiger-Brauer [12] who reported that low concentrations of hydrogen peroxide supplied exogenously can reverse the effect of bFGF. On the other hand, cytokines can also specifically regulate the level of superoxide generation in their specific target cells, thus possibly setting the appropriate oxidation states for transmission of their own signal [3].

The observation that $O_2^{-\cdot}$ can downregulate proliferation of PC3 cells raises the question of possible molecular mechanisms. ROS or lipid peroxidation reduce membrane fluidity, which is known to be essential for the proper biological functioning of membranes [25]. Most of the proteins that play key roles in proliferative signal transduction actually function in a membrane environment, or in close association with membranes, and the activity of integral membrane protein is modulated by ROS [10]. In addition, active oxygen species such as superoxide and hydrogen peroxide would induce DNA damage and particularly DNA breaks [2]. Moreover, inactivation of tumor suppressor activity by mutation or allelic loss seems to be a nearly universal step in the development of prostate cancer [13]. Cellular proto-oncogenes and their activated forms (oncogenes) also play an important role in cell growth and tumorigenesis. In vitro studies showed that p53 mutations are present in prostatic cancer cell lines and that wild-type p53 is able to reduce colony formation [11], indicating that abnormal p53 expression is a frequent event in prostate cancer [26]. ROS is involved in cellular proto-oncogene regulatory processes [22]. Hainaut and Milner [6] showed that the DNA-binding in vitro of p53 appears to to be modulated by oxidizing agents. Ho et al. [9] found that nitric oxide induced nuclear accumulation of p53 protein in a dose- and time-dependent manner in human and rat cancer cells. Our data also demonstrated that $O_2^{-\cdot}$ at the concentrations of 18 μmol/l NADH and 4 μmol/l PMS upregulated the expression of p53 but did not affect c-Ha-*ras*. All these may demonstrate that different redox states of signal transduction proteins are appropriate to different cellular requirements for specific gene activation or expression depending on cell type and situation.

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