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Apocynin, an NADPH oxidase inhibitor, suppresses progression of prostate cancer via Rac1 dephosphorylation

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ABSTRACT

Recently, considerable evidence has been generated that oxidative stress contributes to the etiology and pathogenesis of prostate cancer. The present study focused on the effects of apocynin, an inhibitor of the NADPH oxidase which generates intracellular superoxide, on a rat androgen-independent prostate cancer cell line (PLS10) in vitro and in vivo. Apocynin significantly inhibited cell proliferation of PLS10 cells via G1 arrest of the cell cycle in vitro. Surprisingly, it did not affect reactive oxygen species (ROS) but inhibited phosphorylation of Rac1, one component of the NADPH oxidase complex. A Rac1 inhibitor, NSC23766, also inhibited cell proliferation, and both apocynin and NSC23766 reduced phosphorylation of Rac1 and NF-κB, as well as cyclin D1. Furthermore, in a xenograft model of prostate cancer with PLS10, apocynin suppressed tumor growth and metastasis in a dose dependent manner in vivo, with reduction of cell proliferation and vessel number in the tumors. Expression and secretion of vascular endothelial growth factor (VEGF) were reduced by apocynin treatment in vivo and in vitro, respectively. In conclusion, despite no apparent direct relationship with oxidative stress, apocynin inhibited growth of androgen-independent prostate cancer in vitro and in vivo. Apocynin thus warrants further attention as a potential anti-tumor drug.

1. Introduction

Prostate cancer is the second most frequently diagnosed cancer in males in the world, with especially higher incidences in Oceania, Europe and North America. In Japan, incident and mortality rates of prostate cancer are relatively low but increasing (Jemal et al., 2011; Damber and Aus, 2008). Androgen ablation therapy is a widely used treatment during the initial stage of the disease and may produce an initially favorable outcome, but most patients eventually develop androgen-independent prostate cancers with metastatic foci, which cause patient death. Currently, there is no therapy that is able to cure progressive hormone-refractory metastatic prostate cancer (Damber and Aus, 2008). New therapeutic agents are thus needed urgently.

Reactive oxygen species (ROS) can be important factors for carcinogenesis and tumor progression, not only inducing DNA damage but also producing cellular alterations such as up-regulation of mitogen activated protein kinase (MAPK) and protein kinase C (PKC) (Lee et al., 2006; Wu, 2006). Recently, considerable evidence has been published suggesting oxidative stress contributes to the etiology and pathogenesis of the prostate cancer (Kumar et al., 2008; Khandrika et al., 2009). Therefore, we have focused on inhibition of ROS production as an anti-tumor approach for prostate cancer.

ROS is produced by mitochondria, peroxisomes, cytochrome P-450, and other cellular elements as a by-product, generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is also implicated in a variety of signaling events, including cell growth, cell survival and cell death (Bedard and Krause, 2007). NADPH oxidase consists of phox units ($gp91^{phox}$, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$) and Rac, the small molecular weight G protein (Bedard and Krause, 2007). Apocynin, a methoxy-substituted catechol that inhibits NADPH oxidase by blocking the association of $p47^{phox}$ and $p67^{phox}$ with $gp91^{phox}$ (Stolk et al., 1994), is now used as a standard NOX inhibitor for research purposes (Bedard and Krause, 2007). Additionally, apocynin can be converted by peroxidase-mediated oxidation to a dimer, which has been shown to be more efficient inhibitor than apocynin itself (Stefanska and Pawliczak, 2008). We previously presented evidence that apocynin reduced oxidative stress induced by arsenite treatment of rat urothelium in vivo (Suzuki et al., 2009).

In the present study we focused on NADPH oxidase and tested whether its inhibitor, apocynin, might be able suppression of androgen-independent prostate cancer progression in vitro

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and vivo. The androgen-independent, androgen receptor-negative rat prostate cancer cell line (PLS10), which was established in our laboratory from a 3,2′-dimethyl-4-aminobiphenyl plus testosterone-induced carcinoma in the dorsolateral prostate of a male F344 rat (Nakanishi et al., 1996), was employed for this purpose.

2. Materials and methods

2.1. Cell culture

The PLS10 cell line was cultured in Roswell Park Memorial Institute-1640 Medium (RPMI 1640, Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin, in a humidified incubator with an atmosphere comprising 95% air and 5% CO_2 at 37 °C.

2.2. Animals

All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences. Seven-week-old male athymic nude mice (KSN strain) were purchased from Nihon SLC (Hamamatsu, Japan) and housed in plastic cages with hardwood chip bedding in an air-conditioned room at 23 ± 2 °C and 55 ± 5% humidity with a 12 h light/dark cycle. Oriental MF powder diet (Oriental Yeast Co., Tokyo, Japan) and distilled water were available ad libitum.

2.3. Proliferation assay

PLS10 cells were plated at 2.0×10^2 cells per well in 96-well plates. Twenty-four hours after plating, increasing concentrations of apocynin or NSC23766 (Rac1 inhibitor, COSMO BIO Co., Ltd., Tokyo, Japan) were added. The cells were then incubated for 48 h at 37 °C. Cells cultures were observed microscopically and overall cell number/viability was assessed by WST-1 colorimetric assay (Roche Diagnostics, Mannheim, Germany).

2.4. Cell cycle analysis

After starved conditions with 1% bovine serum albumin instead of 10% FBS, the cells were treated with 250 or 500 μM of apocynin for 48 h, then suspensions were prepared and stained with propidium iodide (Guava® cell cycle reagent, Guava Technologies, Hayward, CA) according to the Guava® Cell Cycle Assay protocol. Cell cycle phase distributions were determined on a Guava® PCA Instrument using CytoSoft Software.

2.5. Invasion assay

For invasion assays, cells were seeded in BD Biocoat™ Matrigel™ invasion chambers (BD Biosciences, San Jose, CA), and treated with apocynin (0, 100 and 200 μM) for 24 h 5% FBS was used as a chemoattractant. Invading cells were fixed with 100% ethanol for 5 min, then stained with 0.5% crystal violet in 20% methanol for 30 min. The number of infiltrating cells was counted under a microscope.

2.6. Detection of ROS production

ROS production was detected by a slightly modification of the method described in a previous report (Rosenkranz et al., 1992). Briefly, 24 h after apocynin treatment, culture supernatant was removed from all wells and the cells were washed twice with warm PBS, then 100 μl of 2′,7′-dichlorofluorescin-diacetate (100 μg/ml, DCFH-DA, Sigma) was added with further incubation at 37 °C for 60 min, in the dark. The cells were washed 3 times with warm PBS, and then were lysed in 100 μl of 2.5% TrironX100. After incubation for 5 min, fluorescence intensity was assessed at 485/535 nm with a spectrofluorometer. Images were also recorded with a fluorescence microscope (BZ-9000; Keyence Corp., Osaka, Japan).

2.7. Detection of vascular endothelial growth factor (VEGF) secretion into the medium

PLS10 cells were plated at 6.0×10^4 cells per well in 24-well plate. Twenty-four hours after plating, they were treated with apocynin or NSC23766 for 24 h. The culture medium of each well was transfered to wells of a Rat VEGF Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN), and VEGF secretion into the medium was determined according to the manufacturer's instructions.

2.8. Immunoblot analyses

After treatment of apocynin or NSC23766, cells were washed with ice-cold phosphate buffer saline (PBS) and scraped with a cell scraper into RIPA buffer (Pierce Biotechnology, Rockford, IL) containing a protease inhibitor (Pierce Biotechnology) on ice. The insoluble matter was removed by centrifugation at 12,000 rpm for 20 min at 4 °C and supernatants were collected. Protein concentrations were determined with a Coomassie Plus™-The Better Bradford Assay Kit (Pierce Biotechnology). Samples were mixed with 2× sample buffer (Bio-Rad Laboratories, Hercules, CA) and heated for 5 min at 95 °C and then subjected to 10% SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes followed by blocking with SuperBlock Blocking Buffer (Thermo Fisher Scientific K.K., Yokohama, Japan) for 1 h at room temperature. Membranes were probed with antibodies for cyclin D1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), cleaved caspase-3, Nuclear Factor Kappa B (NF-κB) p65 and phospho-NF-κB p65 (Ser536) (Cell Signaling, Technology Inc., Danvers, MA), Rac1 (Millipore Corporation, Billerica, MA) and phospho-Rac1 (Upstate, Temecula, CA) in 1× TBS with 0.1% Tween-20 at 4 °C overnight, followed by exposure to peroxidase-conjugated appropriate secondary antibodies and visualization with an enhanced chemiluminescence detection system (GE Healthcare Bio-sciences, Buckinghamshire, NA, UK). To confirm equal protein loading, each membrane was stripped and reprobed with anti-β-actin (Sigma–Aldrich, Co., St. Louis, MO). Band densities of cyclin D1, phospho-NF-κB p65 and phospho-Rac1 were then determined with ImageJ 1.410 (National Institute of Mental Health, MD).

2.9. PLS10 xenograft model

After one week of acclimation, mice were divided into 3 groups of 10 mice each. Two hundred thousand of PLS10 cells were mixed with 50% Matrigel (BD Biosciences) and injected (100 μL) subcutaneously into the back area of each mouse. The animals were given drinking water containing 0, 100 and 500 mg/L apocynin for 30 days and body weights and water consumption were estimated every week. The tumor volumes in each mouse were estimated every other day using the following formula: 0.52 (long axis × short axis × short axis). Mice were sacrificed at experimental day 30, when primary tumors and liver, lung, kidneys and lymph nodes were removed and fixed in 10% buffered formalin. Primary tumors were measured and tumor volume was calculated using the following formula: 0.52 (axis1 × axis2 × axis3). At least 1 section of each tissue and the largest section from each lobe of the lung were processed for hematoxylin and eosin staining, immunostaining and