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Original Contribution

Lung tumor growth-promoting function of peroxiredoxin 6

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ABSTRACT

This study compared lung tumor growth in PRDX6-overexpressing transgenic (Tg) mice and normal mice. These mice expressed elevated levels of PRDX6 mRNA and protein in multiple tissues. In vivo, Tg mice displayed a greater increase in the growth of lung tumor compared with normal mice. Glutathione peroxidase and calcium-independent phospholipase 2 (iPLA2) activities in tumor tissues of Tg mice were much higher than in tumor tissues of normal mice. Higher tumor growth in PRDX6-overexpressing Tg mice was associated with an increase in activating protein-1 (AP-1) DNA-binding activity. Moreover, expression of proliferating cell nuclear antigen, Ki67, vascular endothelial growth factor, c-Jun, c-Fos, metalloproteinase-9, cyclin-dependent kinases, and cyclins was much higher in the tumor tissues of PRDX6-overexpressing Tg mice than in tumor tissues of normal mice. However, the expression of apoptotic regulatory proteins including caspase-3 and Bax was slightly less in the tumor tissues of normal mice. In tumor tissues of PRDX6-overexpressing Tg mice, activation of mitogen-activated protein kinases (MAPKs) was much higher than in normal mice. In cultured lung cancer cells, PRDX6 siRNA suppressed glutathione peroxidase and iPLA2 activities and cancer cell growth, but the enforced overexpression of PRDX6 increased cancer cell growth associated with their increased activities. In vitro, among the tested MAPK inhibitors, c-Jun NH_2-terminal kinase (JNK) inhibitor clearly suppressed the growth of lung cancer cells and AP-1 DNA binding, glutathione peroxidase activity, and iPLA2 activity in normal and PRDX6-overexpressing lung cancer cells. These data indicate that overexpression of PRDX6 promotes lung tumor growth via increased glutathione peroxidase and iPLA2 activities through the upregulation of the AP-1 and JNK pathways.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide [1]. The relative lack of symptoms during the early stages of lung cancer results in delayed diagnosis, thus more than half of patients already have metastatic status by the time of diagnosis [2]. For this reason, lung cancer is a major target of cancer therapy. The most promising curative therapy is complete resection. However, 40–50% of patients with pathological stage I die within 5 years after complete resection [1]. Various genes and proteins, including epidermal growth factor receptor (EGFR), homeodomain transcription factor Nkx2.1, K-RAS (retrovirus-associated DNA sequences), and NF-E2-related factor 2 (Nrf2), are involved in lung cancer development [3]. Overexpression of EGFR, a transmembrane protein receptor, can promote the proliferation, invasion, and metastasis of lung cancer cells [4]. Nkx2.1, a transcription factor, increases tumor growth by regulating cell cycle progression in lung cancer cells [5]. c-Raf, a RAS family member, is associated primarily with malignancies of the lung through elicitation of a K-RAS oncogenic effect [6]. Nrf2 is another transcription factor, which regulates the cellular redox system and which has been associated with the promotion of human lung carcinoma [7].

Peroxiredoxins (PRDXs) are a family of peroxidases [8]. They are divided into two classes: 2-Cys PRDXs and a 1-Cys PRDX [9]. PRDXs destroy peroxides, and cysteine in the PRDX active site is oxidized by the destroyed peroxide. Six PRDX isoforms have been identified. Although their roles in cellular redox regulation seem

Abbreviations:: PRDX, peroxiredoxin; Tg, transgenic; iPLA2, calcium-independent phospholipase 2; AP-1, activating protein-1; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; SAPK2, p38, stress-activated protein kinase 2; ERK1/2, p44/p42, extracellular signal-regulated protein kinase; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; Nrf2, NF-E2-related factor 2; K-RAS, retrovirus-associated DNA sequence; BEL, bromoenol lactone; LLC, Lewis lung carcinoma

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individually different, they catalyze peroxide detoxification and share a common thioredoxin motif [8]. Among the six mammalian members of this family, PRDX6 is the only 1-Cys PRDX and has glutathione peroxidase and phospholipase A2 (PLA2) activities [10]. Whereas other PRDXs utilize thioredoxin as the physiological reductant, human PRDX6 utilizes glutathione [9]. The promoter region of PRDX6 harbors a *cis*-acting antioxidant-response element, which is activated by oxidative stress [10]. As PRDXs are antioxidants, they support survival and tumor maintenance by protecting cells from oxidative stress-induced apoptosis [11]. PRDX6 is often upregulated in malignant tissues of a variety of human organs, including lung [12,13]. High levels of PRDX6 are associated with increased resistance to radiation and some chemotherapeutics, whereas PRDX6 deficiency can sensitize cells to chemotherapy and apoptosis [13]. PRDX6 contributes as an antioxidant to preventing cell death, whereas it contributes to cell cycle arrest and apoptosis via its iPLA2 activity in cultured cancer cells [8,14]. Moreover, Lee et al. [15] and Ho et al. [16] showed lung tumor-promoting activity in cultured cancer cells and in an in vivo xenograft model. However, it is not clear how PRDX6 is involved in lung tumor growth.

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that mediate intracellular signaling associated with many cellular activities, including cell proliferation, differentiation, and survival [17]. The three main members are the stress-activated protein kinase c-Jun NH_2-terminal kinase (JNK), stress-activated protein kinase 2 (SAPK2, p38), and the extracellular signal-regulated protein kinases (ERK1/2, p44/p42) [18]. Because the MAPK signaling cascade mediates the transduction of growth-promoting signals such as growth factors, cytokines, and proto-oncogenes, MAPK signaling is significant in carcinogenesis [17]. Moreover, activation of MAPK in lung cancer specimens is associated with a poor prognosis [18]. Among the MAPK pathways, the JNK pathway is significant in lung tumor growth. Angiotensin-(1–7) has antimigration and anti-invasion effects through inactivation of the JNK signal pathway in lung cancer cells [19]. Acacetin inhibits 12-*O*-tetradecanoylphorbol 13-acetate-induced adhesion, invasion, and migration in lung cancer cells through the inactivation of the JNK signaling pathway [20]. Activation of JNK can phosphorylate c-Jun and activation of ERK can phosphorylate c-Fos [21]. The transcriptional complex AP-1, which is composed of c-Fos and c-Jun proteins, is a regulator of major physiological processes such as cell proliferation, differentiation, organogenesis, and apoptosis [22]. Recruitment of c-Fos and c-Jun to the nucleus by JNK activates transcription factor AP-1 [23]. Growth of lung cancer cells is inhibited by AP-1 blockade using a c-Jun dominant-negative mutant [24]. Enzastaurin inhibits invasion and metastasis by downregulation of AP-1 [25], suggesting that AP-1 plays an essential role in the growth and metastasis of lung cancer cells.

MAPK activation leads to phosphorylation of PRDX6 [26,27] and increases its PLA2 activity [26]. PLA2 activity induces the invasive pathway via accumulation of arachidonic acid and contributes to the metastatic ability of lung cancer cells [12]. 1,4-Phenylenebis(methylene)selenocyanate reduces expression of PLA2 and inhibits cell growth and induction of apoptosis in lung cancer cells [28]. In vivo, iPLA2β expression is involved in ovarian tumorigenesis and metastasis [29]. Glutathione peroxidase overexpression reverses the tumor cell growth inhibition caused by manganese superoxide dismutase overexpression in a variety of cancers [30]. The glutathione peroxidase activity of PRDX6 supports cell growth and the inhibition of peroxidase activity retards cell growth in cancer cells, including lung cancer cells [12]. Atorvastatin can upregulate the activity of glutathione peroxidase, which could provide an alternative angiogenesis mechanism in non-small-cell lung cancer therapy [28]. In this study, we investigated the tumor growth-modulating function of PRDX6 via induction of glutathione peroxidase and PLA2 activities, and the involvement of the AP-1/JNK pathways, in PRDX6-overexpressing Tg mice and in cultured lung cancer cell lines.

Materials and methods

Cell culture

The Lewis lung carcinoma (LLC) mouse lung cancer cell and A549 human lung cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml). Cell cultures were then maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Ethics statement

All experiments were approved and carried out according to the *Guide for the Care and Use of Animals* (Animal Care Committee of Chungbuk National University, Korea (CBNUA-436-12-02)).

Animal experiments

The C57BL/6 J-Tg(Prdx6) mice were purchased from The Jackson Laboratory and the C57BL/6 mice were purchased from Koatech (Pyeongtaek, Korea). The mice were housed and bred under specific-pathogen-free conditions at the Laboratory Animal Research Center of Chungbuk National University, Korea. The mice ($n=5$/cage) were maintained in a room with a constant temperature of 22 ± 1 °C, relative humidity of 55 ± 10%, and 12-h light/dark cycle and fed standard rodent chow (Samyang, Korea) and purified tap water ad libitum. The C57BL/6 J-Tg(Prdx6) mice ($n=10$) and C57BL/6 mice ($n=10$) that were used were matched for age (12 weeks) and weight (16–19 g). LLC cells were implanted subcutaneously (1.2×10^6 tumor cells/0.1 ml phosphate-buffered saline (PBS)/animal) into the upper dorsal region of the mice with a 27-gauge needle. Implanted tumors were visually detected in the injected region on the mice after 7 days. The weight and tumor volume of the animals were monitored twice per week for 21 days. The tumor volumes were measured with Vernier calipers and calculated using the formula $(A \times B^2)/2$, where A is the larger and B is the smaller of the two dimensions. At the end of the experiment, the animals were sacrificed by cervical dislocation. The tumors were separated from the surrounding muscles and dermis, excised, and weighed.

Western blot analysis

Western blot analysis was done as described previously [31]. The membrane was incubated for 2 h at room temperature with specific antibodies: rabbit polyclonal for PRDX6, vascular endothelial growth factor (VEGF) (1:1000 dilution; Abcam, plc., Cambridge UK); caspase-3, caspase-9, Bcl2, p53 (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA); ERK, p-ERK, JNK, p38, c-Fos, c-Jun, cyclin B1, cyclin E (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); Ki67 (1:1000 dilution; Novus Biologicals, Littleton, CO, USA); cyclin-dependent kinase 1 (CDK1; 1:1000 dilution; MBL International, Woburn, MA, USA); rabbit monoclonal for p-p38 (1:1000 dilution; Cell Signaling Technology) and mouse monoclonal for proliferating cell nuclear antigen (PCNA; 1:1000 dilution; Cell Signaling Technology); p-JNK, Bax, CDK2, CDK4, CDK6, cyclin D, matrix metalloproteinase 9 (MMP-9; 1:500 dilution; Santa Cruz Biotechnology); and cyclin A (1:1000 dilution; MBL International). The blot was then incubated with the