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Production of Cyr61 protein is modulated by extracellular acidification and PI3K/Akt signaling in prostate carcinoma PC-3 cells

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ABSTRACT

High expression of Cyr61, an extracellular cysteine-rich heparin-binding protein, has been associated with a malignant cell phenotype and poor outcome in prostate cancers. Although Cyr61 was found by us to be overproduced in androgen-independent PC-3 cells treated with *N*-acetylcysteine (NAC), its significance is still unclear. We therefore aimed to determine how and why Cyr61 protein is overexpressed in NAC-treated cells. Here, we found that Cyr61 protein level markedly increased in cells treated with NAC at high cell seeding density. Silencing of Cyr61 by siRNA induced enhanced activity of caspase-3/7, upregulation of the proapototic Bok, Bim_L and Bim_S, cleavage of apoptosis hallmarkers such as Bax, PARP and caspase-3, and downregulation of antiapoptotic Bcl2, Bcl-xL and Mcl-1 proteins. NAC treatment caused a reduction of extracellular medium pH to acidic and an increase in Akt phosphorylation, after which the replacement with NAC-free medium returned them to control levels within 24 h. Acid stimulation increased the levels of Cyr61 and p-Akt proteins, whereas it suppressed the induction of proapoptotic and antiapoptotic proteins. Overall, our data indicate that PC-3 cells overproduce Cyr61 protein via activation of the PI3K/Akt signaling as a part of the survival mechanisms under the conditions causing extracellular acidity and further cytotoxicity.

1. Introduction

The ability of cells to counteract stressful conditions usually elicits the activation of pro-survival pathways and the production of molecules with antioxidant and antiapoptotic activities. The coordinated action of a variety of molecules likely contributes to this protection, including heat shock proteins (Konishi et al., 1997), nitric oxide synthase (Bhowmick and Girotti, 2012), NF-κB (Lin et al., 2012), Bcl2 (Azad et al., 2010), thioredoxin/thioredoxin reductase system (Calabrese et al., 2007), and Nrf2 (Lee et al., 2012b). In the preliminary study we found that prolonged exposure of PC-3 cells to *N*-acetylcysteine (NAC) suppressed the growth of cells with concomitant overproduction of Cyr61 protein (Lee et al., 2011).

Cyr61 is a secreted cysteine-rich matricellular protein with a broad spectrum of physiologic and pathophysiologic functions, including the promotion of cell survival, cell proliferation, adhesion, tumor growth, and apoptosis in various contexts (Chen and Du, 2007). Upon secretion, Cyr61 is tightly associated with the extracellular matrix (ECM), and its pleiotropic effects are mediated primarily through its chemical interaction with distinct integrins which are cell-surface signaling receptors capable of regulating diverse cellular functions. Cyr61-dependent cell signaling through integrin $\alpha_V\beta_3$ promotes breast cancer cell proliferation, cell survival, and chemoresistance (Babic et al., 1998). Integrin $\alpha_V\beta_5$ is essential for Cyr61-induced metastasis in oral squamous carcinoma cells (Monnier et al., 2008). Cyr61 contributes to the peritoneal dissemination of gastric cancer cells through integrin $\alpha_2\beta_1$ (Lin et al., 2007b). Contrary to these findings, Cyr61 induces p53-dependent apoptosis through interaction with integrin $\alpha_6\beta_1$ and syndecan-4 in fibroblasts (Todorovic et al., 2005). Thus, the biological outcome of Cyr61 signaling is highly context-dependent.

The overexpression of wild-type Cyr61 has been reported in different human cancers, it frequently indicates poor prognosis and is usually linked with the activation of a number of intracellular signaling pathways to regulate tumor cell proliferation, invasion, metastasis, and angiogenesis (Sun et al., 2008; Brigstock, 2003; Planque and Perbal, 2003). Knockdown of Cyr61 expression using

Abbreviations: NAC, *N*-acetylcysteine; ECM, extracellular matrix; PI3K, phosphoinositide 3-kinase; SDS, sodium dodecyl sulfate; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; siRNA, small interfering RNA; PBS, phosphate buffered saline; Akt, serine/threonine protein kinase B; p-Akt, phosphor-Akt; c-FLIP, cellular FLICE-like inhibitory protein.

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small interfering RNA (siRNA) in androgen-independent prostate cancer cells strongly inhibits their proliferation (Franzen et al., 2009), whereas purified Cyr61 protein promotes the migration of LNCaP cells in a dose-dependent manner (Sun et al., 2008). Consistent with a significant role in cell proliferation, Cyr61 induction is triggered by wide range of mitogenic signals and extracellular stimuli, including platelet-derived growth factor, fibroblast growth factor 2, transforming growth factor beta, phorbol esters, and cAMP (O'Brien et al., 1990; Bartholin et al., 2007). In addition to mitogenic signals, synthesis of Cyr61 protein is stimulated by hormones such as angiotensin II and estrogen, as well as by environment changes such as UV light, hypoxia, inflammation and tissue injury (Chen and Lau, 2009).

Despite significant advances in recent years, further factors regulating Cyr61 remain to be identified. The purpose of our current study is to determine the conditions through which Cyr61 expression is regulated in PC-3 cells and, if possible, to identify the significance of Cyr61 overproduction in this cell type in response to NAC toxicity. We show here that NAC treatment at high cell seeding density caused a reduction of extracellular medium pH and activated a pro-survival signaling molecule which is composed of phosphoinositide 3-kinase (PI3K) and the serine/threonine protein kinase B (PKB or Akt), which may contribute to upregulation of Cyr61 protein as an important molecule capable of manipulating cell survival under a cytotoxic microenvironment.

2. Materials and methods

2.1. Reagents and cell culture

Sodium dodecyl sulfate (SDS), phosphate buffered saline (PBS), NAC, and anti-β-actin antibody were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Ly294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] was purchased from Calbiochem (La Jolla, CA, USA). Goat anti-human Cyr61 antibody, horseradish peroxidase (HRP)-conjugated secondary antibodies, and the enhanced chemiluminescence (ECL) system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human Mcl-1, Bcl2, Bcl-xL, Bax, Puma, Bim, Bok, PARP, caspase-3, cleaved caspase-3, Akt and phospho-Akt (p-Akt) antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). NuPAGE 4–12% bis-tris polyacrylamide gels were purchased from Invitrogen (Carlsbad, CA, USA). Cell culture media and reagents were purchased from Gibco (Grand Island, NY, USA). The human prostate carcinoma cell lines PC-3 and LNCaP were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The human prostate carcinoma cell line DU145 and human normal prostate epithelial cell line RWPE-1 were kindly provided by Dr. Jung-Hyun Shim of Soonchunhyang University. PC-3, DU145, and LNCaP cells were maintained in DMEM supplemented with 5% fetal bovine serum, 1 mM glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml. RWPE-1 cells were grown in keratinocyte serum-free medium containing 50 μg of bovine pituitary extract/mL, 5 ng of human epidermal growth factor/mL, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cultures were maintained at 37 °C in a humidified air containing 5% CO_2. The cells were seeded in culture plates at low density (5×10^4 cells/well and 2.5×10^3 cells/well for 6-well and 96-well plates, respectively), moderate density (10×10^4 cells/well and 5×10^3 cells/well for 6-well and 96-well plates, respectively), and high density (2×10^5 cells/well and 10^4 - cells/well for 6-well and 96-well plates, respectively), and grew in a monolayer culture in the medium for 24 h before treatment.

2.2. Cell proliferation assay

Cell proliferation was measured using the Cell Proliferation kit II (XTT) according to the manufacturer's instruction (Roche Diagnostics, Indianapolis, IN, USA). Briefly, cells (10^4 cells/well) were seeded in 96-well microtiter plates and then treated with NAC at 20 mM and/or 8.5% H_3PO_4 or 1.1 M $NaHCO_3$ for the indicated times. After incubation, 50 μL of the XTT labeling mixture was added to each well and incubated for an additional 4 h. The formazan dye that formed was measured spectrophotometrically at 450 nm using a GloMax-Multi Microplate Multimode Reader (Promega, Madison, WI, USA).

2.3. Transient transfection with Cyr61-specific siRNA

RNA interference of Cyr61 was performed using a Cyr61-specific small interfering RNA (siRNA) duplex from Invitrogen (Cat. # 12990). Briefly, cells were seeded in 6-well plate and transfected at 40% confluency with siRNA duplex using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's recommendations. Cells transfected with the Stealth RNAi negative control duplex (Invitrogen) were used as controls. At 6 h after transfection, the media were removed and replaced with fresh growth media. At 24 h, 48 h and 72 h after transfection, cell lysates were analyzed by Western blotting.

2.4. Apoptosis (caspase-3/7) assay

Activation of caspase-3/7 was quantified with the ApoTox-Glo™ Triplex Assay kit according to the manufacturer's protocol (Promega, Madison, WI, USA). Ten nanomoles of Cyr61-specific or control siRNA were transfected into PC-3 cells using lipofectamine RNAiMAX. After 24 h, 48 h and 72 h, cells were incubated with substrate containing Caspase-Glo® 3/7 assay buffer for 30 min. Caspase-3/7 activities were calculated after detection of luminescence by a GloMax-Multi Microplate Multimode Reader. The results were expressed as a percentage, based on the ratio of the luminescence of Cyr61-siRNA to that of control siRNA (100%).

2.5. Western blot analysis

Whole cell lysates were prepared using RIPA buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 μg/ml phenylmethanesulfonylfluoride). Briefly, proteins (40 μg per lane) were separated on NuPAGE 4–12% bis–tris polyacrylamide gels (Invitrogen) and then electrophoretically transferred to Immuno-Blot PVDF membranes. The membranes were incubated with specific antibodies for 2 h at room temperature and then washed and incubated with HRP-conjugated secondary antibodies. The signal was visualized by an ECL detection kit using X-ray films. The blots were then stripped using a stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7) and reprobed with anti-Akt and anti-β-actin antibodies as loading controls.

2.6. Statistical analysis

Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc correction for multiple comparisons using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean ± - SEM. Significant differences were considered with values of $p < 0.05$.

3. Results

3.1. NAC-induced overproduction of Cyr61 protein depends on cell density

In the preliminary study the proliferation of PC-3 cells was suppressed by NAC treatment in a time-dependent manner. On the basis of the observed antiproliferative activity of NAC, we investigated the effect of cell density on cytotoxicity by NAC. The cells were seeded in 96-well culture plates at three different densities (low, moderate, and high) as described in "Section 2". Plates were incubated overnight and were then treated with or without NAC for 72 h. In the XTT assay, NAC treatment at high density suppressed cell growth little than treatment at low density, as compared with their respective controls (Fig. 1A). Next, we utilized these cells to examine the effects of different seeding densities upon the induction of Cyr61 expression. Interestingly, the Cyr61 protein level was markedly enhanced in cells treated with NAC at higher seeding densities, as compared to lower density culture under the same conditions (Fig. 1B).

To quantify the relative expression of Cyr61 protein in prostate cancer cells, we performed Western blot analysis with whole cell lysates extracted from three classic cell lines DU145, PC-3, and LNCaP cells. Fig. 2A shows that the basal level of Cyr61 protein was approximately 1.9-fold higher in DU145 cells and 2.8-fold higher in PC-3 cells, than in normal prostate epithelial RWPE-1 cells. The Cyr61 protein level in LNCaP cells was relatively lower than that of the RWPE-1 cells. Subsequently, cell proliferation increased in a time-dependent manner, and in a cell seeding density-dependent manner, in all the prostate cell lines used (Fig. 2B). It was higher in androgen-independent DU145 and PC-3 cells than in androgen-dependent LNCaP cells, as compared with that in their respective normal RWPE-1 cells.