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Short Research Communication

Differential Roles of Carboxylated and Uncarboxylated Osteocalcin in Prostate Cancer Growth

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Abstract

Serum levels of osteocalcin (OC), a bone matrix non-collagenous protein secreted by osteoblasts, are correlated with pathological bone remodeling such as the bone metastasis of cancer, as well as physiological bone turnover. The pathological roles in prostate cancer growth of the two existing types of serum OC, γ-carboxylated (GlaOC) and lower- (or un-) carboxylated (GluOC), have not yet been discriminatively examined. In the present study, we demonstrate that normal prostate epithelial cell growth was promoted by both types of OC, while growth of cancer cells in the prostate was accelerated by GlaOC but suppressed by GluOC. We suggest that OC regulates prostate cancer growth depending on the γ-carboxylation, in part by triggering reduced phosphorylation of receptor tyrosine kinases.

Key words: osteocalcin, prostate cancer

Introduction

Prostate cancer is one of the most prevalent malignant diseases of men worldwide, and it remains a challenge to distinguish and prevent lethal metastatic prostate cancers, which escape standard therapeutic intervention. Bone is a preferred organ for the metastasis of prostate cancer [1-4], and the metastasis to bone is associated with increased osteoblast activity [4]. In fact, the production of OC by osteoblasts is well correlated with the metastasis of prostate cancer progression [1], and therefore high levels of OC in the serum are assumed to be a metastatic marker of prostate cancer [2,3]. Furthermore, OC has been reported to promote prostate cancer progression [5], but previous studies did not discriminate between the two existing OC types, GlaOC and GluOC. GluOC but not GlaOC has recently been reported to improve glucose metabolism [6-10]. These findings indicate that each form of OC plays a different role depending on the pathophysiological conditions. Therefore, it is important to examine which form of OC is involved in prostate cancer progression. In the present study, GlaOC and GluOC were separately examined to determine their roles in prostate cancer cell growth.

Materials and Methods

Materials

Recombinant GluOC was prepared as described previously [7]. GlaOC was purchased from AnaSpec (Fremont, CA, USA).

Cells

A human prostate cancer cell line (PC-3) and normal prostate epithelial (ProEpi) cells were purchased from ATCC (Rockville, MD, USA) and

Lonza (Basel, Switzerland), respectively. A human prostate carcinoma cell line, PPC-1, was kindly provided by Dr. J.Y. Bahk (Gyeongsang National University, Korea). The two prostate cancer cell lines and ProEpi cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and PrEGM™ Prostate Epithelial Cell Growth Medium BulletKit™ (Lonza), respectively. All cells were cultured at 37°C in humidified air containing 5% CO_2.

Measurement of cell viability

Cell viability was determined with a WST-8 assay (Cell Count Reagent SF; Nakalai Tesque, Kyoto, Japan) and a BrdU uptake assay (BrdU Cell Proliferation ELISA Kit; Exalpha Biologicals, Shirley, MA, USA). Each cell seeding density was optimized before experiments. Prior to assays, cells were treated with vehicle, GluOC or GlaOC (1, 10, 100 ng/ml), in 96-well culture plates in 100 µl for 24 h or 48 h, after preculture in each maintenance medium for 24 h.

RTKs (Receptor Tyrosine Kinases) phosphorylation antibody (phosphor-RTKs) array

An RTKs phosphorylation antibody array was performed using a Human Phospho-RTK Array Kit (R&D Systems, Minneapolis, MN, USA). Following preculture for 24 h, PPC-1 or ProEpi cells were treated with GluOC (10 ng/ml) or GlaOC (10 ng/ml) for 6 h. Cells were then lysed in Lysis Buffer 17 (R&D Systems) including 3.4 µg/ml aprotinin, 10 µg/ml leupeptin, 5 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA). Cell lysate (73.5 µg) was analyzed using the Human Phospho-RTK Array Kit.

Statistical Analysis

Dunnett's test or Student's *t*-test were performed for statistical analysis where appropriate. *P* values < 0.05 were considered to be statistically significant. Results are expressed as mean ± standard deviation (SD).

Results and Discussion

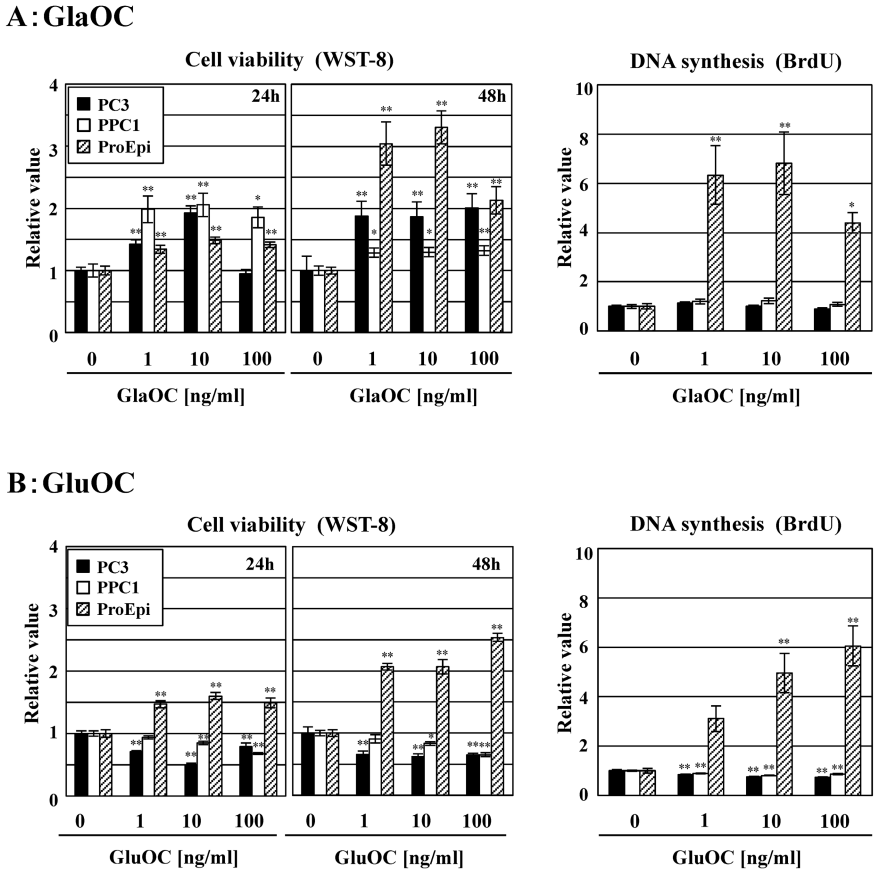


Figure 1. Effects of GlaOC and GluOC on human prostate cell growth. Effect of GlaOC (A) and GluOC (B) on PC-3, PPC-1, and ProEpi cells. Each assay was performed in triplicate. Left and right panels represent WST-8 (cell viability) assays at 24 h and 48 h and BrdU uptake (DNA synthesis) assays at 24 h, respectively. The data represent mean ± SD from three experiments. Mean data are expressed as a ratio of the control. **P* < 0.05 and ***P* < 0.01 *versus* the corresponding value for cells treated with vehicle.

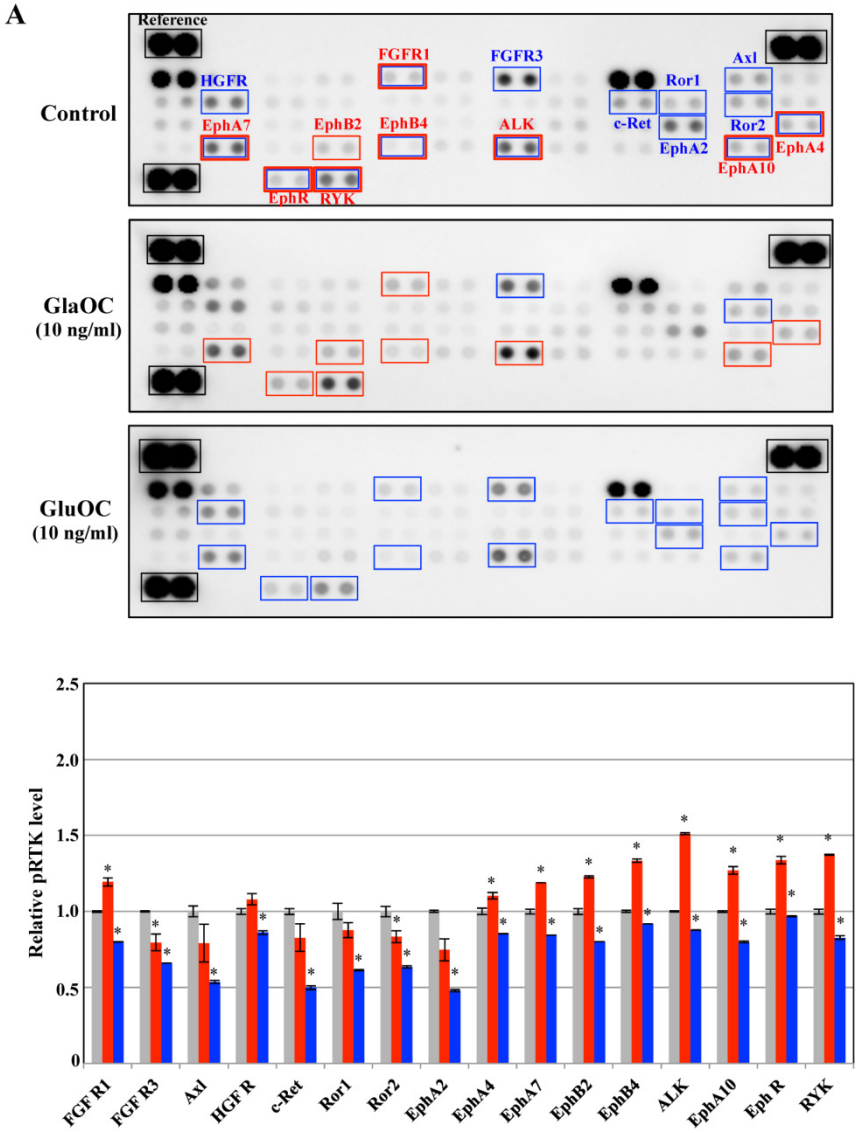
Effects of GlaOC and GluOC on cell viability in human prostate cells

GlaOC at concentration of 1-100 ng/ml dose-dependently promoted the viability of the human prostate cancer cell lines PC-3 and PPC-1 in a WST-8 assay for 24 h, except for PC-3 cells at 100 ng/ml at 24 h (Fig 1A). We also assayed WST-8 for 48 h; more promotion than that for 24 h was observed, except for PPC1 cell line. In contrast, GluOC at the same concentrations inhibited viability of these two cell lines at 24 h and 48 h (Fig 1B). We further investigated a BrdU incorporation assay to examine the differential effects of GlaOC and GluOC on cell proliferation. GluOC dose-dependently decreased the incorporation of BrdU in the two prostate cancer cell lines in a similar manner as in the WST-8 assay, while the effect of GlaOC was not significantly manifested in this assay for unknown reasons (Fig 1AB). In contrast, both GlaOC and GluOC promoted the growth of the ProEpi cell line of

normal prostate epithelial cells, as assessed by WST-8 (at 24 h and 48 h) and BrdU assays (at 24 h) (Fig 1AB). These results indicate that GlaOC and GluOC have stimulatory and inhibitory effects on prostate cancer cell growth, respectively, but that normal prostate epithelial cells respond in a similar manner to both forms.

Regulation of the phosphorylation levels of RTKs by GlaOC and GluOC in human prostate cells

Receptor tyrosine kinases (RTKs), a family of cell surface receptors for growth factors, mediate an initial signaling for cell proliferation [11]. We therefore assayed phospho-RTKs arrays using PPC-1 as cancer cells and ProEpi as normal cells to elucidate the mechanism by which GlaOC or GluOC affects cell growth. GlaOC enhanced the phosphorylation levels of nine RTKs, including FGFR1 (fibroblast growth factor receptor 1) [12], EphA4 (ephrin receptor A4) [13], EphA7 [14], EphA10 [15], EphB2 [16], EphB4 [17], EphR (ephrin receptor) [18], ALK (anaplastic lymphoma kinase) [19], and RYK [20] (Fig. 2A), which are all closely related to prostate cancer progression in PPC-1 cells [12-20]. In contrast, GluOC did not enhance any RTKs, but rather reduced phosphorylation levels of fifteen RTKs, including FGFR1 [12], EphA4 [13], EphA7 [14], EphA10 [15], EphB4 [17], EphR [18], ALK [19], RYK [20], HGFR (hepatocyte growth factor receptor) [21], FGFR3 [22], c-Ret [23], Ror1 [24], Ror2 [24], Axl [25], and EphA2 [13] (Fig. 2A), whose expression or activation are also closely related to prostate cancer progression [12-15,17-25].



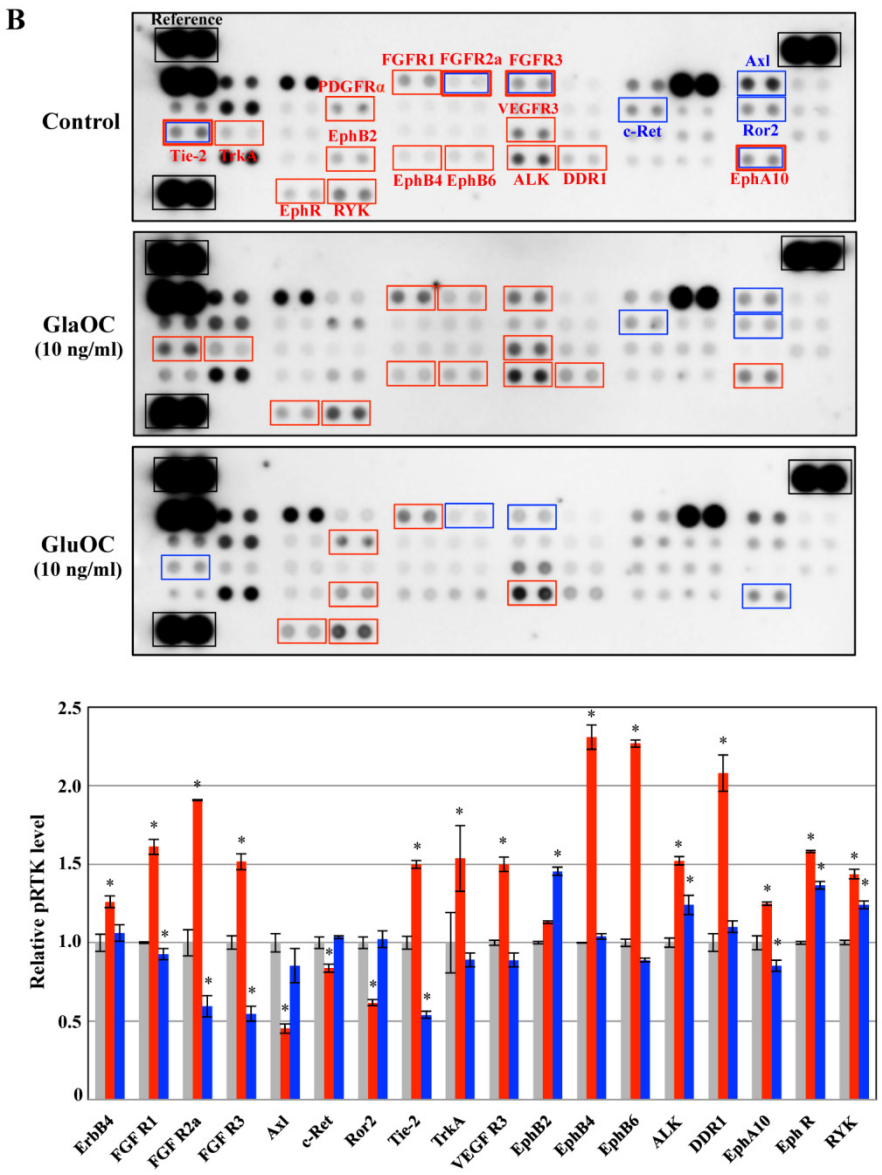


Figure 2. Phospho-RTKs array in PPC-1 (A) and ProEpi (B) cells. Quantitation of the dot densities of phospho-RTKs was performed using scanning images and ImageQuant LAS 4000 software (GE Healthcare UK, Buckinghamshire, England). Each pair of the kinase dots that increased (red) or decreased (blue) compared with the controls is enclosed in a square. Each assay was repeated three times. Four separate results are summarized in the graphs. *$P < 0.05$ *versus* the corresponding value for the control.

These results indicate that GlaOC promotes prostate cancer cell proliferation through the activation of nine RTKs, although it remains unclear whether GlaOC is a direct ligand for those RTKs. The mechanisms for GlaOC effect are currently unknown because the receptors for GlaOC and the signaling cascade have not yet been identified, but the tyrosine kinases are involved in the growth promotion since LY294002, an inhibitor for PI3 kinase and U0126, an inhibitor for MEK1/2 kinase, down-stream kinases after tyrosine kinase activation completely blocked the growth promotion by GlaOC (data not shown). On the contrary, GluOC is recognized by a tentative receptor, GPRC6A, followed by activation of cAMP production and protein kinase A [10], which might be implicated in the inhibition of several tyrosine kinases. We here suggest that GluOC could be a potential therapeutic agent against cancer, as it might inhibit the RTK-related signal transduction that is constitutively active in malignant cells [11].

Phosphorylation of RTKs using ProEpi cells, normal cells displayed different patterns, except for EphA10 (Fig. 2B). The most distinctive feature was that GluOC triggered increased phosphorylation of six RTKs in ProEpi cells, FGFR1 [12], EphB2 [16], EphR [18], ALK [19], RYK [20], and PDGFRα (platelet-derived growth factor receptor alpha) [26], which may be correlated with the proliferative activity triggered by GluOC in normal cells. On the other hand, GlaOC also triggered increased

phosphorylation of the same RTKs as GluOC, including FGFR1 [12], EphR [18], ALK [19], and RYK [20]. Additionally, GlaOC promoted the phosphorylation of additional nine RTKs, EphA10 [15], EphB4 [17], EphB6 [27], FGFR2a [28], FGFR3 [22], DDR1 (discoidin domain receptor 1) [29], VEGFR3 [30], Tie-2 [31], and TrkA [32] (Fig 2B). The mechanisms for GluOC to promote the growth in normal cells, likely by activation of tyrosine kinases are currently unknown.

These findings indicate that serum OC may contribute to prostate tissue homeostasis by regulating the RTK phosphorylation status, and that GlaOC and GluOC are discriminatively involved in prostate cell proliferation. In order to advance future clinical studies, GlaOC and GluOC have to be considered discriminatively.

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Competing Interests

The authors have declared that no competing interest exists.

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