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Prolyl oligopeptidase inhibition-induced growth arrest of human gastric cancer cells

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ABSTRACT

Prolyl oligopeptidase (POP) is a serine endopeptidase that hydrolyzes post-proline peptide bonds in peptides that are <30 amino acids in length. We recently reported that POP inhibition suppressed the growth of human neuroblastoma cells. The growth suppression was associated with pronounced G_0/G_1 cell cycle arrest and increased levels of the CDK inhibitor $p27^{kip1}$ and the tumor suppressor p53. In this study, we investigated the mechanism of POP inhibition-induced cell growth arrest using a human gastric cancer cell line, KATO III cells, which had a p53 gene deletion. POP specific inhibitors, 3-({4-[2-(*E*)-styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) and benzyloxycarbonyl-thioprolyl-thioprolinal, or RNAi-mediated POP knockdown inhibited the growth of KATO III cells irrespective of their p53 status. SUAM-14746-induced growth inhibition was associated with G_0/G_1 cell cycle phase arrest and increased levels of $p27^{kip1}$ in the nuclei and the pRb2/p130 protein expression. Moreover, SUAM-14746-mediated cell cycle arrest of KATO III cells was associated with an increase in the quiescent G_0 state, defined by low level staining for the proliferation marker, Ki-67. These results indicate that POP may be a positive regulator of cell cycle progression by regulating the exit from and/or reentry into the cell cycle by KATO III cells.

1. Introduction

Prolyl oligopeptidase (POP, EC 3.4.1.26) is an oligopeptidase that cleaves proline-containing peptides that are <30 amino acids in length and belongs to the POP family of serine proteases (family S9 of clan SC). In mammals, high POP activities have been detected in the brain, kidney, and testis [1]. In general, POP has been considered to be a cytosolic enzyme, but it has been detected as a membrane-bound form and in the nucleus of non-neuronal cell lines and neuronal cell cultures [2]. POP may play a role in numerous biological processes, such as learning and memory [3,4], signal transduction [5], and protein secretion [6]. However, no conclusive results have yet been reported, and the primary physiological role of POP remains to be elucidated.

POP involvement in cell division was suggested in several studies [7–10]. POP was shown to be involved in cell proliferation and DNA replication in a mouse Swiss 3T3 cell line [7]. In addition, POP activity is commonly elevated in many cancers [8,9]. More recently, POP was found in various cell types in both the cytoplasm and nuclei in mouse whole-body sections and at the cellular level in peripheral tissues by immunohistochemical study. The nuclear colocalization of the POP protein and Ki-67, a proliferation marker protein, suggested that POP is involved in cell proliferation [10].

We recently reported the effects of POP inhibitors, 3-({4-[2-(*E*)-styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) and benzyloxycarbonyl-thioprolyl-thioprolinal (Z-TT-CHO), and small interfering RNAs directed against human POP on the growth of NB-1 human neuroblastoma cells [11]. Our results indicated that POP inhibition suppressed the growth of NB-1 cells without inducing cell death. SUAM-14746-induced growth inhibition was associated with pronounced G_0/G_1 cell cycle arrest and reduced levels of phosphorylated retinoblastoma protein (pRb), cyclin E, and cyclin dependent kinase (CDK) 2, and increased levels of the CDK inhibitor $p27^{kip1}$ and the tumor suppressor p53. SUAM-14746 also induced a transient inhibition of S and G_2/M cell cycle phase progression, which was correlated with retarding the decreases in the levels of cyclins A and B. RNAi-mediated POP knockdown also resulted in inhibition of NB-1 cell growth, and this effect was accompanied by G_0/G_1 cell cycle arrest.

p53-Dependent G1 cell cycle arrest is well documented. After cells are exposed to stress, the half-life of the p53 protein significantly increases, and p53 accumulates in the nucleus of treated cells. A primary mechanism by which p53 negatively controls cell cycle progression is through the transcriptional activation of p21WAF1/CIP1 [12]. p21WAF1/CIP1 inhibits cyclin-dependent kinases, which block the phosphorylation and subsequent

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inactivation of pRb and causes the arrest of cell cycle progression in the G1/S phase. p53 has also been reported to be involved in G2/M cell cycle arrest as well as G1 arrest [13]. Because POP inhibition not only arrests the cell cycle at the G0/G1 phase but also impedes S and G2/M phase progression, we hypothesized that POP inhibition-induced growth suppression may be mediated by a p53-dependent pathway.

Thus, in the present study, we investigated the mechanism of cell growth inhibition by POP inhibition using a human gastric cancer cell line, KATO III, which had a p53 gene deletion [14]. In this cell line, the p53 pathway in POP inhibition-induced cell growth arrest was not a primary contributor to the inhibition mechanism. Our results also indicated that POP inihibitor-mediated cell cycle arrest in KATO III cells was associated with an increase in the proportion of cells in the quiescent G_0 state. This suggests that POP is a positive regulator of cell cycle progression, which regulates the exit from and/or reentry into the cell cycle by KATO III cells.

2. Materials and methods

2.1. Cells and reagents

KATO III (JCRB0611) human gastric cancer cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's Medium (DMEM) and RPMI-1640 medium (Sigma–Aldrich) supplemented with 50 μg/ml of kanamycin and 10% fetal calf serum (FCS). T98G (RCB1954) human glioblastoma cells were from the RIKEN RRC cell bank (Ibaraki, Japan) and cultured in DMEM supplemented with 50 μg/ml of kanamycin and 10% FCS. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The POP inhibitor 3-({4-[2-(*E*)-Styrylphenoxy]butanoyl}-L-4-hydroxyprolyl)-thiazolidine (SUAM-14746) was obtained from the Peptide Institute (Osaka, Japan).

Benzyloxycarbonyl-thioprolyl-thioprolinal (Z-TT-CHO) was synthesized by a previously reported method [15]. Anti-CDK2 and anti-pRb2/p130 antibodies were purchased from BD Bioscience (San Jose, CA, USA). Anti-cyclin E and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-cyclin D3, anti-CDK4, anti-CDK6, anti-p27Kip1, anti-pRb, anti-mouse IgG, and anti-rabbit IgG HRP-conjugated antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). An anti-Ki-67 antibody was purchased from Thermo Scientific (Waltham, MA, USA). An Alexa Fluor 488 anti-mouse IgG antibody was obtained from Molecular Probes (Eugene, OR, USA).

2.2. Cell growth assay and cell cycle analysis

Cell growth was assessed using a WST-1 assay (Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 3×10^3 cells/well. After incubation for 24 h, cells were treated with 0.5% DMSO (control) or with a range of doses of a POP inhibitor. After different treatment periods, the WST-1 reagent was added to cells for 4 h at 37 °C. The amount of formazan produced was determined using a spectrophotometric microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Cell viability was assessed using a trypan blue exclusion assay. Cells were seeded in 24-well plates at a concentration of 3×10^4/ml and incubated for 24 h. Cells were then treated with different concentrations of a POP inhibitor and stained with trypan blue.

Cell cycle stages were assessed by staining with propidium iodide (PI). Cells were treated with a POP inhibitor (60 μM) or 0.5% DMSO. At the indicated times, cells were harvested, washed with PBS, and gently fixed with 70% cold ethanol. Cells were then washed with PBS, treated with PBS containing 0.1 mg/ml of RNase at room temperature (RT) for 30 min, and resuspended in PBS containing 50 μg/ml of PI. Stained cells were analyzed with a FACScan (BD Bioscience) flow cytometer. Data were analyzed using ModFit software (Verity Software House, Topsham, ME, USA).

2.3. Ki-67 and PI staining for quiescent G_0 cell cycle status

Quiescent cell status was assessed using Ki-67/PI staining as previously described [16], with some modifications. Briefly, cells were gently fixed with 70% cold ethanol, washed with PBS containing 10% FCS, and then incubated with an anti-Ki-67 antibody at RT for 30 min. They were then washed, incubated with an Alexa Fluor 488 anti-mouse IgG antibody at RT for 30 min in the dark, washed, and resupended in PBS containing 10% FCS and 50 μg/ml of PI. Stained cells were analyzed with a FACScan flow cytometer.

2.4. Western blot analysis

Cells were rinsed with ice-cold PBS, suspended in CelLytic™ M lysis/extraction reagent (Sigma–Aldrich Co., St Louis, MO, USA) supplemented with 0.5 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, bestatin, E-64, pepstatin A, and leupeptin. Cells were lysed using three freeze–thaw cycles. Cell extracts were centrifuged and the supernatants were analyzed by 12% SDS–PAGE. The proteins in gels were transferred to nitrocellulose membranes (Bio-Rad), and blocked for non-specific binding with 5% skim milk in Tris-buffered saline, pH 7.4, with 0.1% Tween-20 (TBST) at RT for 1 h. The membranes were incubated with a primary antibody, followed by incubation with a corresponding HRP-conjugated secondary antibody. Immune complexes were detected using LumiGLO® Reagent (Cell Signaling). Immunoblotting for actin was used to verify equivalent amounts of loaded protein. Protein concentrations were determined using a BCA™ Protein Assay Kit (Thermo Scientific).

2.5. POP silencing by siRNA

The 25-nucleotide modified synthetic RNAs (stealth RNAi) were custom synthesized (Invitrogen, Carlsbad, CA, USA). The primer sequences were: hPOP-siRNA sense, 5′-GGGUGGAGCUGAGUUAUCUGAUGAU-3′, and antisense,

5′-AUCAUCAGAUAACUCAGCUCCACCC-3′. Stealth RNAi negative control was directly ordered from Invitrogen. Cells were seeded on a 60-mm dish and incubated for 24 h. Cells were transfected with 33 nM of hPOP-siRNA or negative control using Lipofectamine 2000 (Invitrogen). After 48 h, transfected cells were used for cell growth determinations.

3. Results

3.1. POP inhibition effects on cell proliferation and cell cycle progression

To investigate the effects of POP inhibition on KATO III cells' proliferation, we used POP specific inhibitors, SUAM-14746 and Z-TT-CHO, and POP silencing with siRNA. SUAM-14746 is a POP inhibitor that was obtained by screening numerous thiazolidine derivatives [17,18]. KATO III cells were treated with different concentrations of SUAM-14746, and then assessed for proliferation every 24 h by WST-1 assay. Fig. 1A shows cell growth was inhibited after treatment with SUAM-14746 in a time- and dose-dependent manner. The viability of cells treated with SUAM-14746