Research in Pharmaceutical Sciences, June 2015; 10(3): 200-205
Received: June 2014
Accepted: Aug 2014

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

uPAR peptide antagonist alters regulation of MAP kinases and Bcl-2 family members in favor of apoptosis in MDA-MB-231 cell line

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Abstract

Urokinase plasminogen activator receptor (uPAR) and its ligands play a major role in many tumors by mediating extracellular matrix degradation and signaling cascades leading to tumor growth, invasion and metastasis. Recently we introduced uPAR decapeptide antagonist with cytotoxic effect on MDA-MB-231 cell line. In this study we assessed the alteration in uPAR downstream signaling following treatment with the peptide antagonist. In this regard, extracellular-signal-regulated kinase (ERK) and p38 from mitogen-activated protein kinase family and Bcl-2, Bim and Bax from Bcl-2 protein family were investigated. Our data revealed that the peptide caused p38 activation and low ERK activation. On the other hand, the peptide induced down-regulation of Bcl-2 and up-regulation of Bim without Bax modulation. Changes in target protein expression/activation explain the apoptotic property of the peptide and highlight its potential to be used as a therapeutic agent in cancerous cells expressing high levels of uPAR.

Keywords: uPAR; Peptide antagonist; Bim; ERK; Bcl-2 family

INTRODUCTION

The urokinase plasminogen activator receptor (uPAR) is a cell surface protein which binds to the cell membrane via glycosyl phosphatidylinositol anchor which has been implicated in progression, metastasis and angiogenesis in multiple cancer types (1,2). These activities are initiated through interaction with N-terminal growth factor like-domain of its proteolytic ligand; urokinase plasminogen activator (uPA) (3,4), resulting in extracellular matrix (ECM) degradation and intracellular signaling (1,5,6). High expression of uPAR and uPA has been associated with poor prognosis (7,8). The uPAR-uPA is involved in cell proliferation (9,10), adhesion (1,10), angiogenesis (11,12) and invasion (10,13,14) of tumors. Other cell surface proteins such as integrin and vitronectin are also crucial in inducing uPAR signaling cascade (15,16).

Several signaling pathways are activated via uPAR including extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), p38 MAPK, Tyr kinases focal adhesion kinase (FAK) and the small G-protein, Rac (17-21). ERK pathway is over-activated in many cancer types, accelerating cell proliferation and metastasis (22). In MDA-MB-231 breast cancer cell line, block of uPA/uPAR by anti-uPA antibody decreases the level of phosphorylated ERK (p-ERK) and thereby promotes apoptosis (23).

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P38 is a stress-activated MAP kinase which can act as a tumor suppressor and apoptosis regulator (21,24). uPAR binding to uPA and integrin regulates tumor cell proliferation by ERK/MAPK activation and phospho-p38 (p-p38) down-regulation (20).

Bcl-2 family members are regulators of cell fate having aberrant expression in many cancer types (25-27). These members can function either as pro-survival or pro-apoptotic molecules, harboring conserved regions termed Bcl-2 homology domains (28,29).

It has been proposed that the relative sensitivity of cells to cytotoxic stimuli is governed by the ratio of Bax to Bcl-2 and other anti-apoptotic Bcl-2 family proteins (30). Hence, having a better understanding of apoptotic stimuli such as uPAR antagonists, it is crucial to screen the treated cells for any possible modulation of Bcl-2 family expression.

In this regard, we investigated the effects of decapeptide antagonist designed for uPAR on ERK and p38 activation as well as probable changes in Bcl-2 family levels. These data shed some light on uPAR downstream signaling pathways affected by our newly designed uPAR antagonist.

MATERIALS AND METHODS

Peptide sequences and preparation

The peptide with 10 amino acids was designed based on the interaction site of uPA with its receptor and was synthesized (GenScript, USA). Lyophilized peptide was dissolved in sterile water at pH=7 based on analysis data sheet. Peptide was stored as a 10 mM stock solution at -20 °C.

Cell culture

MDA-MB-231, human breast cancer cell line was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin (all from PAA, Austria) in a humidified air containing 5% CO_2 at 37 °C.

Western blot analysis

To evaluate phospho-ERK (p-ERK), p-p38, Bcl-2 and BimEL expression, MDA-MB-231 cells were seeded in 6-well plates and induced to enter a quiescent state by serum deprivation (1% FBS) for 24 h. Following serum starvation, the cells were treated with 250 µM of the peptide and incubated for 15 and 60 min for the expression of mentioned proteins and 24 h for bcl-2 family. Subsequently, the cells were harvested into Laemli buffer containing Tris 62.5 mM, pH 6.8, dithiothreitol 50 mM, sodium dodecyl sulphate (SDS) 2%, Glycerol 10%, and bromophenol blue 0.25% (w/v). Prepared lysates were boiled for 9 min and 40 µg of each samples were loaded on 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and subjected to gel electrophoresis. Afterwards, proteins were transferred onto PVDF membrane (Roche, Germany) and blots were blocked gently with casein blocking buffer (10% casein in tris-buffered saline (TBS) and 0.1% tween 20) at room temperature for 2 h and probed thereafter with respective p-ERK, Bcl-2, Bim and p-p38 primary antibodies (all from cell signaling, MA, USA) at 4 °C overnight. As an internal control, the correspondent blots were stripped and reprobed with ERK primary antibody, p38 primary antibody (both from Cell Signaling, MA, USA) and β-Actin (Santa Cruz, USA). Incubated blots were further rinsed with TBS comprising 0.1 % Tween 20 followed by exposure to goat anti mouse/rabbit horse radish peroxidase conjugate secondary antibody (Biorad, USA) for an hour at room temperature. Protein bands were detected by BM chemiluminescence western blotting kit (Roche, Germany) followed by exposure to X-ray film. Densitometry quantification was analyzed using ImageJ software (NIH, USA). The protein expression was normalized to internal control and calculated as ratio to control.

RNA isolation, reverse transcription and real-time polymerase chain reaction

Expressed levels of Bcl-2 and Bax were quantified using real-time polymerase chain reaction (RT-PCR) assay. The total RNA was purified by the aid of TriPure isolation reagent

(Roche, Germany) following manufacturer's protocol. The extracted RNA was then checked for quality and integrity by measuring OD 260/280 ratio and denaturing gel electrophoresis respectively. One microgram of RNA was used for cDNA synthesis using PrimeScript first strand cDNA synthesis kit (TAKARA, Japan), where RT master mix (containing dNTPs, random primers and RTase) along with the RNA was incubated at 42 °C for 60 min.

Quantitative RT-PCR (qRT-PCR) was performed exploiting a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and a SYBR Premix Ex Taq (Tli RNase H Plus) kit (TAKARA, Japan). Standard reaction mixture (20 µl) was assembled with 10 µl SYBR Premix Ex Taq 2x, 2 µl of template cDNA, 7 µl ultra-pure water and primers at the concentration of 250 nM. The primers for the target genes were designed by Primer Express software that spans exon-exon boundaries. Primer sequences for Bcl-2 were forward 5'- CCAGGATAACG GAGGCTGGGAT-3' and reverse 5'- GGCA GGCATGTTGACTTCACTTGT-3'; for Bax were forward 5'- CCCTGGACCCGGTGCCT CA-3' and reverse 5'- CACGGCGGCAATCA TCCTCTG-3'; for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were forward 5'-GAAGGTGAAGGTCGGAGTCAAC-3' and reverse 5'- CAGAGTTAAAAGCAGCCCTG GT-3'. GAPDH was served as internal control. A total of 40 amplification cycles were performed, and each cycle consisted of heating the mixtures to 94 °C for 5 s followed by cooling them to 60 °C for 30 s. Experiments were performed in triplicate for each data point and quantitative values were expressed in terms of threshold cycle and were analyzed by REST software.

RESULTS

Effect of uPA antagonist on the pattern of p38 and ERK activation

The pattern of ERK and p38 activation in the presence of decapeptide antagonist of uPA was studied on MDA-MB-231 cell line. This human breast cancer cell line expresses high levels of uPAR (23). The cells were treated with 250 µM of peptide for 15 and 60 min and protein expression was analyzed by western blotting.

As it is shown in (Fig. 1A), uPA antagonist decreased p-ERK up to 40% after 60 min. On the other hand, p-p38 level was drastically accumulated by the peptide even after 30 min and remained unchanged after 60 min compared to control (Fig. 1B).

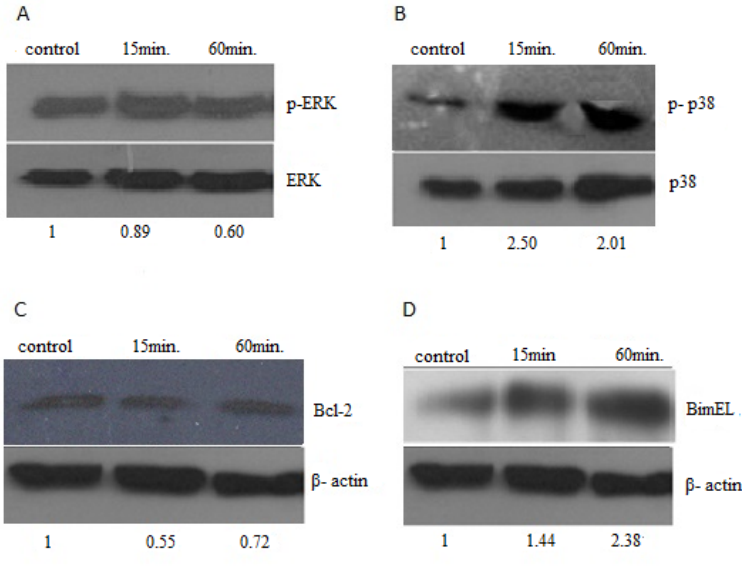

Fig.1. The effect of uPAR antagonist decapeptide on the activation of ERK, P38 and expression of Bcl-2 and BimEL. The protein expression of A; p-ERK, B; p-p38, C; Bcl-2 and D; BimEL were determined by western blot analysis after 15 and 60 min treatment with peptide in MDA-MB-231 cell line. The expression ratios were calculated compared to control and presented bellow each blot. ERK, p38, and β-actin were served as internal controls.

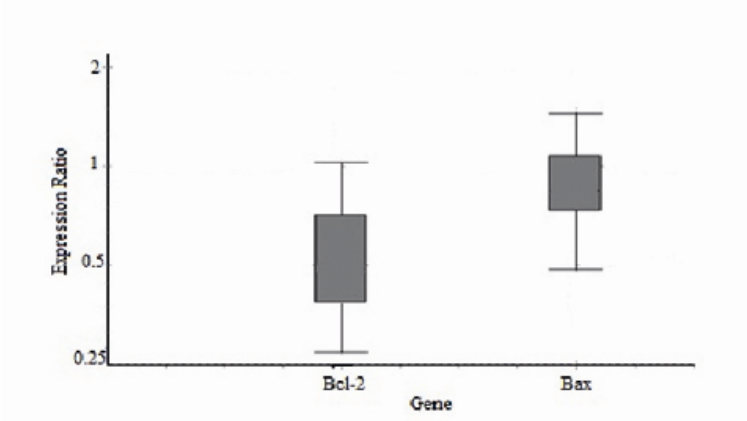

Fig.2. Effect of uPAR antagonist decapeptide treatment on expression of Bcl-2 and Bax. Bcl-2 is down regulated by 50% (expression to control=0.518, *P*-value=0.047) in treated samples, however; Bax remained unchanged (expression to control=0.878, *P*-value=0.468). Boxes represent the interquartile range, or the middle 50% of the observations. The dotted line represents median gene expression. Whiskers represent the minimum and maximum observations.

Effect of uPA antagonist on bcl-2 and BimEL

Protein expression of Bcl-2 and BimEL (Bim extra long, 23 kd) was evaluated by western blot analysis on MDA-MB-231. Bcl-2 as a pro-apoptotic member of Bcl-2 family was down-regulated around 30% after 60 min (Fig. 1C) and continued up to 24 h (data not shown). However, the peptide augmented the expression level of anti-apoptotic member Bim by two fold within 60 min of treatment (Fig. 1D), but this alternation did not last for 24 h (data not shown).

Effect of uPA antagonist on Bcl-2 mRNA level and Bax expression

To determine the consequences of peptide treatment on Bcl-2 and Bax mRNA expression levels, we measured expression quantitative RT-PCR with GAPDH as an endogenous normalization control. Results indicated that transcription of Bcl-2 was reduced significantly in peptide treated cells by 50%, compared to the control group using GAPDH as the reference gene. In contrast, Bax mRNA expression was not changed in treated cells (Fig. 2).

DISCUSSION

The signaling pathways initiated through uPAR have been implicated in invasion, proliferation and metastasis in cancerous cells (31). Likewise its upregulation is restricted to tumor tissues and rarely expressed in adjacent normal tissues (32). Therefore, uPAR has been an attractive therapeutic agent for cancer treatment. We tested the downstream signaling of a decapeptide as uPAR antagonist that inhibited proliferation of MDA-MB-231 breast cancer cell line (unpublished data).This peptide antagonist contacts all three domains of uPAR in the central cavity of the receptor.

Our data showed that the activated ERK (p-ERK) was decreased after treatment of the cell with peptide antagonist. On the other hand, p-p38, the active form of p38 MAPK, was up-regulated simultaneously following the treatment. It has been distinguished that the switch between dormancy and cell proliferation is dependent on the relative ratio of p-ERK and p-p38; as shift in balance toward p-p38 induces tumor cell death whereas p-ERK accumulation results in cell proliferation (21). Hence, the antagonist peptide of uPAR could cause MDA-MB-231 breast cancer cells to undergo apoptosis.

Bcl-2 protein family members have been proposed to play a central role in regulating apoptosis (29). Following treatment with decapeptide, the level of Bcl-2 decreased at both transcript and protein levels along with a significant increase in BimEL expression. These findings are in line with p-ERK down-regulation; since it has been shown that Bcl-2 level has a direct relation on ERK activation (33). Up-regulation of BimEL, the most abundant isoform of Bim, can be in part due to

the ERK deactivation, as p-ERK is known to act as an inducer of BimEL ubiquitination and its degradation (34-37). As a result, the novel peptide antagonist can alter equilibration of Bcl-2-ERK-BimEL axis in favor of apoptosis. Our data indicate that the cell death induction by decapeptide is mediated via two mechanisms including reduced Bcl-2 expression and enervation of Bcl-2 by Bim up-regulation. Bcl-2 is known to act as a sequesterant of Bax and could lead to Bax multimerization and as a result predispose cells to enter apoptosis (38). This property of the peptide can be used to specifically target cancer cells, since Bcl-2 is overexpressed in multiple cancer types (39). Bim regulates Bax-Bcl2 interaction in a competitive manner, whereby, increased Bim level overcomes the restraint of activated Bax by Bcl-2 (30,40-43).

CONCLUSION

Accordingly, our observations suggest that uPAR decapepide antagonist might be beneficial for therapeutic intervention in tumors overexpressing uPAR. Thus this peptide can be used as an antagonist for uPA receptor activation as well as a template for peptidomimetic drug design. Further *in vivo* studies as well as characterizing its ability to target other cancerous cells could possibly lead to introducing a novel drug.

ACKNOWLEDGMENTS

This work has been supported by a grant from Tehran University of Medical Sciences.

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