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Inhibition of Human Breast Cancer Cell (MBA-MD-231) Invasion by the Ea4-Peptide of Rainbow Trout Pro-IGF-I

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Abstract It was shown previously that Ea4-peptide of trout pro-IGF-I exerted mitogenic activity in non-transformed cells and inhibited colony formation in a soft agar medium of established human cancer cells. Here we report that the same peptide inhibits the invasion of human breast cancer cells (MDA-MB-231) through a matrigel membrane in a dose-dependent manner. The expression of urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor 1 (PAI1) genes in MDA-MB-231 cells were downregulated by treatment with rtEa4-peptide. The inhibition of expression of these genes in response to rtEa4-peptide treatment was reduced to the control level when inhibitors for c-Jun N-terminal kinase 1/2 (JNK1/2), mitogen activated protein kinase kinase 1/2 (Mek1/2), p38 mitogen activated protein kinase (p38 MAPK), phosphatidylinositol 3-kinase (PI3K), and phosphokinase C (PKC) were used. These results suggest that inhibition of invasion of MDA-MB-231 cells by rtEa4-peptide may be mediated via the suppression of *uPA*, *tPA*, and *PAI1* gene activities through signal transduction pathways. J. Cell. Biochem. 99: 1363–1373, 2006.

Key words: rainbow trout Ea4-peptide; pro-IGF-I; cancer cell invasion

Many peptide hormones are initially synthesized as the pro-peptide precursors, which are subsequently processed through the post-translational modification to yield mature peptide hormones [Steiner et al., 1980]. In this fashion, the mature insulin-like growth factors (IGFs) [IGF-I and IGF-II], containing the B, A, C, and D functional domains, are produced from their pro-hormones by proteolytic removal of the E-peptide domains [Duguay, 1999]. Multiple forms of pro-IGF-I peptides have been identified in various animal species from fish to human with differences only in their C-terminal E-peptides [Rotwein et al., 1986; Shamblott and Chen, 1993; Chew et al., 1995]. In human, three isoforms of pro-IGF-I (i.e., pro-IGF-Ia, pro-IGF-Ib, and pro-IGF-Ic) have been identified [Chew et al., 1995]. These three pro-IGF-I isoforms contain identical mature IGF-I [70 amino acid (AA) residues] but different E-peptides (hEa, 35 AA; hEb, 77 AA; and hEc, 40 AA). Similarly, four isoforms of pro-IGF-I E peptides with identical mature IGF-I but different lengths of E-peptides (35, 47, 62, and 74 amino acid residues, respectively) were also identified in rainbow trout (*Oncorhynchus mykiss*) [Shamblott and Chen, 1993]. These E-peptides were designated as rtEa1, rtEa2, rtEa3, and rtEa4.

It was generally believed that E-peptides of IGFs played little or no obvious biological function except for their possible role in facilitating the biosynthesis of mature IGFs. Recently, hEa-, hEb-, and hEc-peptides were shown to possess mitogenic activity in established human cell lines [Hylka et al., 1985; Siegfried et al., 1992; Yang and Goldspink, 2002]. Similar to the E-peptides of human pro-IGF-I, rtEa4-, rtEa3-, and rtEa2-peptides but not rtEa1 were shown to exert mitogenic activity in a non-transformed mouse cell line, NIH3T3, and primary caprine mammary gland cells in culture [Tian et al., 1999]. To our surprise, in addition to exerting mitogenic activity, studies conducted in our laboratory

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showed recently that rtEa4-peptide also exhibited other biological activities in many established human malignant cells such as breast cancer cells (MDA-MB-231 and ZR75-1), neuroblastoma cells (SK-N-F1), colon cancer cells (HT-29), prostate cancer cells (PC-3), and ovarian cancer cells (SKOV and VASCOR) [Chen et al., 2002; Kuo and Chen, 2002]. These activities include: (i) inducing morphological differentiation and increasing cell attachment; (ii) inhibiting anchorage-independent cell division; and (iii) inhibiting cancer cell invasion through matrigel membrane in vitro [Chen et al., 2002; Kuo and Chen, 2002]. Although, many biological activities of rtEa4-peptide have been identified in recent years, nearly nothing is known about the action of this peptide at the molecular level. Kuo and Chen [2003] have recently shown that rtEa4-peptide bound specifically to two-membrane binding components with the binding affinities at approximately 10^{-11} and 10^{-6} M, respectively in SK-N-F1 cells. Furthermore, they also showed that the action of rtEa4-peptide on the induction of morphological differentiation in SK-N-F1 cells may have been mediated through phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated protein kinase 1/2 (ERK1/2) of the mitogen activated protein kinase (MAPK) pathways [Kuo and Chen, 2002].

Cancer metastasis, spreading of cancer cells from the initial sites to distant locations, is the primary cause of mortality in cancer patients [Engers and Gabbert, 2000]. Cancer metastasis involves multi-sequential steps [Engers and Gabbert, 2000], and among these steps, cell adhesion and invasion are the two most critical steps of tumor metastasis. In previous studies [Siri et al., 2006], we have shown that rtEa4-peptide promoted attachment of established MDA-MB-231 cells to the substratum, suggesting that rtEa4-peptide may inhibit cancer cell invasion. To investigate whether rtEa4-peptide can inhibit cancer cell invasion, we have determined the inhibitory activity of rtEa4-peptide on invasion of MDA-MB-231 cells through matrigel in vitro. Since cancer cell invasion requires specific proteolytic enzymes such as urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) [Stetler-Stevenson et al., 1993], we hypothesized that rtEa4-peptide may down-regulate the expression of these genes, and consequently, resulting in an inhibition of cancer cell invasion. We further hypothesized that if rtEa4-peptide could inhibit cancer cell invasion, it might act through the activation of signaling molecules of FAK/MAPK in the signal transduction pathway. In this article, we report results of studies conducted to prove these hypotheses.

MATERIALS AND METHODS

Cell Cultures

Human breast cancer cells, MDA-MB-231, were obtained from American Type Culture Collection (ATCC, Rockville, MD). Single-cell subclones of MDA-MB-231 cells were isolated from the ATCC stock and routinely maintained in DMEM/F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY) at 37°C under a humidified atmosphere with 5% CO_2.

Preparation of Recombinant rtEa4-Peptide

Recombinant rtEa4-peptide was prepared from *E. coli* cells containing an expression vector (pET15b) with the cDNA of rtEa4, and the control protein was prepared from *E. coli* cells containing the expression vector (pET15b) without the rtEa4 cDNA by the method described by Tian et al. [1999] and Kuo and Chen [2002].

Matrigel Invasion Assay

Cell invasion assay was conducted in a 24-well BD BioCoat matrigel invasion chamber (Becton-Dickinson, Bedford, MA) following the protocol recommended by the manufacturer. About 2.5×10^4 MDA-MB-231 cells in 500 μl of DMEM/F12 supplemented with 1.25% FBS and 0–5.12 μM rtEa4-peptide or 5.12 μM of control peptide were plated onto each insert of matrigel or control invasion chambers, and the inserts were placed into a 24-well plate containing DMEM/F12 medium supplemented with 10% FBS. MDA-MB-231 cells were allowed to invade through the matrigel insert or control insert for 22 h at 37°C in a humidified atmosphere with 5% CO_2. After 22 h of incubation, non-invasive cells on the upper layer of each insert were removed with cotton swabs and the invaded cells underneath the inserts were stained with Quick-Diff stain kit (Becton-Dickinson Labware), observed under an Olympic inverted microscope (200× magnification). Pictures of cells at the approximate center views of each

insert were taken. The number of invaded cells was counted and the average data from three invasion inserts were used to represent each data point. Two independent experiments were conducted to ensure the reproducibility of the results. The percent of cells invasion was calculated as following:

$$\text{Percent cell invasion} = \frac{\text{Number of cells invaded through matrigel insert}}{\text{Number of cells invaded through control insert}} \times 100\%$$

Isolation of Permanent Transformants

Two gene constructs, CMV-Ea4-cDNA-IRES-EGFP and CMV-IRES-EGFP, cloned in the backbone of pIRES2-EGFP (Clontech, Palo Alto, CA) were used in the transfection studies. A signal peptide sequence of hIGF-I was fused to the N-terminus of the Ea4-peptide cDNA to ensure that the synthesized E-peptide would be secreted out of the cell. The expression of these gene constructs was driven by cytomegalovirus (CMV) promoter. Single-cell subclone of MDA-MB-231 cells were cultured to 90% confluence in DMEM/F12 (1:1) medium supplemented with 10% FBS. About 5×10^6 cells were harvested and resuspended in 1 ml of serum-free DMEM/F12 (1:1) medium containing 20 μg of constructs. The cells were electroporated in a BRL Cell-Porator (Life Technologies) using the following settings: low Ω, 1,180 micro Faraday (μF) capacitance, and two pulses at 200 volts. Following electroporation, cells were resuspended in 12 ml of DMEM/F12 (1:1) medium supplemented with 10% FBS and seeded into two T-25 flasks to recover. Permanent transfectants expressing green fluorescence protein (GFP) were enriched in a medium containing neomycin (G418) at 1 mg/ml for 10 days and followed with 500 μg/ml for continuous maintenance. Individual green cell clones of transfectants were isolated from the enriched population by the method of serial dilution.

Measurement of Gene Expression by Real-Time RT-PCR

The expression levels of *uPA*, *tPA*, *PAI1*, and *TIMP1* genes were determined by a 2-step real-time RT-PCR with SYBR Green I incorporation using an iCycler thermocycler (Bio-Rad, Hercules, CA). Primers specific for each gene were designed using vector NTI software. The fidelity of the primers was examined by a melting curve analysis and by electrophoresing the PCR products on agarose gels. The melting curve was performed after the regular 50 cycles of amplification by decreasing the temperature from 95°C to 25°C at 0.5°C decrements per 10 s. The primer sequences used in this study are listed in Table I. Optimum annealing temperature, $MgCl_2$ concentration, and SYBR Green I concentration were also tested to obtain the amplification efficiency greater than 95%.

RNA was extracted from cells using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). To confirm that the RNA samples were free of DNA contamination, RNA samples were used as templates for direct amplification of β-actin sequence without prior reverse transcription. One microgram of total RNA was reverse transcribed in a 20 μl reaction volume containing 100 ng oligo dT, 10 mM dNTP, 200 mM DTT, 1× buffer, and 1 μl PowerScript reverse transcriptase (Clontech) at 42°C for 1.5 h. The PCR reaction was carried out in a 96-well plate. One tenth of each cDNA reaction was used in a PCR reaction volume of 100 μl containing 0.2 mM dNTP, 3 mM $MgCl_2$, 0.4 μM of each primer, 0.01 μM fluorescein, 1:20,000 dilution of 10,000× SYBR Green I, and

TABLE I. Primer Sequences

Accession number	Gene	Sequences	Amplicon size (bp)
XM_044354	Urokinase-type plasminogen activator (uPA)	Upper primer 5′ATGCTGTGTGCTGCTGACC3′ Lower primer 5′CTTCCTTGGTGTGACTGCG3′	222
XM_005024	Tissue-type plasminogen activator (tPA)	Upper primer 5′CAGGACTTGGTCTGCTGATTTC3′ Lower primer 5′GCTGCTCACGGTGACAGGTC3′	153
XM_051249	Plasminogen activator inhibitor, type 1 (PAI1)	Upper primer 5′GCCATTACTACGACATCCTG3′ Lower primer 5′CAGCCTGAAACTGTCTGAAC3′	261
XM_033879	Tissue inhibitor of metalloproteinase 1 (TIMP1)	Upper primer 5′GCGGATACTTCCACAGGTCC3′ Lower primer 5′GCATTCCTCACAGCCAACAG3′	183
BC014861	β-actin	Upper primer 5′TCGTGCGTGACATTAAGGAG3′ Lower primer 5′AGGAAGGAAGGCTGGAAGAG3′	178

1× PCR buffer. Twenty microliters of each reaction mixture was used per well and the average data of four wells were collected to represent each data point. At least two independent experiments were performed to ensure the reproducibility of the results. The amplification profile contained the following cycles: 1 cycle of 95°C for 3 min, 50 cycles of 95°C for 15 s, desired annealing temperature for 15 s, and 72°C for 30 s. The cycle threshold, C_T, was determined from the fluorescence value, which was 10 times the mean standard deviation of fluorescence of the base line cycles. The relative gene expression was determined using arithmetic formula: $2^{-[S\Delta CT - C\Delta CT]}$. ΔCT is the difference in C_T value between the gene of interest and the housekeeping gene (β-actin). SΔCT−CΔCT is the difference between the sample ΔCT and control ΔCT.

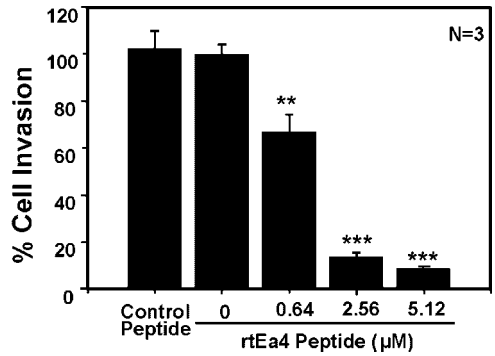

Fig. 1. Effect of rtEa4-peptide on the invasion of MDA-MB-231 cells through the matrigel. The cell invasion assay was carried out in a 24-well Biocoat matrigel plate (Becton-Dickinson) following the method described in ''Materials & Methods.'' Percents of MDA-MB-231 cells, which invaded through the matrigel chamber, were calculated and the average results from three matrigel inserts were calculated for each data point. Two independent experiments were conducted and similar results were obtained. $^{***}P \leq 0.001$ and $^{*}P \leq 0.01$.

Gene Regulation

The regulation of expression of *uPA*, *tPA*, *PAI1*, and *TIMP1* genes in response to rtEa4-peptide treatment via the signaling molecules of FAK/MAPK pathway was studied using the following inhibitors: inhibitor for mitogen activated protein kinase kinase, PD98095 (Promega, Madison, WI); inhibitor for MAPK, SB202190 (Promega); inhibitor for Jun kinase, SP600125 (Promega); inhibitor for Protein kinase C, Tamoxifen (Sigma, St. Louis, Mo), and inhibitor for PI3K, Wortmannin (Sigma). Approximately 1×10^6 cells were treated with 0–10 μM of the inhibitors for 2 h prior to the addition of 2.56 μM of rtEa4-peptide. Cells were incubated for an additional 1.5 h and RNA was extracted from the cells as mentioned previously. The expression of the above genes was determined using a 2-step real-time RT-PCR as described earlier. At least two independent experiments were performed to ensure the reproducibility of the results.

RESULTS

Rainbow trout Ea4-peptide reduced cell invasion and suppressed the expression of protease genes. To test whether rtEa4-peptide exhibited anti-invasion activity in MDA-MB-231 cells, the invasive activity of MDA-MB-231 cells were assessed in a matrigel invasion chamber in the presence of various concentrations (0–5.12 μM) of rtEa4-peptide. As shown in Figure 1, a dose-dependent inhibition of MDA-MB-231 cell migration across the matrigel membrane over a period of 22 h was observed in the presence of rtEa4-peptide. Percentages of MDA-MB-231 cells that had invaded through the matrigel were $66.9 \pm 7.2\%$, $13.8 \pm 1.5\%$, and $8.6 \pm 0.9\%$ when cells were treated with 0.64, 2.56, and 5.12 μM of rtEa4-peptide, respectively. In contrast to rtEa4-peptide, the control proteins did not exhibit any significant inhibition on MDA-MB-231 cell migration across the matrigel membrane.

Results of preliminary microarray screening conducted in our laboratory (Chen et al., unpublished results) showed that *uPA*, *tPA*, and *PAI1* genes were downregulated in MDA-MB-231 cells transfected with the rtEa4-peptide gene whereas tissue inhibitor of metalloproteinase 1 (*TIMP1*) gene was upregulated. To confirm these preliminary results, we determined the levels of uPA, tPA, PAI1, and TIMP1 mRNAs in MDA-MB-2s31 cells transfected with rtEa4-peptide gene and the control construct by comparative real-time RT-PCR. Similar to the results of microarray analysis, the levels of uPA, tPA, and PAI1 mRNAs were suppressed in rtEa4 gene transfected cells by 0.50 ± 0.05, 0.64 ± 0.03, and 0.39 ± 0.04 fold, respectively (Fig. 2). However, unlike the result of microarray analysis, the level of TIMP1 mRNA was reduced by 0.77 ± 0.19 fold in cells transfected with rtEa4-peptide gene (Fig. 2). Furthermore, the levels of these mRNA species were also

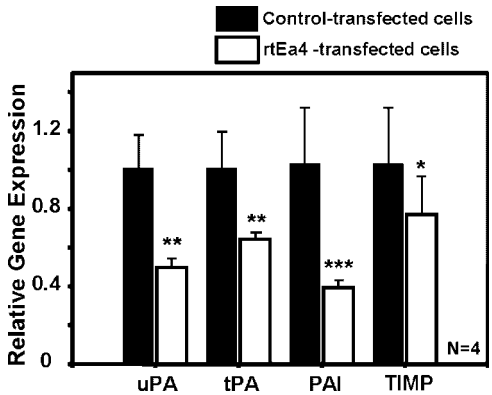

Fig. 2. Expression of *uPA, tPA, PAI1*, and *TIMP1* genes in rtEa4- and control-transfected cells. The levels of the expression of *uPA, tPA, PAI1*, and *TIMP1* genes were determined using a two-step real-time RT-PCR with SYBR Green I incorporation. RNA was isolated from cells transfected with rtEa4-peptide or control gene construct using Trizol reagents following the recommended protocol from the manufacturer (Invitrogen). First-stranded cDNA was obtained from the reverse transcription reaction and was used as the template in the PCR amplification reaction. The expression of the above genes was normalized with the expression of β-actin gene and the relative gene expression of cells transfected with rtEa4-peptide or control gene construct was determined using an arithmetic formula: $2^{-[S\Delta CT-C\Delta CT]}$ as described in the Materials and Methods. At least four independent experiments were conducted and similar results were obtained. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$.

determined in MDA-MB-231 cells treated with rtEa4-peptide in culture. Similar to the results from rtEa4-peptide gene transfected cells, the levels of uPA, tPA, PAI1, and TIMP1 were suppressed when cells were treated with rtEa4-peptide for 1.5 h (Fig. 3). As the increasing amounts of rtEa4-peptide were added to the cells, lower levels of mRNAs were detected for each gene, suggesting that the suppression of uPA, tPA, PAI1, and TIMP1 gene expression by rtEa4-peptide was dose dependent.

Since MDA-MB-231 cells treated with rtEa4-peptide resulted in reduction of levels of uPA and tPA mRNAs, these results suggest that suppression of uPA and tPA mRNA levels by rtEa4-peptide might reduce the invasive activity of MDA-MB-231 cells to migrate through the matrigel membrane. We further analyzed the expression levels of *uPA* and *tPA* genes in MDA-MB-231 cells treated with Ea4-peptide over a time course of 24 h. The levels of expression of *uPA* and *tPA* genes dramatically decreased in the first 1.5 h of the peptide treatment, and their expression levels gradually increased to control levels at 20 h after the treatment (Fig. 4). We hypothesized that a threshold level of rtEa4-peptide in the culture medium might be essential to maintain the effectiveness of rtEa4-peptide to downregulate the expression of *uPA* and *tPA* genes. To confirm this possibility, MDA-MB231 cells were treated with one dose of rtEa4-peptide for 24 h, and then fresh rtEa4-peptide was re-introduced into the culture medium. As expected, when the second dose of rtEa4 peptide was given to the cells at 24 h, the expression level of *uPA* gene was reduced to a level similar to the first dose of rtEa4-peptide at 1.5 h after initiation of the peptide treatment (data not shown). Since the levels of uPA and tPA mRNAs in cells transfected with rtEa4-peptide gene were much lower than those cells transfected with the control gene construct, we would expect the invasion activity of the rtEa4-peptide gene transfected cells be much lower or totally abolished when compared to cells transfected with the control construct. The invasion activity of MDA-MB-231 cells transfected with the rtEa4-peptide gene was reduced to below 8% when compared to the cells transfected with the control gene construct. Together, these results point to the possibility that rtEa4-peptide suppresses the invasive activity of MDA-MB-231 cells by downregulating the expression of *uPA* and *tPA* genes.

Regulation of uPA gene expression by rtEa4-peptide. Previously, we have demonstrated that the expression of fibronectin 1 gene in MDA-MB-231 cells induced by rtEa4-peptide may be mediated via the signaling molecules of FAK/MAPK transduction pathway (Siri and Chen, submitted for publication). We hypothesized that the downregulation of expression of *uPA* and *tPA* genes in MDA-MB-231 cells in response to rtEa4-peptide treatment could also be mediated through the same signaling molecules. To prove this hypothesis, we investigated the effect of PD98095, SB202190, SP600125, Tamoxifen, and Wortmannin on downregulation of *uPA* gene in MDA-MB-231 cells in response to rtEa4-peptide treatment. As shown in Figure 5, a dose-dependent inhibition of rtEa4-induced suppression of uPA gene expression was observed in MDA-MB-231 cells treated with each of these inhibitors. Without the inhibitors, MDA-MB-231 cells responded to rtEa4-peptide treatment by showing a 0.5-fold reduction of uPA mRNA level. However, by treating MDA-MB-231 cells with 0.1 μM of PD98095 or SB202190, 0.01 μM of SP600125, 1 μM of Tamoxifen, or 0.01 μM of Wortmannin,

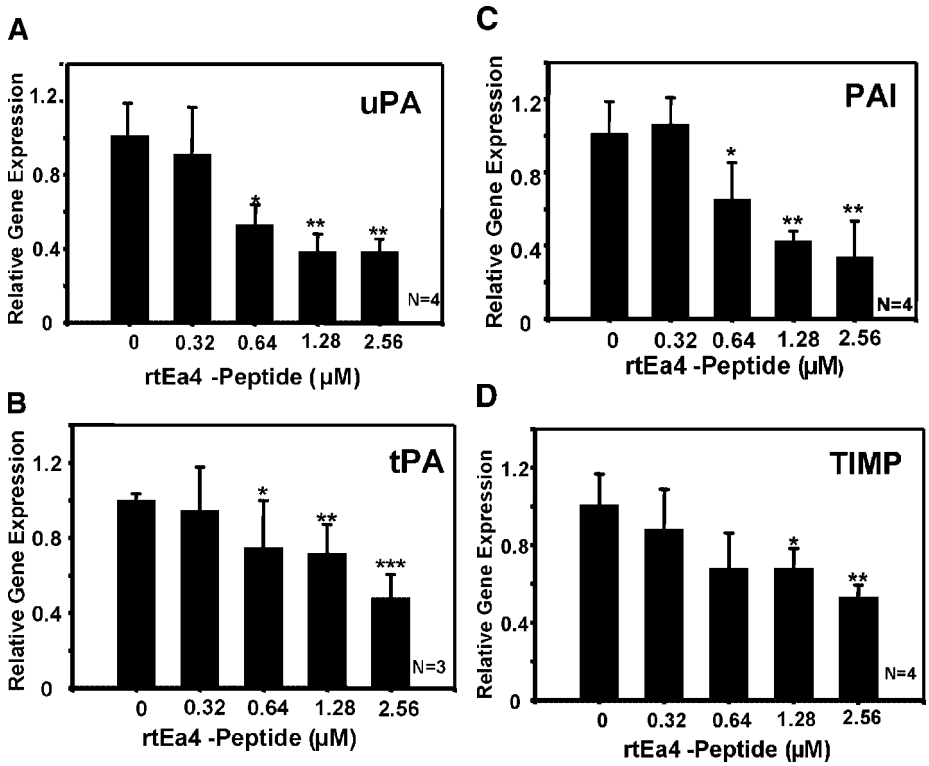

Fig. 3. Expression of urokinase-type plasmaminogen activator (uPA), tissue-type plasminogen activator (tPA), plasminogen activator inhibitor tyoe 1 (PAI1) and tissue inhibitor of metalloproteise 1 (TIMP1) genes in MDA-MB-231cells treated with various concentrations of rtEa4-peptide. Approximately 5×10^5 cells each were resuspended in DMEM/F12 and were treated with 0, 0.32, 0.64, 1.28, and 2.56 μM of rtEa4-peptide for 1.5 h. RNA was isolated from the treated cells using Trizol reagents and the expression of *uPA, tPA, PAI1,* and *TIMP1* genes were determined using a two-step real-time RT-PCR with SYBR Green I incorporation as described in Figure 2. The expression of the above genes was normalized to the expression of β-actin gene. The relative gene expression in cells treated with various concentrations of rtEa4-peptide was compared to that of cells treated with the control proteins. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$.

respectively, the levels of uPA mRNA in response to rtEa4-peptide treatment were indistinguishable from those in the control cells.

DISCUSSION

The first step of tumor metastasis involves the secretion of several proteolytic enzymes specific for degrading extracellular matrix (ECM) by cancer cells and thus allowing cancer cells to invade through basement membranes and migrate to other locations [Stetler-Stevenson et al., 1993; Koblinski et al., 2000]. uPA, which is a serine protease, was reported to be present at high levels in human breast cancer cell [Duffy et al., 1990; Spyratos et al., 1992; Duggan et al., 1995; Seddighzadeh et al., 1999]. Among three human breast cancer cell lines, MDA-MB-231, MDA-MB-435, and MCF-7, MDA-MB-231 cells express the highest level of uPA [Holst-Hansen et al., 1996]. Furthermore, high level of uPA expression was believed to correlate well to high invasive activity of MDA-MB-231 cells [Holst-Hansen et al., 1996]. In this study, we have demonstrated that MDA-MB-231 cells treated with rtEa4-peptide resulted in a reduction of invasion activity as well as suppression of the expression of *uPA* and *tPA* genes. These results are in good agreement with the following observations. Treatment of MDA-MB-231 cells with an anti-uPA monoclonal antibody resulted in the inhibition of cell invasion in a dose-dependent manner [Holst-Hansen et al., 1996]. Diaz et al. [2002] showed that RWP1 pancreatic tumor cells transfected with a tPA-antisense transgene resulted in a significant reduction of cell invasion. Therefore, our results strongly suggest that rtEa4-peptide inhibits the invasion of MDA-MB-231 cells by reducing the expression *uPA* and *tPA* genes. Upon closer examination, we observed that the reduction of uPA and tPA gene expression following rtEa4-peptide treatment lasted for 6 h, and thereafter levels of expression of both genes gradually

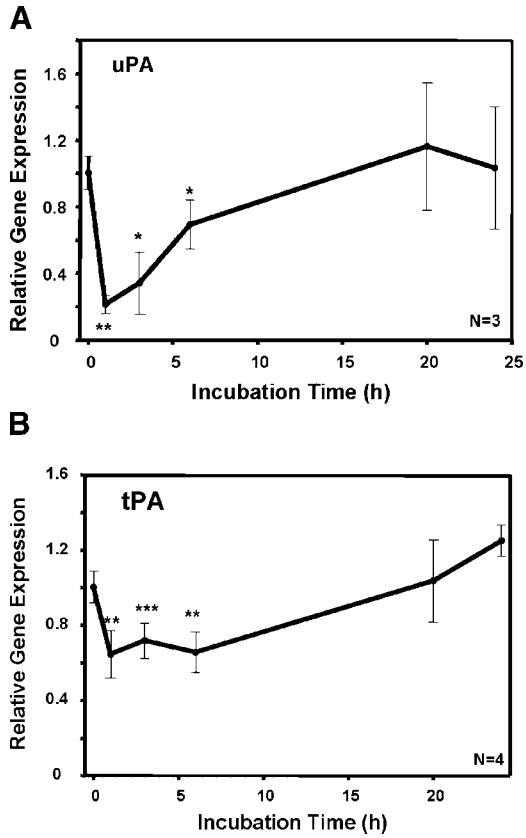

Fig. 4. Expression of urokinase-type plasmaminogen activator (uPA) and tissue-type plasminogen activator (tPA) genes in MDA-MB-231 cells treated with rtEa4-peptide in a time course of 24 h. About 5×10^5 cells each in DMEM/F12 medium were treated with or without 2.56 μM of rtEa4-peptide. RNA was collected from cells after the incubation of 0, 1, 3, 6, 20, and 24 h. RNA was isolated using Trizol and the levels of uPA and tPA mRNAs were determined by the real-time RT-PCR assay with SYBR Green I incorporation. The level of expression of each gene was normalized with that of β-actin gene and the relative gene expression of cells treated with rtEa4-peptide was compared to that of cells without the treatment. Each data point was the average results from four PCR amplification reactions, and at least three independent experiments were conducted, and similar results were obtained. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$.

returned to the control levels (Fig. 4). We hypothesized that an effective dose of rtEa4-peptide might be required to suppress the expression levels of *uPA* and *tPA* genes. This hypothesis was supported by the following several lines of evidence. When a second dose of rtEa4-peptide was added to MDA-MB-231 cells 20 h after the first dose, the level of uPA gene expression returned to the same level as that of the first dose of rtEa4-peptide treatment. We have determined that the half-life of rtEa4-peptide in culture was approximately 40 min (data not shown). Furthermore, constitutive production of rtEa4-peptide in MDA-MB-231 cells transfected with an rtEa4-peptide gene resulted in production of reduced level of uPA mRNA and the cells were unable to invade through matrigel in an invasion chamber assay (Figs. 2 and 5). It is conceivable that reduction of MDA-MB-231 cells invading through matrigel membrane in the invasion chamber by rtEa4-peptide treatment could be the consequence of cytotoxicity of the peptide. However, results of our studies disproved this possibility since treatment of MDA-MB-231 cells with 20 μM of rtEa4-peptide for 24 h did not result in any difference in the viability of cells when compared to controls (data not shown).

Previously we have shown that rtEa4-peptide induced upregulation of fibronectin 1 and laminin receptor genes in MDA-MB-231 cells may be mediated via the signaling molecules of FAK/MAPK transduction pathway [Siri and Maria Chen, 2006]. In the current studies we showed that the rtEa4-peptide induced down-regulation of uPA and tPA gene expression might also be mediated through the same signal transduction pathway. This conclusion was arrived from results of studies involving inhibitors of JNK1/2, Mek1/2, p38 MAPK, PI3K, and PKC. Several growth factors such as basic fibroblast growth factor [Roghani et al., 1996], epidermal growth factor [Laiho and Keski-Oja, 1989; Aguirre Ghiso et al., 1997], hepatocyte growth factor [Pepper et al., 1992], vascular endothelial growth factor [Mandriota and Pepper, 1997], and IGF-I and IGF-II [Guerra et al., 1996] have been shown to upregulate the expression of *uPA* gene via binding to tyrosine kinase receptors. Furthermore, addition of inhibitors to JNK1/2, Mek1/2, p38 MAPK, PI3K, and PKC, to cancer cells (i.e., human breast carcinoma cells, human dermal fibroblast cells, squamous carcinoma cells, and hepatocellular carcinoma cells) resulted in further reduction of uPA gene expression or suppression of cell invasion through matrigel membrane in the invasion chambers [Reifel-Miller et al., 1996; De Petro et al., 1998; Montero and Nagamine, 1999; Sliva et al., 2002a,b]. Kuo and Chen [2002] in our laboratory demonstrated earlier that addition of rtEa4-peptide resulted in the activation of ERK1/2 phosphorylation, and in this article we showed that rtEa4-peptide suppressed the expression of *uPA* gene. Therefore, we believe the mechanism that underlies the upregulation of uPA gene expression by basic fibroblast growth factor, epidermal growth

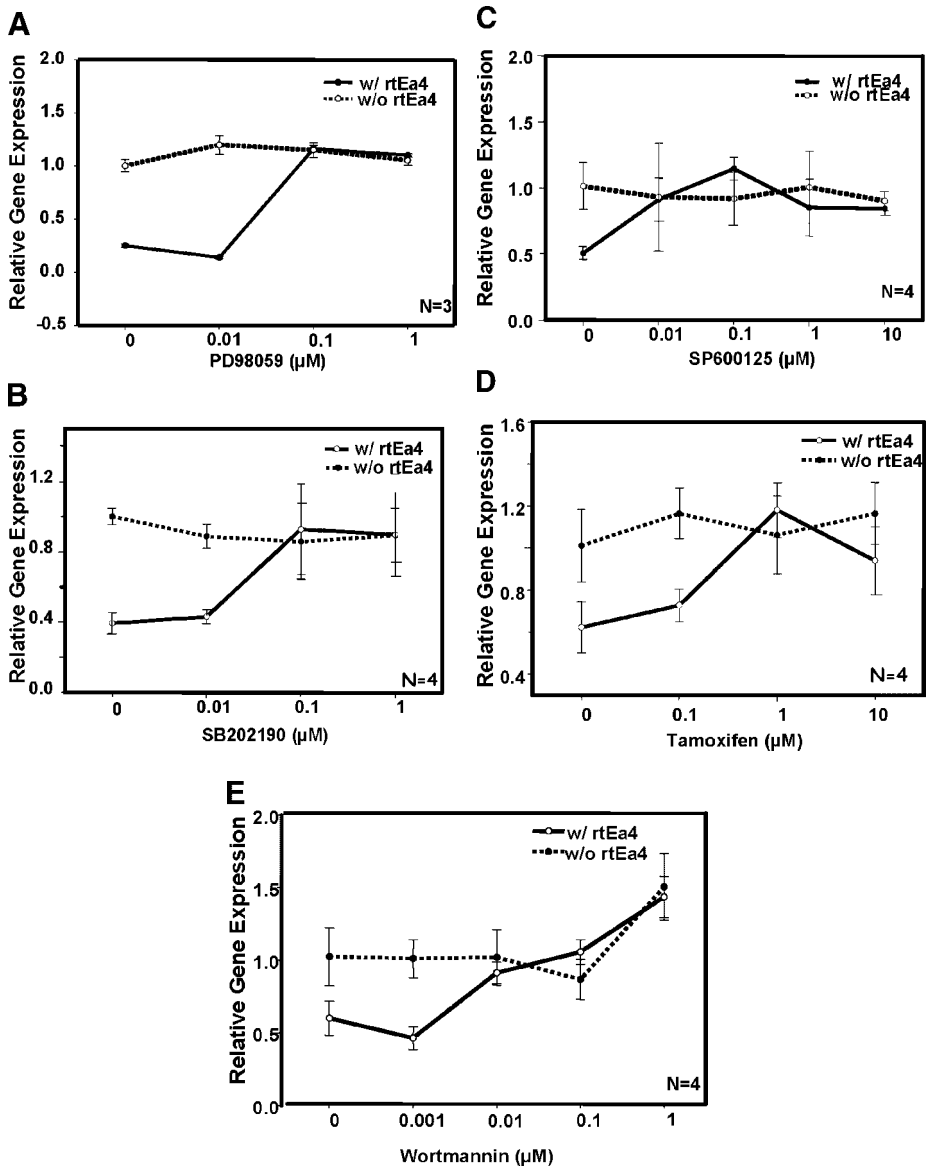

Fig. 5. Effects of inhibitors of signaling molecules in the focal adhesion kinase (FAK)/mitogen activated protein kinase (MAPK) pathway on the expression of urokinase-type plasmaminogen activator (uPA) gene in MDA-MB-231 cells treated with rtEa4-peptide. MDA-MB-231 cells (5×10^5 cells) in DMEM/F12 medium were treated for 2 h with 0–10 μM of Tamoxifen or SP600125 or with 0–1 μM of PD98095, SB202190, or Wortmannin. After 2 h of the treatment with inhibitors, cells were treated with or without 2.56 μM of rtEa4-peptide for 1.5 h at 37°C under humidified atmosphere with 5% CO_2. RNA was isolated from the treated cells or controls using Trizol reagent and the expression of uPA gene was normalized to the expression of β-actin. The expression of *uPA* gene in cells treated with the inhibitors, rtEa4-peptide, or both inhibitors and rtEa4-peptide were compared to that of cells without any treatment. Each data point was the mean value from four PCR amplification reactions and at least two independent experiments were performed with similar results.

factor, hepatocyte growth factor, vascular endothelial growth factor, or IGF-I and-II is different from rtEa4-peptide induced down-regulation of *uPA* gene.

Based on the results presented in this article and those reported by Siri and Maria Chen [2006], we proposed a molecular mechanism to account for the action of rtEa4-peptide in inhibiting invasion of MDA-MB-231 cells across matrigel membrane in an in vitro invasion assay chamber (Fig. 6). Since the effective concentrations of rtEa4-peptide in reducing cell invasion were similar to those in promoting cell attachment, we proposed that rtEa4-peptide might bind to specific cell membrane receptors (α2/ß1 integrin or other receptors). Activation of

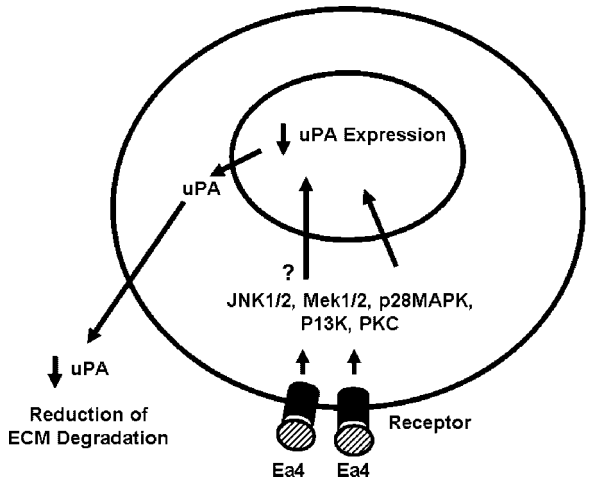

Fig. 6. A proposed model of action of rtEa4-peptide on cancer cell invasion. rtEa4-peptide binds to integrin receptor or other receptor and triggers the activation of FAK/MAPK signal cascades to downregulate the expression of *uPA* and *tPA* genes, and thus resulted in inhibition of invasion of cancer cells.

the receptors by rtEa4-peptide may result in the activation of JNK1/2, Mek1/2, p38 MAPK, PI3K, and PKC transduction cascades. As a result of activating the above-mentioned signaling molecules, the expression of fibronectin1 gene is induced. The secretion of fibronectin 1 peptide will assist the binding of MDA-MB-231 cells to extracellular matrices, thus restraining cells from the migration. In addition, the activation of these signaling molecules also resulted in the suppression of the expression of *uPA* gene. The reduction of uPA and other proteases may result in the reduction of plasmin and matrix metalloproteinases [Reuning et al., 1998] and, thus, leading to reducing the number of MDA-MB-231 cells that could invade through matrigel membrane. Unarguably more studies are required to prove this hypothesis.

Cancer cell attachment and cell invasion are critical processes of cancer metastasis [Roghani et al., 1996]. The activity of rtEa4-peptide in promoting cell attachment and reducing cell invasion suggest that rtEa4-peptide may possess anti-metastatic activity of cancer cells. Supporting this hypothesis, we demonstrated that seeding of invasive cancer cells (MDA-MB-231) transfected with rtEa4-peptide gene onto the chorioallantoic membrane of the developing chicken embryos resulted in no development of cancer nodules throughout the embryos when compared to that the invasive cancer cells transfected with a control construct without rtEa4-peptide gene (Chen et al., unpublished results). These results further suggest that rtEa4-peptide has the potential to be developed into a cancer therapeutic agent.

Over the past two decades, the roles of IGF-I in regulating numerous developmental processes and cellular activities have been intensively investigated. However, the biological functions of E-peptides that are part of the pro-IGF-I have never been addressed until recently. Tian et al. [1999] showed earlier that Ea4-peptide of trout pro-IGF-I possessed mitogenic activity in non-transformed cells. More recently Chen et al. [2002] and Kuo and Chen [2002] showed that the same peptide exerted inhibitory activities on anchorage-independent growth and invasion in established human cancer cells in vitro. In the present studies we have shown that rtEa4-peptide inhibited invasion of the invasive breast cancer cells (MDA-MB-231) by suppressing the expression of *uPA*, *tPA*, and *PA11* genes. It is intriguing to find that an E-peptide of the pro-IGF-I not only is biological active, but also elicits functions that are entirely different from its mature hormone under certain conditions. This is similar to the C-peptide of pro-insulin that elicits functions different from the mature insulin [Johansson et al., 1992a,b; Ido et al., 1997; Wahren et al., 2000]. Although the physiological relevance of E-peptide activities remain unclear, Kuo and Chen [2002, 2003] have demonstrated the presence of a high affinity binding component specific for rtEa4-peptide on the membrane of human neuroblastoma cells (SK-N-F1), and the E-peptide induced morphological differentiation and inhibition of anchorage-independent growth of SK-N-F1 cells is reversed by signal transduction inhibitors involving MAP kinase/PI3-kinase cascades. Overall, these new findings highlight the presence of additional levels of control over the functionality of the IGF system. Further studies on the newly emerged E-peptide functions might prove that it plays important roles in regulating normal and abnormal growth and development as part of the IGF system. Together with the recent reports on the function of pro-insulin C-peptide, these findings could potentially revolutionize our perspectives on peptide hormone precursors. Although more studies are required to address the biological roles of the rtEa4-peptide, our novel discoveries point to the importance of questioning the previously established concepts of IGF actions.

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