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Induction of Apoptosis in Human Cancer Cells by Human Eb- or Rainbow Trout Ea4-Peptide of Pro-Insulin-Like Growth Factor-I (Pro-IGF-I)

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1. Introduction

Insulin-like growth factors (IGFs)-I and -II are members of the insulin family that play essential roles in regulating growth, development and metabolism in all vertebrates (de Pablo et al., 1990, 1993; Stewart and Rotwein, 1996). Like many peptide hormones, IGFs are initially produced as pre-pro-hormones containing an amino-terminal signal peptide, followed by the mature peptide of B, C, A, and D domains, and a carboxyl terminal E-domain. Via post-translational processing, the signal peptide and the E-peptide are proteolytically cleaved off from the pre-pro-peptide, and both the mature IGFs and E-peptides are secreted into the circulation (Rotwein et al., 1986; Duguay 1999). From molecular characterization of IGF-I gene and its transcription products, multiple isoforms of IGF-I mRNA were identified from fish to mammals. In human, three different species of IGF-I mRNAs which encode three isoforms of pro-IGF-I (i.e., pro-IGF-Ia, -Ib and Ic) were identified (Rotwein et al., 1986; Duguay 19995). These three isoforms of pro-IGF-I contain an identical mature IGF-I with 70 amino acid residues (aa) and different E-peptides with 30 aa (Ea), 77 aa (Eb) and 40 aa (Ec), respectively. The a-type E-domains are highly conserved among vertebrates but the b-type E-peptides are conserved in the first 15 amino acids and different thereafter among human, rats and mice (variable region).

In fish, multiple isoforms of IGF-I transcripts have also been identified. These different isoforms of IGF-I transcripts encode an identical mature IGF-I but different E-peptides. Shamblott and Chen (1992, 1993) reported the presence of four different IGF-I transcripts encoding four different isoforms of pro-IGF-I for rainbow trout (*Oncorhynchus mykiss*). Similar to the isoforms of human pro-IGF-I, the four different isoforms of the rainbow trout pro-IGF-I contain an identical mature IGF-I (70 aa) and four different lengths of E-peptides (i.e., rtEa1, 35 aa; rtEa2, 47 aa; rtEa3, 62 aa; rtEa4, 74 aa). The first 15 amino acid residues of these four E-peptides are identical among themselves and the a-type E-peptides of mammals, and the 20 amino acid residues at the C-termini share 70% identity with their human counterparts. The rtEa1-peptide is composed of the first 15 and the last 20 amino acid residues. Insertion of either 12 or 27 amino acid residues between the first and the last segments of rtEa1-peptide results in rtEa2- or rtEa3-peptide, whereas insertion of both segments into rtEa1-peptide gives rise to rtEa4-peptide. Similar to rainbow trout, four different forms of Ea-peptides of pro-IGF-I have also been identified in Chinook salmon,

Coho salmon (Duguay et al., 1992: Willis and Devlin, 1993), and red drum (Faulk et al., 2010). However, not all teleosts possess four different E-peptides. While gilthead seabream possesses three different E-peptides (namely Ea1, Ea2 and Ea4), zebrafish and grouper each has only one Ea-peptide: Ea2-peptide in zebrafish and Ea4-peptide in grouper (Chen et al., 2001; Shi et al., 2002; Tiago et al., 2008).

Although the biological activity of the mature IGF-I has been extensively studied, the biological activity of E-peptides has been over-looked until recently. It was generally assumed that E-peptides of pro-IGF-I may be biologically inert; however the following lines of evidence suggest that E-peptides may possess biological activity. First, many peptide hormones are initially synthesized as complex pro-hormone molecules and following post-translational processing to generate multiple peptides with distinct or similar biological activities. Examples are pro-opiomelanocortin (Civelli et al., 1986), pro-glucaogon (Bell et al., 1983) and pro-insulin (Ido et al., 1997), just to name a few. In a similar manner, generation of E-peptides from pro-IGF-I may suggest a pluripotential role for these peptides. Second, the E-domains of pro-IGF-I are evolutionarily conserved. Third, different isoforms of pro-IGF-I transcripts are expressed in a tissue-specific and developmental stage-specific manner, and exhibiting differential responses to growth hormone (Shamblott and Chen 1993; Duguay 1994; Yang and Goldspink 2002). Siegfried et al. (1992) reported the first evidence that hEb-peptide contained biological activity. In their studies, Siegfried and colleagues showed that a synthetic peptide amide with 23 amino acid residues (Y-23-R-NH$_2$) of the hEb-peptide (a.a. 103-124) at 2-20 nM exerted mitogenic activity in normal and malignant human bronchial epithelial cells. They further demonstrated that Y-23-R-NH$_2$ bound to specific high affinity receptors (K_d = 2.8 ± 1.4 x 10^{-11}M) present at 1-2 x 10^4 binding sites per cell and the ligand binding was not inhibited by recombinant insulin or IGF-I. Several investigators have reported recently that the Eb-peptide of rodent pro-IGF-I peptide (same sequence as hEc-peptide) possessed activity in promoting proliferation of rodent myoblasts, whereas Ea-peptide stimulated differentiation of mature myoblasts (Yang and Goldspink 2002; Matheny et al., 2010). Besides exerting mitogenic activity in rodent myoblasts by rodent Eb-peptide, murine Ea-peptide of pro-IGF-I also modulates the entry of mature IGF-I protein into C2C12, a murine skeletal muscle cell line (Pfeffer et al., 2009).

Evidence documenting the biological activity of E-peptides of rainbow trout pro-IGF-I came from studies conducted by Tian et al. (1999). Tian et al. reported that recombinant rtEa2-, rtEa3- and rtEa4-peptides but not Ea1-peptide exhibited a dose dependent mitogenic activity in NIH 3T3 cells and carprine mammary gland epithelial cells (CMEC). Recently, Mark Chen in Taiwan also demonstrated that Ea2-peptide of zebrafish exerted a stimulatory effect on incorporation of ^{35}S-sulfate into zebrafish gill bronchial cartilage in a sulfation assay (personal communication). These results are consistent with those reported in mammals (Siegfried et al., 1992; Matheny et al., 2010; Yang and Goldspink 2002). Further studies conducted in our laboratory showed that both recombinant rtEa4- or synthetic hEb-peptide exerted unexpected anti-cancer cell activities in established human cancer cell lines such as MDA-MB-231, HT-29, HepG2, SK-N-F1, SKOV-3A, PC-3 and OVCAR-3B (Chen et al., 2002, 2007; Kuo and Chen 2002). These activities include: (i) induction of morphological differentiation and inhibition of anchorage-independent growth, (ii) inhibition of invasion and metastasis, and (iii) inhibition of cancer-induced angiogenesis.

Programmed cell death, i.e., apoptosis, is a crucial process of eliminating unwanted cells in animal life, and it is vital for embryonic development, homeostasis and immune defense (Elmore 2007, for review). Apoptosis is characterized by typical morphological and

biochemical hallmarks, such as cell shrinkage, nuclear DNA fragmentation and plasma membrane blebbing (Hengartner, 2000). Results of extensive studies revealed that two pathways, extrinsic pathway (death receptor triggered pathway) and intrinsic pathway (mitochondrial pathway) can lead to apoptosis (Hengartner, 2000; Fulda and Debatin, 2006). Furthermore, there is ample evidence indicating that these two pathways are linked, and molecules from one pathway can influence the other pathway. One striking feature of cancer cells is that they do not readily undergo apoptosis due to reduction of expression or mutation of pro-apoptotic genes such as caspase genes (Ghavani et al., 2009) while increasing expression of anti-apoptotic genes such as Bcl-2 family genes (Youle and Strasser, 2008). Certain anti-cancer drugs and agents which have been identified as potential effective cancer treatment can restore normal apoptotic pathways (Fulda and Debatin, 2006; Yang et al., 2008). Since rtEa4- and hEb-peptides have been shown to possess anti-cancer activities in vitro in a variety of human cancer cells (Kuo and Chen, 2002; Chen et al., 2007), it would be of great interest to determine if these peptides can also induce apoptosis in human cancer cells. In this paper, we report that rtEa4- and hEb-peptide of pro-IGF-I induces apoptosis in various human cancer cell lines via both extrinsic and intrinsic pathways.

2. Materials and methods

2.1 Cell culture

Single-cell subclones were isolated from the aggressive breast cancer cells (MDA-MB-231), ovarian cancer cells (OVCAR and SKOV), neuroblastoma cells (SK-N-SH and SK-N-F1) and non-cancerous foreskin cells (CCD-1112SK) [all purchased from ATCC, Mannassas, VA] were routinely maintained in F12/DMEM (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) at 37oC under a humidified atmosphere of 5% CO_2. Each cell line was sub-cultured every 3 days.

2.2 Preparation of rtEa4- and hEb-peptides

Recombinant rtEa4-peptide, hEb-peptide and control protein were prepared following the method described by our laboratory (Tian et al., 1999; Kuo and Chen, 2002; Chen et al., 2007). Briefly, E. coli cells, transformed with an expressing vector pET-15b (Novagen, EMD Chemicals, Gibbstown, NJ) containing the coding sequence of rtEa4-peptide or hEb-peptide, were cultured in 5 ml of LB broth for 4 h, diluted to 500 ml LB broth and allow the culture to grow at 37 oC. The culture was induced with 1 mM isopropyl β-D-thiolactopyranoside (IPTG) at an OD_{600} of 0.6-0.8 for 2 h. Induced cells were harvested by low speed centrifugation and resuspended in 10 ml of a binding buffer (50 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Following ultrasonication, the cell lysate was obtained by centrifugation at 39,000g for 20 min. The recombinant protein, containing His-tags, was isolated by affinity chromatography on His-bind resin (Novagen) and followed by extensive dialysis in 1X PBS buffer to remove immidazole. The purity of the recovered recombinant protein was accessed to be about 80% by electrophoresis on SDS-polyaccrylamide gels. The control protein was prepared from E. coli cells harboring the backbone of the expression vector without E-peptide insert following the same method as described above.

2.3 Microscopic observation of apoptotic cells induced by recombinant rtEa4-peptide

Cancer cells (MDA-MB-231C and SKOV-3A) in serum free medium were treated with various concentrations (1.4 μM or 2.8 μM) of recombinant rtEa4-peptide or control protein

for 2 h, and were observed under an inverted phase contrast microscope (Olympus IX50) to determine apoptotic cells. SKOV-3A cells, after treated with 1.5 µM or control protein for 3 days, were stained with H33258 and observed under an inverted phase contrast microscope (Olympus IX50).

MDA-MB-231C cells in serum free medium were treated with rtEa4-peptide (3.0 µM) or same concentration of control protein for 48 h. One sample was treated with H_2O_2 for 16 h to induce apoptosis to serve as a positive control. TUNEL assay was carried out following the protocol supplied by the manufacturer (Roche Diagnostics Corp., Indianapolis, IN). MDA-MB-231C cells treated with rtEa4-peptide or control protein were stained with Rodamine Red labeled mono-specific polyclonal rabbit anti-activated capase 3 immunoglobulin (Biocompare, South San Francisco, CA) following conditions provided by the manufacturer. All images were observed under an inverted microscope (Olympus IX50) equipped with epifluorescence attachment.

2.4 Quantitative relative real-time RT-PCR analysis

Cancer cells (MDA-MB-231C, SKOV-3A, OVCAR-3B, SK-N-SH, and SK-N-F1) and non-immortalized foreskin cells (CCD-1112SK) were treated with recombinant rtEa4-peptide (2.0 µM), hEb-peptide (0.5 µM) or control protein for 6 h and RNA samples were prepared from these cells using Trizol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Each RNA sample was treated with RNase-free DNase to remove any DNA contamination during isolation. 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 micrograsm of total RNA was reverse transcribed in a 20 µl reaction volume, containing 100 ng of oligo(dT)$_{18}$, 10 nM dNTP, 200mM DTT, 1 x reverse transcription buffer, and 1 µl SuperScrip^R III reverse transcriptase (Invitrogen) for 90 min at 42 °C, and the reverse transcription product was diluted to 100 µl with buffer. The PCR reaction was carried out in a 96-well plate with a final volume of 20 µl per well. The reaction mixture consists 1 X Sso fast EvaGreen Supermix (BioRad) containing Sso7d-fusion polymerase (BioRad), 500 nM of each forward and reverse primers (Table 1), and 4 µl of cDNA products. The amplification profile is as the following: 1 cycle of 95 °C for 2 min, 40 cycles of 95 °C for 2 sec and 60 °C for 10 sec annealing and synthesis (conditions provided by BioRad). The cycle threshold, CT, was determined from the fluorescence value which was 10 times the mean standard deviation of fluorescence of the base line cycles, and the efficiency of amplification in all of the genes determined is 95-98%. The relative expression was determined using the arithmetic formula:

$2^{-[(S\Delta CT-C\Delta CT)]}$, where S$\Delta$CT is the difference in CT values between the gene of interest in cells treated with rtEa4- or hEb-peptide and the house keeping gene (hGAPDH) in the sample, and CΔCT is the difference in CT values between the gene of interest in cells treated with control protein and the housekeeping gene (hGAPDH) of the control sample. Each experiment was repeated three times (n=3).

3. Results and discussion

Earlier studies conducted in our laboratory showed that recombinant rtEa4- or synthetic hEb-peptide of pro-IGF-I inhibited anchorage-independent growth and invasion of established human breast cancer cells (MDA-MB-231C), colon cancer cells (HT-29), ovarian cancer cells

(OVCAR-3A and SKOV-3B), neuroblastoma cells (SK-N-F1), human hepatoma cells (HepG-2) and rainbow trout hepatoma cells (RTH) in vitro (Chen et al., 2002; Kuo and Chen 2002). Chen et al. (2007) also showed that seeding of aggressive human breast cancer cells (MDA-MB-231C) onto the chorioallantoic membrane (CAM) of five-day old chicken embryos resulted in rapid growth of MDA-MB-231C cells into cancer nodules, invasion of the cancer cells and induction of the blood vessel formation in and around the cancer mass. The growth, invasion and angiogenesis by MDA-MB-231C cells on the chicken CAM was inhibited by treatment with a single or multiple doses rtEa4 or hEb-peptide. By microarray and quantitative relative real-time RT-PCR analyses, Chen et al. (2007) have further shown that a group of genes related to cancer cell activities were up- or down-regulated in MDA-MB-231C cells transfected with a cDNA encoding rtEa4-peptide. Together, these results suggest that rtEa4-or hEb-peptide may be developed as therapeutics for treating human cancers.

Gene	Sequence	Amplification Size (bp)
hGAPDH	(Fd): 5'GAAGGTGAAGGTCGGAGT-3'	207
	(Re): 5'-GAAGATGGTGATGGGATTTC-3'	
hBcl-XL	(Fd): 5'-GCCACTTACCTGAATGACCACC-3'	222
	(Re): 5'-GGGAGGGTAGAGTGGATGGTCAG-3'	
hCaspase-8	(Fd): 5'-GGTCTCACTCTGTTGCCCA-3'	223
	(Re): 5'-CAGGCGGATCACTTGAGGTC-3'	
hCaspase-9	(Fd): 5'-GGGACAGATGAATGCCGTGGA-3'	229
	(Re): 5'-GGCACCACTCAGGAAGACGC-3'	
hFADD	(Fd): 5'-CAGAGCGTGTGCGGGAGTC-3'	208
	(Re): 5'-GCTTCGGAGGTAGATGCGTC-3'	
hMCL-1	(Fd): 5'-AGGAGGAGGGAGTGGTGGG-3'	279
	(Re): 5'-GTCCTAACCCTTCCTGGCACA-3'	
hTRAIL-R1	(Fd): 5'-GCTGTTGGTGGCTGTGCTGA-3'	179
	(Re): 5'-CAGAGACGAAAGTGGACAGCGA-3'	
hCytochromeC	(Fd): 5'-GGCAGGCAGATCACTTGAGG-3'	220
	(Re): 5-TCACTCGTCACCCAGGCTTG-3'	
hBcl-2A	(Fd): 5'-TCCGACCACTAATTGCCAAGC-3'	176
	(Re): 5'-TCTCCCCAGCCTCCAGCAGC-3'	
hCaspase-3	(Fd): 5'-GCAATAAATGAATGGGCTGAG-3'	139
	(Re): 5'-GCGTATGGAGAAATGGGCTG-3'	
P53	(Fd): 5'-CCCAGCCAAAGAAGAAACCA-3'	81
	(Re): 5'-CTCGGAACATCTCGAAGCG-3'	
hPTEN	(Fd): 5'-CAAGATGATGTTTGAAACTATTCCAATG-3' 77	
	(Re): 5-CCTTTAGCTGGCAGACCACAA-3'	

Table 1. Gene specific primers used in comparative real-time RT-PCR analysis

Cancer cells do not undergo apoptosis because they bypass apoptosis through a series of complex mechanisms involving dynamic interplays between cancer causing genes, oncogens, and/or mutated suppressor genes (Fulda and Debatin, 2004; Lowe and Lin, 2000). Apoptosis dysregulation in many cancers is one of the major hindrances towards the destruction of cancers in cancer therapy. Therefore, drugs and agents which can restore the normal apoptosis signaling pathways are considered as effective therapeutic agents for treating cancers (Evan and Vousden, 2001; Finkel 1999; Max 2002). Since rtEa4- or hEb-peptide suppresses anchorage-independent growth, invasion and angiogenesis of many different cancer cells in vitro (Chen et al., 2002, 2007; Chen and Kuo, 2002), it would be of

great interest to know whether the protein can induce apoptosis in cancer cells. Treatment of ovarian cancer cells (SKOV-3A) or breast cancer cells (MDA-MB-231C) cells with recombinant rtEa4-peptide (1.4 and 2.8 μM) for 2 h, many cells exhibited distinct morphology of membrane blebbing, cell shrinkage, and chromatin condensation and disintegration (Fig 1). These morphological characteristics are consistent with those reported for apoptosis (Hacker 2000, for review). While these morphological changes in the rtEa4-peptide treated cells were further enhanced with time, no such morphological changes were observed in untreated cells or cells treated with control protein even after 48 h of culture. To confirm that rtEa4-peptide treated cancer cells exhibited apoptosis, MDA-MB-231C cells were treated with 3.0 μM of recombinant rtEa4-peptide for 48 h, and the treated cells were subjected to TUNEL assay or immuno-cytochemical staining with a mono-specific antibody against activated caspase 3. As shown in Figure 2, after incubation for 48 h in a serum free medium containing 3.0 μM of recombinant rtEa4-peptide, most of the cells showed positive in TUNEL assay (Fig 2A) as well as in immuno-cytochemical staining for activated caspase 3 (Fig 2B). Furthermore, apoptosis was also observed in ovarian cancer cells, SKOV 3A, treated with 1.5 μM of recombinant rtEa4-peptide (Fig 3). These results confirmed that rtEa4-peptide induced apoptosis in human breast cancer cells and ovarian cancer cells.
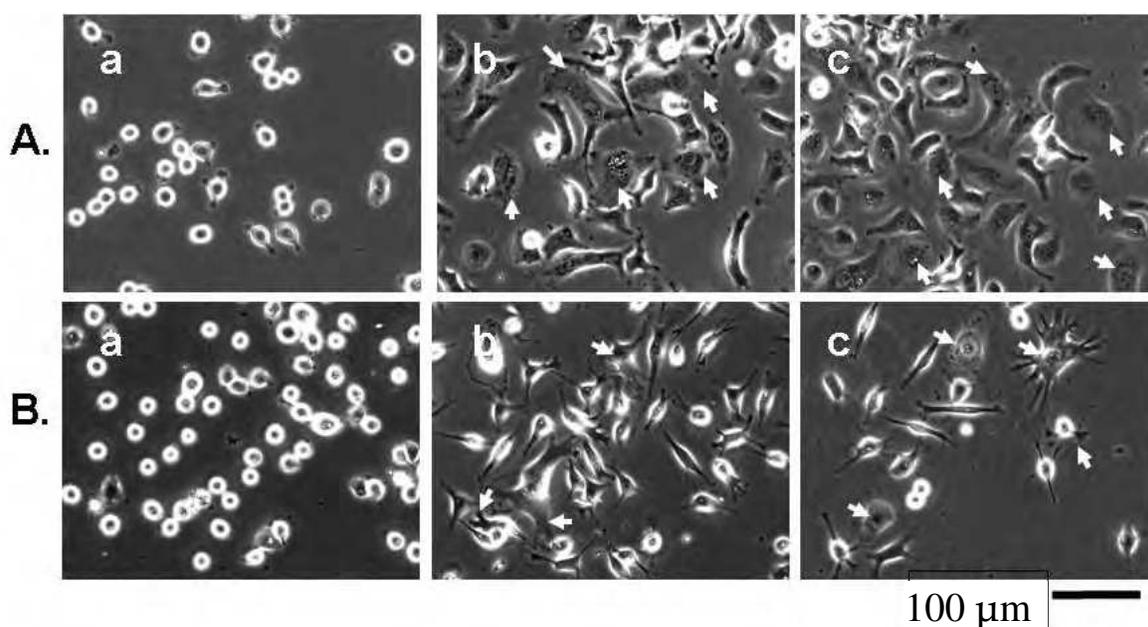

Fig. 1. Induction of apoptosis in ovarian cancer cells (A, SKOV-3A) and breast cancer cells (B, MDA-MB-231C) by recombinant rtEa4-peptide. Cancer cells in serum free medium were treated with or without rtEa4-peptide for 2 h, and observed under an inverted phase contrast microscope (Olympus IX50). a, control protein; b, 1.4 μM rtEa4-peptide ; c, 2.8 μM rtEa4-peptide. Arrows indicate apoptotic cells.

Generally speaking, apoptosis can be stimulated via extrinsic and intrinsic pathways. Does rtEa4-peptide or hEb-peptide induce apoptosis in cancer cells via both extrinsic and intrinsic pathways? It has been reported by many investigators that if apoptosis is induced via extrinsic pathway, up-regulation of expression of *TRAIL-RI, FADD* and *pro-Casp-8* genes will be observed. On the other hand, if apoptosis is induced via intrinsic pathway, down-regulation of expression of *Bcl-2, Bcl-XL and Mcl-1* genes and up-regulation of expression of
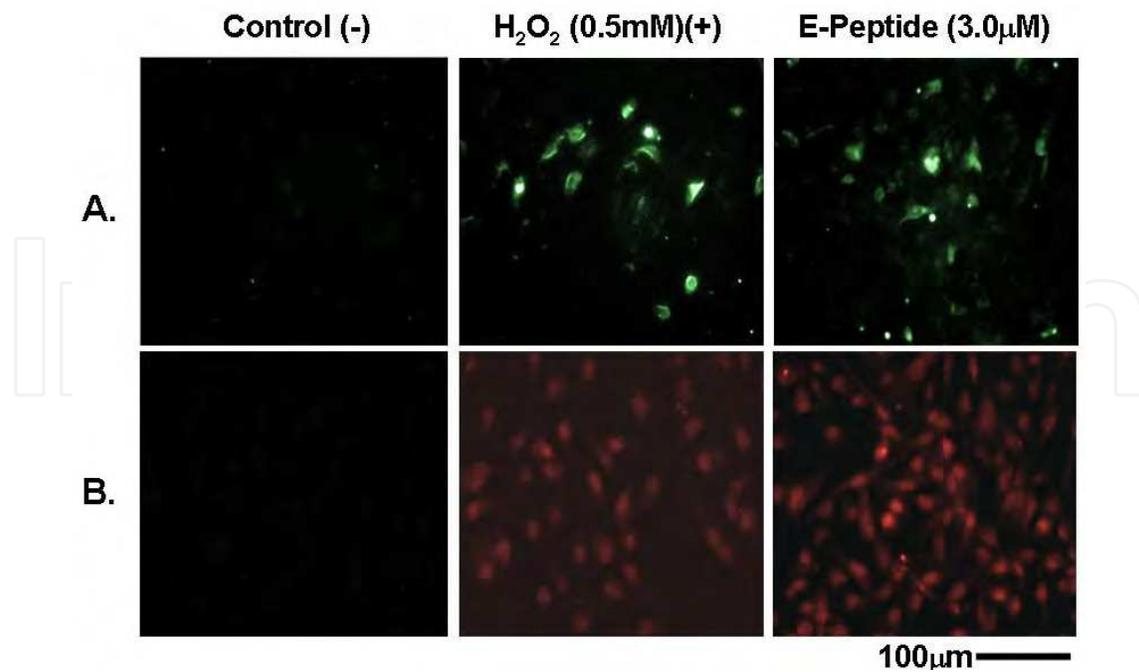
Fig. 2. Apoptotic cells identified by TUNEL assay (A) and immunostaining with mono-specific antibody to activated capase-3 (B). MDA-MB-231C cells in serum free medium were treated with (3.0μM) rtEa4-peptide or control protein for 48 h. One sample was treated with H_2O_2 for 16 h to induce apoptosis as a positive control. TUNEL assay was carried out following the protocol supplied by the manufacturer. Monospecific polyclonal rabbit anti-activated caspase 3 immunoglobulin was labeled with Rodamine Red and used to stain recombinant rtEa4 peptide treated or positive control cells. The immuno-stained cells were observed under an inverted microscope (Olympus IX50) with epifluorescence attachment.
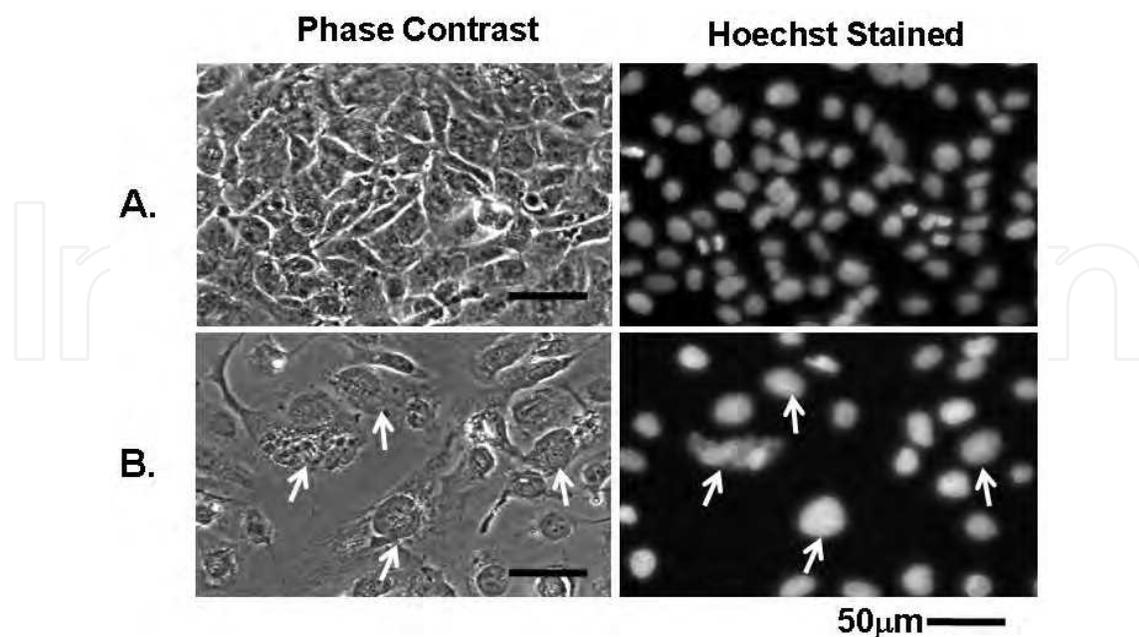
Fig. 3. Induction of apoptosis in ovarian cancer cells (SKOV-3A) by rtEa4-peptide. SKOV-3A cells in serum free medium were treated with control protein (A) or 1.5 μM of rtEa4-peptide (B) for 3 d, and cells were stained with H33258. Arrows indicate apoptotic cells.
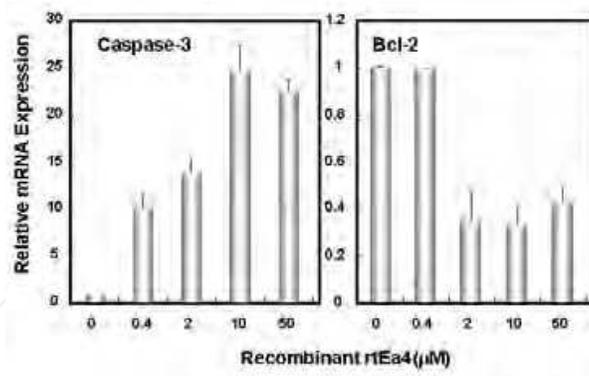
A
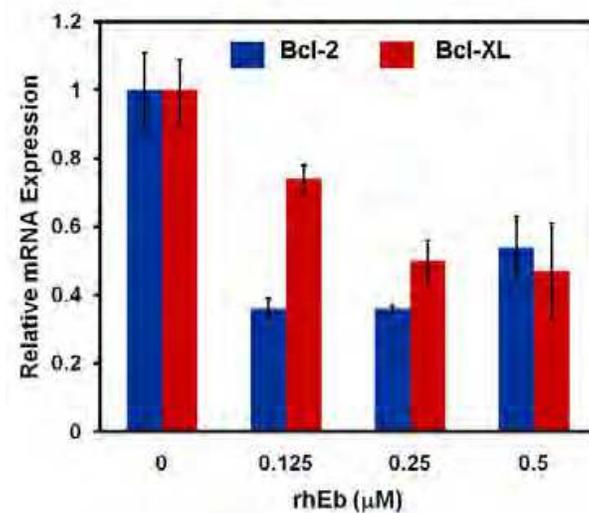
B
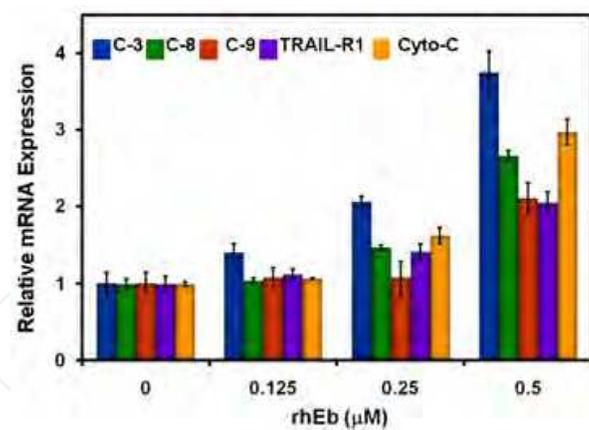
C

Fig. 4. Effect of recombinant rtEa4- (A) or hEb-peptide (B and C) on levels of Casp-3, Casp-8, Casp-9, TRAIL-R1 and Cyt-C mRNAs in MDA-MB-231C cells. MBA-MD-231C cells in serum free medium were treated with various concentrations of recombinant rtEa4- or hEb-peptide for 6 h, and total RNA samples were isolated from the treated cells. The levels of mRNA were determined by quantitative relative real-time RT-PCR analysis. The level of GAPDH mRNA was used as an internal control. Relative Expression Level = $2^{-(S\Delta CT-C\Delta CT)}$, where S$\Delta$CT is the difference between the CT number of the sample (cancer cells treated with E-peptide) and GAPDH, and the CΔCT is the difference between the CT of cancer cells without E-peptide treatment and the CT of GAPDH. Error bars indicate standard deviation (n=3).
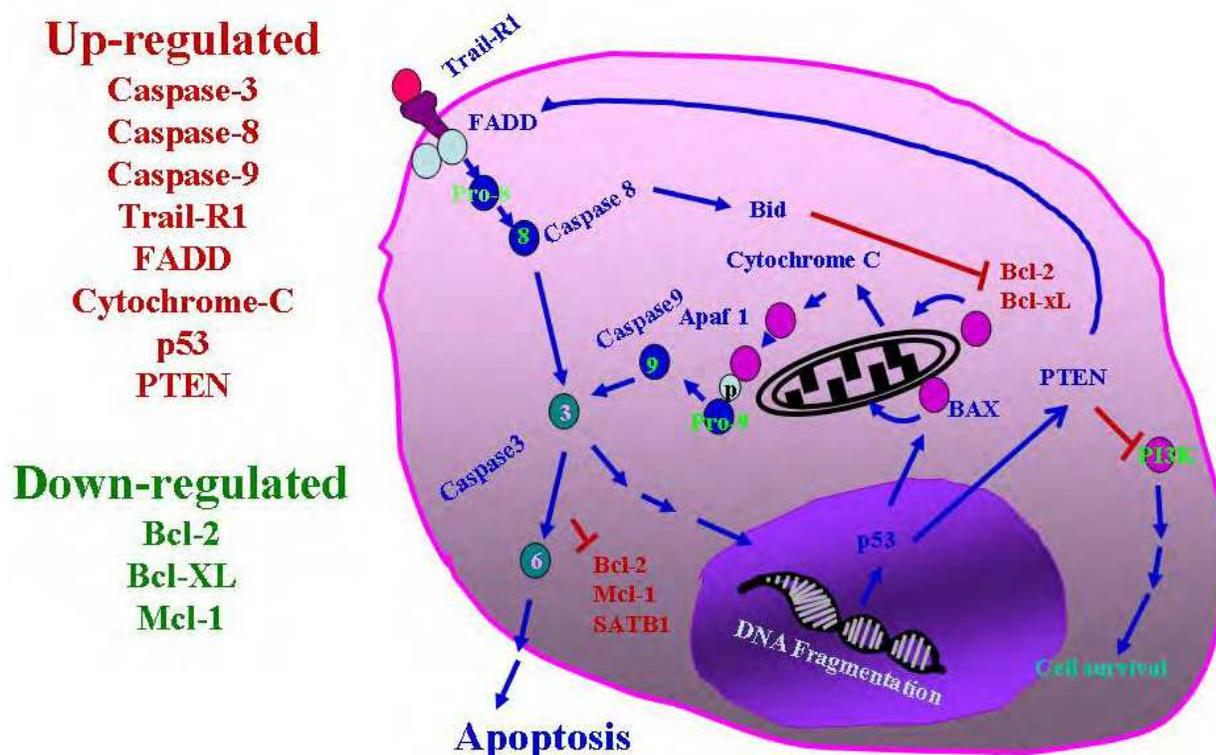
Fig. 5. A schematic presentation of extrinsic and intrinsic pathways of apoptosis. Apoptosis can be initiated via stimulation at the plasma membrane by death receptor (extrinsic pathway) or at mitochondria (intrinsic pathway). Stimulation of death receptors will result in aggregation and recruitment of the adaptor molecule Fas-associated protein with death domain (FADD) and pro-caspase 8. Upon recruitment, pro-caspase 8 is activated to caspase 8 and initiates apoptosis by direct activating downstream caspases (caspases 3 and 9). Intrinsic pathway is initiated by a variety of stress stimuli in which the mitochondrial membrane permeability is regulated by balance of opposing action of pro-apoptotic and antiapoptotic Bcl family members (Bax, Bak, Bcl2, Bcl-XL and Mcl-1). Following mitochondril permeabilization, mitochondrial proapoptotic proteins such as cytochrome C, Samc/Diablo, Omi/HrtA2, AIF and Endo G are released via transmembrane channels across the mitochondriasl outer membrane. This figure was redrawn from information provided by Fulda and Debatin (2006).

Cyt-C gene will be observed (Figure 5) (Fulda and Debatin, 2006; O'Brien and Kirby, 2008 for review). Therefore, to address the question whether rtEa4-peptide and hEb-peptide can induce apoptosis in cancer cells through both extrinsic and intrinsic pathways, levels of Bcl-2, Bcl-XL, Casp-3, Casp-8, Casp-9, TRAIL-R1 and Cyt-C mRNAs were measured by quantitative relative real-time RT-PCR analysis in MBA-MD-231C cells following treatment with recombinant rtEa4- or hEb-peptide. As shown in Figures 4A, 4B and 4C, upon treatment of MDA-MB-231C cells with various doses of recombinant rtEa4- or hEb-peptide, a dose-dependent up-regulation on levels of Casp-3, Casp-8, Casp-9, TRAIL-R1 and Cyt-C mRNAs and a dose-dependent down-regulation on levels of Bcl-2 and Bcl-XL mRNAs were observed. These results suggest that E-peptide may induce apoptosis in breast cancer cells via both extrinsic and intrinsic pathways. To further confirm that rtEa4- or hEb-peptide can also induce apoptosis in other cancer cells, human ovarian cancer cells (OVCAR-3A, SKOV-

3B), neuroblastoma cells (SK-N-SH and SK-N-F1) and non-cancerous foreskin cells (CCD-1112SK) were treated with 2.0 μM of rtEa4-peptide or 0.5 μM hEb-peptide for 6 h and RNA samples were extracted for determination of levels of Bcl-2, Bcl-XL, Mcl-1, Casp-3, Casp-8, Casp-9, TRAIL-R1, FADD, Cyt-C, p53 and PTEN mRNAs by quantitative relative real-time RT-PCR analysis. As shown in Table 2, while the levels of Bcl-2, Bcl-XL and Mcl-1 mRNAs in rtEa4-peptide or hEb-peptide treated cancer cells were down-regulated significantly, those of Casp-3, Casp-8, Casp-9, TRAIL-R1, FADD, Cyt-C, p53 and PTEN mRNAs were up-regulated. Results presented in Table 2 not only provide a strong evidence to support the hypothesis that rtEa4- or hEb-peptide induces apoptosis in cancer cells via both extrinsic and intrinsic pathways, but also confirm the notion that E-peptide can induce apoptosis in many cancer cells other than MBA-MD-231C cells.

Genes	Cell Lines (Relative Expression Levels)						
	rtEa4 (2μM)						hEb (0.5μM)
	CCD-1112SK*	MDA-MB-231C	OVCAR-3B	SKOV-3A	SKNSH	SKNF1	MDA-MB-231C
Bcl-2	1.08 ± 0.55	0.19 ± 0.03	0.66 ± 0.10	0.20 ± 0.05	0.79 ± 0.34	0.08 ± 0.02	0.54 ± 0.09
Bcl-XL	0.96 ± 0.04	0.33 ± 0.00	0.49 ± 0.01	0.21 ± 0.01	0.50 ± 0.04	0.18 ± 0.00	0.47 ± 0.14
Mcl-1	0.92 ± 0.04	0.60 ± 0.00	0.29 ± 0.01	0.15 ± 0.02	0.38 ± 0.08	0.07 ± 0.01	-
Casp-3	0.96 ± 0.12	3.48 ± 0.27	26.07 ± 2.50	12.18 ± 2.61	3.32 ± 0.10	13.91 ± 1.81	3.75± 0.28
Casp-8	0.91 ± 0.03	3.74 ± 0.28	2.44 ± 0.04	2.67± 0.06	5.49 ± 0.56	7.96 ± 0.01	2.66± 0.07
Casp-9	1.08 ± 0.01	2.18 ± 0.10	3.13 ± 0.021	1.98 ± 0.02	6.16 ± 0.18	8.70 ± 1.12	2.11 ± 0.2
TRAIL-R1	1.01 ± 0.11	2.04 ± 0.16	2.24 ± 0.01	1.37 ± 0.06	6.50 ± 0.16	7.47 ± 0.78	2.05± 0.14
FADD	1.04 ± 0.11	3.61 ± 0.09	1.42 ± 0.03	2.67 ± 0.03	3.31 ± 0.05	8.33 ± 1.14	1.49± 0.22
Cyt-C	1.02 ± 0.05	2.54 ± 0.18	1.40 ± 0.00	1.72 ± 0.01	4.31 ± 1.02	10.59 ± 8.20	2.97± 0.17
p53	1.08 ± 0.06	2.37 ± 0.05	3.09 ± 0.29	1.03 ± 0.51	3.99 ± 0.47	6.93 ± 1.75	1.57 ± 0.03
PTEN	--	8.30 ± 0.73	--	4.61 ± 0.11	4.09 ± 0.20	30.80 ± 0.15	--

Relative Expression Level $2^{-[(S\Delta CT-C\Delta CT)]}$ and standard deviation (n=3) vhere SΔCT is the difference between the CT number of the sample (cancer cells treated with E-peptide) and the house keeping gene (GAPDH), and the CΔCT is the difference between the CT of cancer cells without E-peptide treatment and the CT of GAPDH $2^{-[(S\Delta CT-C\Delta CT)]}>1$, up-regulation; $2^{-[(S\Delta CT-C\Delta CT)]}<1$, down-regulation *CCD-1112SK, non-immortalized human fibroblast cells.

Table 2. Relative Expression Levels of Apoptosis Genes by E-peptide of Pro-IGF I

It is very interesting to note that treatment of non-cancerous foreskin cells (CCD-1112SK) with the same concentration of recombinant rtEa4-peptide or hEb-peptide (result not shown) did not result in significant changes in the levels of Bcl-2, Bcl-XL, Mcl-1, Casp-3, Casp-8, Casp-9, TRAIL-R1, FADD, Cyt-C, p53 and PTEN mRNAs when compare to non-treatment control (Table 2). This observation suggests that rtEa4- or hEb-peptide only induces apoptosis in cancer cells. The resistance of cancer cells to apoptosis is one of the major concerns in cancer therapy. So in searching for effective chemotherapeutic drugs, the

effectiveness of the drugs to induce apoptosis in a wide variety of cancer types will be the top choice. Although there are numerous chemotherapeutic drugs available on the market that have been shown to induce apoptosis in cancer cells, unfortunately these drugs also induce apoptosis in non-cancerous cells. In this study we have shown that rtEa4- or hEb-peptide can induce apoptosis in a variety of human cancer cells but not in non-cancerous cells. Therefore, we believe that rtEa4- or hEb-peptide could be developed as an ideal therapeutic agent for treating human cancers.

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5. References

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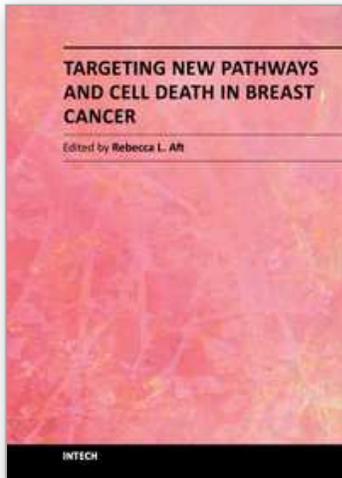
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