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Suppression of Growth and Cancer-Induced Angiogenesis of Aggressive Human Breast Cancer Cells (MDA-MB-231) on the Chorioallantoic Membrane of Developing Chicken Embryos by E-peptide of Pro-IGF-I

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Abstract E-peptide of the pro-Insulin-like growth factor-I (pro-IGF-I) is produced from pre-pro-IGF-I by proteolytic cleavage in the post-translational processing. Previous in vitro studies conducted in our laboratory showed that Ea4-peptide of rainbow trout (rt) pro-IGF-I or Eb-peptide of human (h) pro-IGF-I exhibited activities including induction of morphological differentiation, inhibition of anchorage-independent cell growth and suppression of invasion of several well established human cancer cell lines such as MDA-MB-231, HT-29, SK-N-F1, and HepG-2 (Chen et al. [2002] Gen Comp Endocrinol 126:342–351; Kuo and Chen [2002] Exp Cell Res 280:75–89). Seeding of aggressive human breast cancer cells, MDA-MB-231, on the chorioallantoic membrane (CAM) of 5 days old chicken embryos resulted in rapid growth and invasion of the cells and induction of blood vessel formation around the MDA-MB-231 cell mass in the chicken embryos. The invasion of MDA-MB-231 cells in the chicken embryos was further confirmed by immunocytochemistry. The rapid growth and invasion of MDA-MB-231 cells and the induction of blood vessel formation by MDA-MB-231 cells on chicken CAM are inhibited by treatment with a single or multiple doses of rtEa4- or hEb-peptide. Furthermore, a dose-dependent inhibition of angiogenesis by rtEa4- or hEb-peptide was also demonstrated by the chicken CAM assay. Results of microarray analysis of human gene chips (containing 9,500 unique cDNA clones) and confirmation by comparative real-time RT-PCR analysis showed that a group of genes related to cancer cell activities are up- or down-regulated in MDA-MB-231 cells transfected with a rtEa4-peptide gene. Together these results confirm the anti-tumor activity of rtEa4- and hEb-peptides, and further suggest that these peptides could be developed as therapeutics for treating human cancers. J. Cell. Biochem. 101: 1316–1327, 2007.

Key words: rtEa4-peptide; shEb-peptide; invasion; antiangiogenesis; inhibition of cancer cell growth; chorio-allantoic membrane (CAM) of chicken embryo; rtEa4-peptide regulated genes

Insulin-like growth factors (IGFs)-I and -II, are members of the insulin gene family and play essential roles in growth, development, and metabolism in vertebrates [de Pablo et al., 1990; de Pablo et al., 1993; Stewart and Rotwein, 1996]. IGFs are initially produced as the pre-pro-IGFs containing an amino terminal signal peptide, followed by the mature peptide of B, C, A, and D domains, and a carboxyl-terminal E-domain. The signal peptide and the E-domain peptide are proteolytically cleaved from the pre-pro-peptide to yield mature IGFs during post-translational processing [Rotwein et al., 1986; Duguay, 1999]. To date, multiple forms of pro-IGF-I have been identified from fish to mammals. In human, three different isoforms of pro-IGF-I (i.e., pro-IGF-Ia, Ib, and Ic) have been identified [Rotwein et al., 1986; Duguay, 1999]. These three pro-IGF-I isoforms contain an identical mature IGF-I of 70 amino acid

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residues (aa) and a different E-domain peptide sequence of 35, 77, and 40 aa, respectively. Similarly, multiple forms of pro-IGF-I E peptides, designated as pro-IGF-I Ea1 (35 aa), Ea2 (47 aa), Ea3 (62 aa), and Ea4 (74 aa), were also identified in rainbow trout (*Oncorhynchus mykiss*) [Shamblott and Chen, 1993].

While the biological activity of the mature IGF-I peptide has been extensively studied, the biological activity of E-peptides has been overlooked until recently. Siegfried et al. [1992] showed that a synthetic peptide amide with 23 aa residues of the human Eb-peptide (hEb) exerted mitogenic activity in human bronchial epithelial cells. We have reported earlier that recombinant rtEa2-, rtEa3-, and rtEa4- but not rtEa1-peptides possessed mitogenic activity in cultured BALB/3T3 fibroblasts and primary caprine mammary epithelium cells [Tian et al., 1999]. However, in oncogenic transformed cell lines such as human breast cancer cells (MDA-MB-231), colon cancer cells (HT-29), neuroblastoma cells (SK-N-F1) and trout hepatoma cells, rtEa2-, rtEa4-, and hEb-peptides induced morphological differentiation and inhibited anchorage-independent cell growth [Chen et al., 2002, 2004; Kuo and Chen, 2002]. These results showed that rtEa4- and hEb-peptides may inhibit cancer cell growth and invasion in vitro.

The chicken chorio-allantoic membrane (CAM) is formed on days 3–4 upon incubation by fusion of chorine and allantois, and the resulting membrane consists of chorion (ectoderm), mesoderm with blood vessels, and allantois (endoderm) [DeFouw et al., 1989]. Since there is extensive development of capillary plexus on the CAM before day 6 of chicken embryonic development, the CAM has been widely used experimentally to study factors regulating angiogenesis [Folkman and Shing, 1992; Ribatti et al., 1997; Ribatti and Vacca, 1999] or factors exerting antiangiogenic activity [Ribatti and Vacca, 1999]. Furthermore, since chicken embryos in the early phase of development lack mature immune system, the CAM provides ideal but inexpensive in vivo environments for studying molecules that suppress cancer cell growth, metastasis, apoptosis and cancer cell induced angiogenesis [Kamosfky et al., 1952; Leighton, 1964].

To confirm whether the effects of E-peptide on human cancer cells observed from in vitro studies are consistent with those of in vivo studies, we have adopted the chicken CAM system as a model to determine the effects of rtEa4- or hEb-peptide on an aggressive human breast cancer cell line, MDA-MB-231. In this paper we report that rtEa4- or hEb-peptide inhibits growth, invasion, and angiogenesis of MDA-MB-231 cells on the CAM of developing chicken embryos.

MATERIALS AND METHODS

Preparation of rtEa4- and hEb-Peptides

Recombinant rtEa4-peptide was prepared following the method described by our laboratory earlier [Tian et al., 1999; Kuo and Chen, 2002]. A single colony of BL21(DE3), transformed with an expression construct pET-15b (Novagen) containing the coding sequence of rtEa-4-peptide, were cultured in 5 ml of LB broth for 4 h, diluted to 500 ml LB broth and allow to grow at 37°C. The culture was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6–0.8. After 2 h of further incubation, the cells were spun down and resuspended in 10 ml of a binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Cell lysates were obtained by sonication followed by centrifugation at 39,000*g* for 20 min. The recombinant protein, containing his-tags (6 histidine residues), were purified by affinity chromatography with His-bind resin (Novagen) followed by extensive dialysis to remove imidazole. Following affinity chromatography on His-bind resine, the purity of the recombinant rtEa4-peptide was estimated about 60% by SDS-polyacrylamide gel electrophoresis. The control proteins were prepared from BL21(DE3) cells transformed with the expression vector pET-15b without rtEa4-peptide insert, and purified following the same procedures. Synthetic hEb-peptide was purchased from Genemed Synthesis, Inc. (South San Francisco, CA).

Cell Culture

Single-cell subclones isolated from the aggressive human breast cancer cells (MDA-MB-231) purchased from ATCC (Manassas, VA), were routinely maintained in F12/DMEM (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) at 37°C under a humidified atmosphere of 5% CO_2. MDA-MB-231 cells expressing enhanced green fluorescence protein (EGFP) were prepared by

transfecting the cells with a construct, pCMV-IRES-EGFP, following conditions described by Chen et al. [2002], individual green cell subclones of transfectants were isolated from the enriched population by the method of serial dilution.

Growth of Cancer Cells on the Chick Chorioallantoic Membrane (CAM)

Fertilized white Leghorn chicken eggs, purchased from Charles River SAPFAS, Inc. (N. Flanklin, CT) were incubated at 37°C under 65% relative humidity. On the 3rd day after initiation of incubation, the egg shell was opened and the embryo was carefully transferred into a paper cup lined with a piece of "all purpose" laboratory wrap. The cup was then covered with another layer of the "all purpose" laboratory wrap and the cup was returned to the incubator. Twenty embryos were used in experimental and control groups, respectively. On the 5th day, experimental group I and II embryos were seeded with 1×10^7 MDA-MB-231 cells in 50 μl of F12/DMEM at one site on the CAM of a chicken embryo with or without 600 μg of the recombinant rtEa4-peptide, and the control embryos were seeded with same amount of control proteins alone. The group II embryos continued to receive three additional doses (600 μg/dose) of rtEa4-peptide at 2-day intervals. The embryos were observed at day 13 and photographed with a digital camera (Olympus). In a separate set of experiments, each embryo was seeded with MDA-MB-231 cells (1×10^7 cells) on the 5th day. Starting on the 9th day, each embryo received three doses of synthetic hEb peptide or BSA (600 μg per dose per embryo) at every 2-day interval, and the embryos were examined on day 15. Ten embryos were used in each experimental and control groups, respectively. The CAM that contained cancer cell mass was carefully dissected and examined under an inverted microscope (Olympus IX50 with a filter set to give excitation wavelength at 490 nm and emission wavelength at 520 nm) at 40× magnification and photographed.

Immunocytochemistry

CAM containing cancer cell mass was dissected from the embryo and fixed in 50% methanol for 30 min and 3.7% formaldehyde overnight. The fixed tissue was embedded in paraffin with CAM facing up, and 5 μm cross sections were prepared. Following deparaffinization and rehydration, the sections were incubated in a blocking solution [TBS (10 mM Tris-HCl, pH 8.0 and 150 mM NaCl) containing 1% BSA and 0.1% Tween-20] for 2 h at room temperature. The sections were then incubated in a TBS solution containing a rabbit polyclonal antibody specific for green fluorescence protein (1/1,000 dilution, BD Biosciences) and 0.2% Triton X-100 at room temperature for 2 h. Following washing in TBS three times, the sections were incubated in 0.2% Triton X-100/TBS containing goat anti-rabbit IgG labeled with FITC (1/200 dilution, BD Biosciences) at room temperature for 2 h in the dark. After washing, the sections were examined under a fluorescence microscope (Olympus IX50 with an appropriate filter set to give excitation wavelength at 490 nm and emission wavelength at 520 nm) at 200× magnification. The photographs were taken along the sections and composite photos were complied.

In Vivo Angiogenesis Assay

Fertilized White Leghorn Chicken eggs, purchased from Charles River SAPFAS, Inc. (N. Flanklin, CT) were incubated at 37°C under 65% relative humidity. On the 3rd day after initiation egg incubation, the egg shell was opened and the embryo was carefully transferred into a paper cup lined with a piece of "all purpose" laboratory wrap. A piece of sterile 3 M filter paper (0.5 cm × 0.5 cm) soaked with various amounts [2 nmol (20 μg) to 16 nmol (160 μg)] of rtEa4- or synthetic hEb-peptide or the control proteins was placed on each embryo. The embryo cups were covered with a layer of the "all purpose" laboratory wrap and return to the incubator for another 2 days. On the 5th day, the 3 M filter paper was carefully removed from the embryos with a pair of forceps and the embryos were photographed with a digital camera (Nikon Coolpix 8700). The photographs were transferred to Adobe Photoshop and the numbers of the intersections of CAM vessel (vessel density) on the embryos were scored. Endostatin was used as a positive control to ascertain that the chicken CAM system that we set up is responsive to a known antiangiogenic factor. The vessel density on each embryo was counted three times. Each data point was the average of 10 embryos per dose of the E-peptide tested, and the data were presented as mean ± standard error (SE of the mean).

Screening of a High Density Human cDNA Microarry

A high density (9,500 spots) unique sequence human cDNA microarray chip was used to determine genes in MDA-MB-231 cells that are up- or down-regulated by rtEa4-peptide, following the method described eleswhere [Chen et al., 1998; Yoneda et al., 2001], and the hybridization signals were detected by colorimetry. Poly(A)$^+$-RNA samples were isolated from MDA-MB-231 cells and MDA-MB-231 cells transfected with the secreted form of rtEa4-peptide gene [Chen et al., 2002]. Once MDA-MB-231 cells were transfected with the secreted form of the rtEa4-peptide gene, the resulting transfectants lost the malignant properties [Chen et al., 2002], that is similar to direct treatment of MDA-MB-231 cells with rtEa4-peptide in vitro. One microgram of each mRNA sample was labeled with biotin or digoxigenin [Chen et al., 1998; Yoneda et al., 2001], and the products were used as probes for hybridization to microarray chips. A microarray chip containing 9,500 unique sequence human double stranded cDNA spotted on nylon membranes was prehybridized in 1 ml hybridization buffer (5× SSC, 0.1% SDS, 1% BM blocking solution and 50 μg/ml salmon sperm DNA) for 1 h at 68°C. To initiate the hybridization, two cDNA probes (one from mRNA of MDA-MB-231 cells and the other from mRNA of rtEa4-peptide transfected MDA-MB-231 cells) were mixed in equal quantities in 10 μl of hybridization solution containing 200 μg/ml of $d(A)_{10}$, and the hybridization mixture was sealed in an assembly (SureSeal, Hybiaid, Middlesex, UK) with the microarray membrane. The assembly was incubated at 95°C for 2 min and then at 68°C for 12 h.

Following hybridization, membrane was washed with 2× SSC containing 01% SDS for 5 min at room temperature and followed by three washes with 0.1× SSC containing 0.1% SDS at 65°C for 15 min each. The membrane was incubated in 1 ml of 1% blocking reagent (Boehringer Manheim) containing 2% dextran sulfate at room temperature for 1 h and then rinsed with 1× TSB buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl ands 0.3% BSA). To visualize the hybridization signal on the membrane, the membrane was incubated in 1 ml of 1× TSB buffer containing antibodies of β-galactosidase-conjugated streptavidin and alkaline phosphatase-conjugated digoxigenin, 4% polyethylene glycol 8000 and 0.3% BSA for 2 h and then washed three times in 1× TSB buffer (5 min each). To visualize the hybridization signal with cDNA labeled with biotin, the membrane was incubated in an 1 ml β-gal substrate solution containing 1.2 mM X-gal, 1 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM K4Fe(CN)6 in 1× TSB buffer at 37°C for 45 min; to visualize the hybridization signal with cDNA labeled with digoxigenin, the membrane was reacted in 1 ml Fast red TR/naphthol AS-MX substrate solution (Pierce, Rockford, IL). The color reactions were then stopped by incubating the membrane in 1× PBS containing 20 mM EDTA. After color development, the image of each DNA dot was digitized by scanning the membrane on a high resolution flat-bed scanner [UMAX (Fremont, CA) MagieScan at 3,000 dpi], and the resulting data were analyzed by GenePix Pro software program (Axon Instruments, Foster City, CA).

Comparative Real-Time RT-PCR Analysis

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, Palo Alto, CA) 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 specific gene primer (Table I), 0.01 μM fluorescein, 1:20,000 dilution of 10,000× SYBR Green I, and 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 three 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 sec, desired annealing temperature for 15 sec, and 72°C for 30 sec. 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. The

TABLE I. Gene Specific Primers Used in Comparative Real-Time RT-PCR Analysis

Gene	Sequence	Amplicon size (bp)
β-actin	(Fd): 5′-TCGTGCGTGACATTAAGGAG-3′ (Re): 5′-AGGAAGGAAGGCTGGAAGAG-3′	178
Plasminogen activator, urokinase (uPA)	(Fd): 5′-ATGCTGTGCTGTGCTGACC-3′ (Re): 5′-CTTCCTTGGTGTTGACTGCG-3′	222
Plasminogen activator inhibitor, type 1 (PAl1)	(Fd): 5′-GCCATTACTACGGACATCCTG-3′ (Re): 5′-CAGCCTGAAACTGTCTGAAC-3′	261
Fibronectin 1	(Fd): 5′-gcttcctggcacttctggtc-3′ (Re): 5′-AGGAAGGAAGCTGGAAGAG-3′	146
Laminin receptor 1	(Fd): 5′-TTGATGTGGTGGATGCTGG-3′ (Re): 5′-TCCTTGTCACTGCCTTCTC-3′	158
BCL 2	(Fd): 5′-TCCGACCACTAATTGCCAAGC-3′ (Re): 5′-TCTCCCCAGCCTCCAGCAGC-3′	176
Caspase 3	(Fd): 5-′GCAATAAATGAATGGGCTGAG-3′ (Re): 5′-GCGTATGGAGAAATGGGCTG-3′	208
Ctsteine-rich angiogenesis inducer 61 (CYR61)	(Fd): 5′-GCCTTCAACCACTCAGACCT-3′ (Re): 5′-CGTGGCTGCATTAGTGTCCAT-3′	66
Tumor-associated Ca^{++} signal transducer 2 (TACSTD 2)	(Fd): 5′-GCCTTCAACCACTCAGACCT-3′ (Re): 5′-ATGGTGGGCTGCTCGTAGTG-3′	87
TYPO3 protein tyrosine kinase	(Fd): 5′-CGGGTCACACAAAGGCAT-3′ (Re): 5′-GCGTGCTCAGATTGTGGGT-3′	140
Tumor rejection antigen 1 (Gp96)	(Fd): 5′-CTAAGGAGAGTCGTGAAGCAGTT-3′ (Re): 5′-TGTTGCCAGACCATCCGT-3′	144
Heat shock 90 kDa protein 1α	(Fd): 5′-GCTGTAACTGAAGAAATGCCAC-3′ (Re): 5′-GGTAAGTCATCCCTCAGCCA-3′	73
Heat shock 70 kDa protein 10	(Fd): 5′-CCACCATTCCTACCAAGCAG-3′ (Re): 5′-ACACGCTCGCCTTCATAAACC-3′	70
Glyceralaldehyde-3-phosphate dehydrogenase (GAPDH)	(Fd): 5-′GAAGGTGAAGGTCGGAGT-3′ (Re): 5′-GAAGATGGTGATGGGATTTC-3′	206

efficiency of amplification in all of the genes determined is 95–98%. The relative gene expression was determined using the arithmetic formula: $2^{-[(S\Delta CT-C\Delta CT)]}$. SΔCT is the difference in CT value between the gene of interest and the housekeeping gene (β-actin or GAPDH) in the sample, and CΔCT is the difference between the gene of interest and the housekeeping gene (β-actin) of the control sample.

RESULTS AND DISCUSSION

Inhibition of Tumor Cell Growth and Invasion

Results of earlier studies reported by our laboratory showed that rtEa4-peptide or synthetic hEb-peptide inhibited anchorage-independent growth of established human cancer cells such as MDA-MB-231, HT-29, SK-N-F-1 and HepG-2, and rainbow trout hepatoma cells (RTH) in vitro [Chen et al., 2002, 2004; Kuo and Chen, 2002]. It would be of great interest to verify if rtEa4-peptide could exhibit the same inhibitory effect on cancer cell growth in an in vivo system. Chicken embryos are immune deficient during embryonic development, and thus the CAM has been adapted as a convenient model to evaluate many different parameters of tumor growth [Chamber et al., 1982; Chambers et al., 1992] and in antineoplastic drug screening by many investigators [Brooks et al., 1994; Stan et al., 1999]. Furthermore, the CAM model has been considered as an ideal alternative model system for cancer research because it can conveniently and inexpensively reproduce many of the tumor characteristics in vivo such as tumor mass formation, tumor-induced angiogenesis, infiltrative growth and metastasization [Chamber et al., 1982; Chambers et al., 1992; Brooks et al., 1994; Stan et al., 1999]. To test whether rtEa4-peptide exhibits an inhibitory effect on cancer cell growth in vivo, we have adopted the CAM model to assess the growth inhibition of MDA-MB-231 cells on the CAM by rtEa4-peptide. In this assay, about 1×10^7 cells of MBA-MD-231 cells labeled with EGFP were seeded on the top of the CAM of a 5-day-old embryo with 600 μg of rtEa4-peptide or the same amount of the control proteins, and the embryo was scored at day 13. As shown in Figure 1, MBA-MD-231 cells seeded on the CAM with the control proteins grew into tumor mass in 8 days (Fig. 1a,b) whereas MDA-MB-231 cells seeded together with 600 μg of rtEa4-peptide did not grow into tumor mass (Fig. 1c). No obvious toxic effect was observed in CAM treated with the same amount of the control proteins (Fig. 1c,d). These results suggest that the growth of MDA-MB-231 cells on the chicken CAM was arrested by rtEa4-peptide. Similar results were obtained

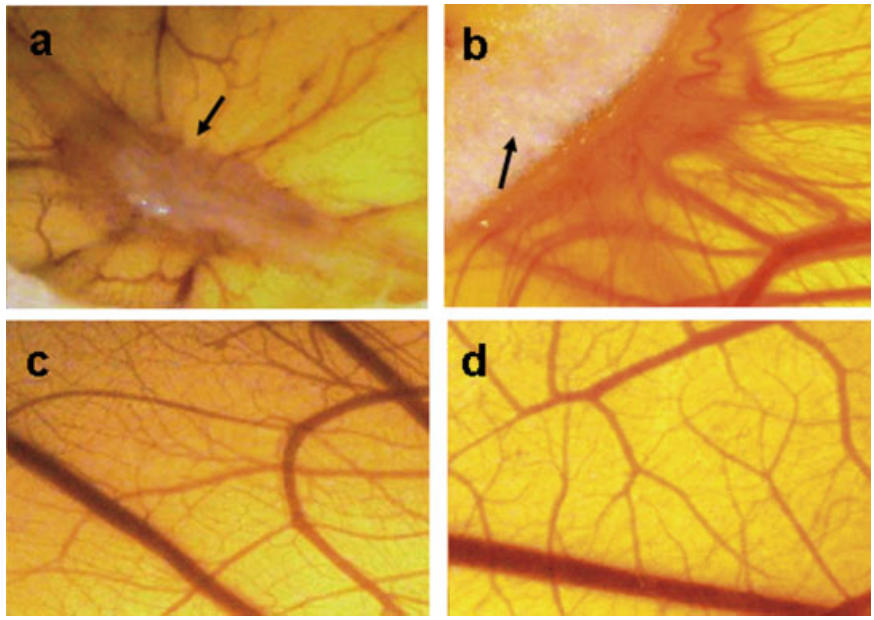

Fig. 1. Effect of rtEa4-peptide on growth of MDA-MB-231 cells on the chorioallantoic membrane (CAM) of chicken embryos. Five-day-old chicken embryos were seeded with MDA-MB-231 cells (1×10^7 cells) and treated with one dose of recombinant rtEa4-peptide (600 μg) or the same amount of the control proteins. After incubation for 8 days, embryos were observed under a dissecting microscope and photographed with a digital camera. **a**,**b**: CAM seeded with MBA-MD-231 cells and 600 μg/embryo of control proteins, (**c**) CAM seeded with MBA-MD-231 cells mixed with 600 μg/embryo of rtEa4-peptide, (**d**) CAM without any treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

when similar doses of synthetic hEb-peptide were used instead of rtEa4-peptide (results not shown). Results in Table II further showed that although about 20% embryos seeded with MDA-MB-231 cells and one dose of rtEa4-peptide (600 μg/embryo) still resulted in the development of a small tumor mass, none of the embryos showed tumor mass development when the embryos received an additional three doses (600 μg/embryo/dose) of rtEa4-peptide at every 2-day intervals. It is of great interest to note that 2.4 mg of rtEa4- or synthetic hEb-peptide did not affect the normal development of chicken embryos. Since no special effort was made to re-fold the recombinant rtEa4-peptide in our preparation, the active form of the rtEa4-peptide might be much lower than the 600 μg/dose that we used in these studies. Work is underway in our laboratory to refold and purify recombinant rt Ea4-peptide to homogeneity.

Since MDA-MB-231 cells are highly invasive breast cancer cells, one would expect to detect metastasis of the cells in chicken embryos when the cells are seeded on the CAM. The distribution of MDA-MB-231 cells in the CAM over a period of 8 days was determined by immunocytochemical staining of GFP in the MDA-MB-231 cells with GFP specific antibodies in cross sections of CAM and the associated embryonic materials. Figure 2a,b shows the composite images complied from images taken along the cross sections of the samples. In embryos seeded with MDA-MB-231 cells and the control proteins, cancer cells were identified not only on the CAM but also in the embryos behind the CAM (Fig. 2a). This observation suggests that

TABLE II. Inhibition of Growth of MDA-MB-231 Cells in Chicken CAM by rtEa4-Peptide

Treatment	#Embryos treated	#Embryos with tumors	% of tumor formed
1×10^7 cells seeded	20	20	100
1×10^7 cells seeded, treated with one dose of rtEa4-peptide (600 μg)	20	4	20
1×10^7 cells seeded, treated with 4 doses of 600 μg rtEa4-peptide	20	0	0

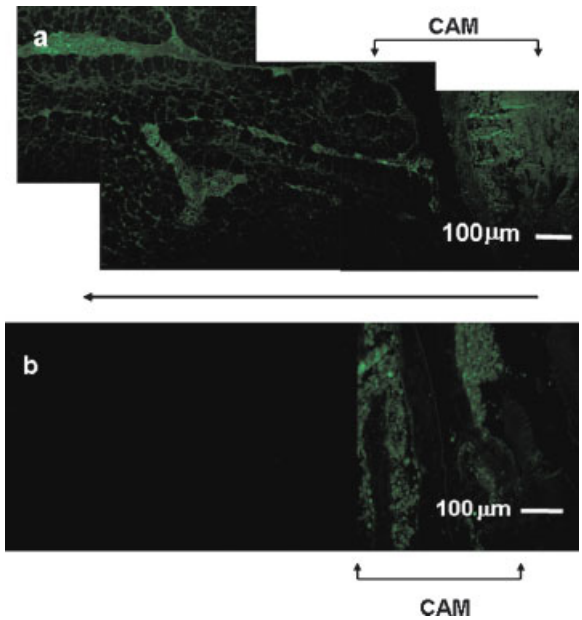

Fig. 2. Effect of recombinant rtEa4-peptide on invasion of MDA-MB-231 cells in developing chicken embryos. Five-day-old chicken embryos were seeded with MDA-MB-231 cells expressing enhanced green fluorescence protein (EGFP) and treated with rtEa4-peptide or the control proteins as in Figure 1. The CAM with MDA-MB-231 cells were dissected, fixed sequentially in 50% ethanol and 3.7% formaldehyde and embedded in paraffin with CAM facing up. Serial cross sections of 5 μm were prepared and stained with anti-green florescence protein IgG and FITC labeled goat anti-rabbit IgG sequentially. The stained sections were observed under a fluorescence microscope (Olympus IX50 with an appropriate filter set to give excitation wavelength at 490 nm and emission wavelength at 520 nm) at 200× magnification. Images were taken along the sections from CAM and inward and composite photos were compiled the serial images. **a**: Sections of CAM with MDA-MB-231 cells and control proteins and (**b**) sections of CAM with MDA-MB-231 cells treated with rtEa4-peptide. Arrow indicates the orientation of CAM and direction of sectioning. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MDA-MB-231 cells have invaded through CAM the chicken embryos. In the embryos seeded with MDA-MB-231 cells together with 600 μg/embryo of rtEa4-peptide, a small amount of cancer cells were only identified on the surface of the CAM membrane, suggesting that the invasive activity of MDA-MB-231 cells was inhibited by the rtEa4-peptide (Fig. 2b). These results are in good agreement with the results of a matrigel invasion chamber assay, in which a dose-dependent inhibition of MDA-MB-231 cell invasion across the matrigel membrane was observed by rtEa4-peptide [Chen et al., 2002; Siri et al., 2006].

It is possible that the results observed in Figure 1c could be the consequence of E-peptide killing of MDA-MB-231 cells that are not in active proliferation. Could rtEa4- or synthetic hEb-peptide kill MDA-MD-231 cells that are in the state of active proliferation in chicken embryos? To answer this question, MDA-MB-231 cells were seeded at one location on the CAM of 5-day-old embryos, allowing the cells to establish their growth on the CAM for 4 days. On day 9, each embryo was treated with 600 μg of synthetic hEb, and the treatment was repeated 2 additional times at every 2-day interval. In these studies, MDA-MB-231 cells transfected with EGFP gene were used to facilitate data scoring by fluorescence microscopy in order to increase its sensitivity and specificity. As shown in Figure 3Ba,Bb, while numerous tumor masses were observed below the CAM in the embryos without hEb-peptide treatment, no visible tumor mass was observed in embryos treated with multiple doses of hEb-peptide. Furthermore, multiple treatments of chicken embryos with hEb-peptide did not exhibit any toxic effect to the embryos, since no obvious apparent defects were observed in

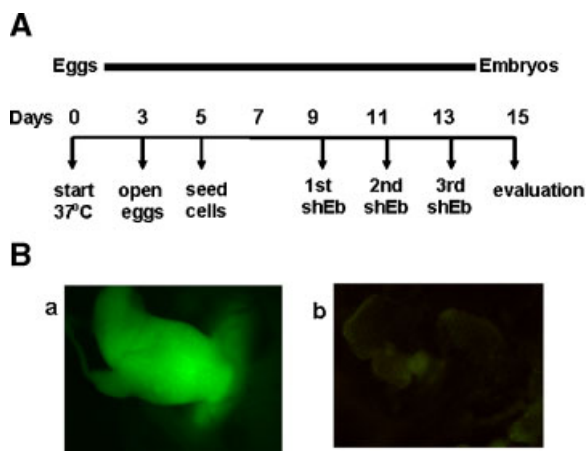

Fig. 3. Suppression of MDA-MB-231 cell growth on CAM of developing chicken embryos by synthetic human Eb-peptide. MDA-MB-231 cells (1×10^7 cells) were seeded on CAM of 5-day-old embryos. From day 9 to day 13, 3 doses of synthetic hEb-peptide or BSA (0.6 mg/dose/embryo) were added to each embryo. On day 15, the CAM was dissected and observed under an inverted microscope (Olympus IX50 with an appropriate filter set to give excitation wavelength at 490 nm and emission wavelength at 520 nm) at 40× magnification. **A**: Treatment scheme; (**B**) breast cancer cell mass on CAM. **a**: MDA-MB-231 cell mass with the control proteins on CAM; (**b**) MDA-0MB-231 cells on CAM treated with synthetic hEb-peptide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the embryos treated in the hEb-peptide. These results further support the conclusion that both growth and invasion of MDA-MB-231 cells in the CAM of chicken embryos are inhibited by rtEa4-or synthetic hEb-peptide.

Inhibition of Angiogenesis by rtEa4- or Synthetic hEb-Peptide on CAM

Upon careful examination of CAM bearing MDA-MB-231 cell masses, numerous blood vessels radiated from the cell mass were observed (Fig. 1a,b). When MDA-MB-231 cells on the CAM were treated with rtEa4-peptide, neither visible cancer cell mass nor blood vessels associated with the cancer cell mass, like those observed in Figure 1a,b, were observed (Fig. 1c), and the distribution of blood vessels on the CAM membrane of the rtEa4-peptide treated embryos was not obviously different from that of the embryos treated with the control proteins (Fig. 1d). These results suggest that rtEa4-peptide may possess an antiangiogenic activity. The chicken embryo CAM assay is considered to be the simplest, inexpensive and most reproducible among many of the

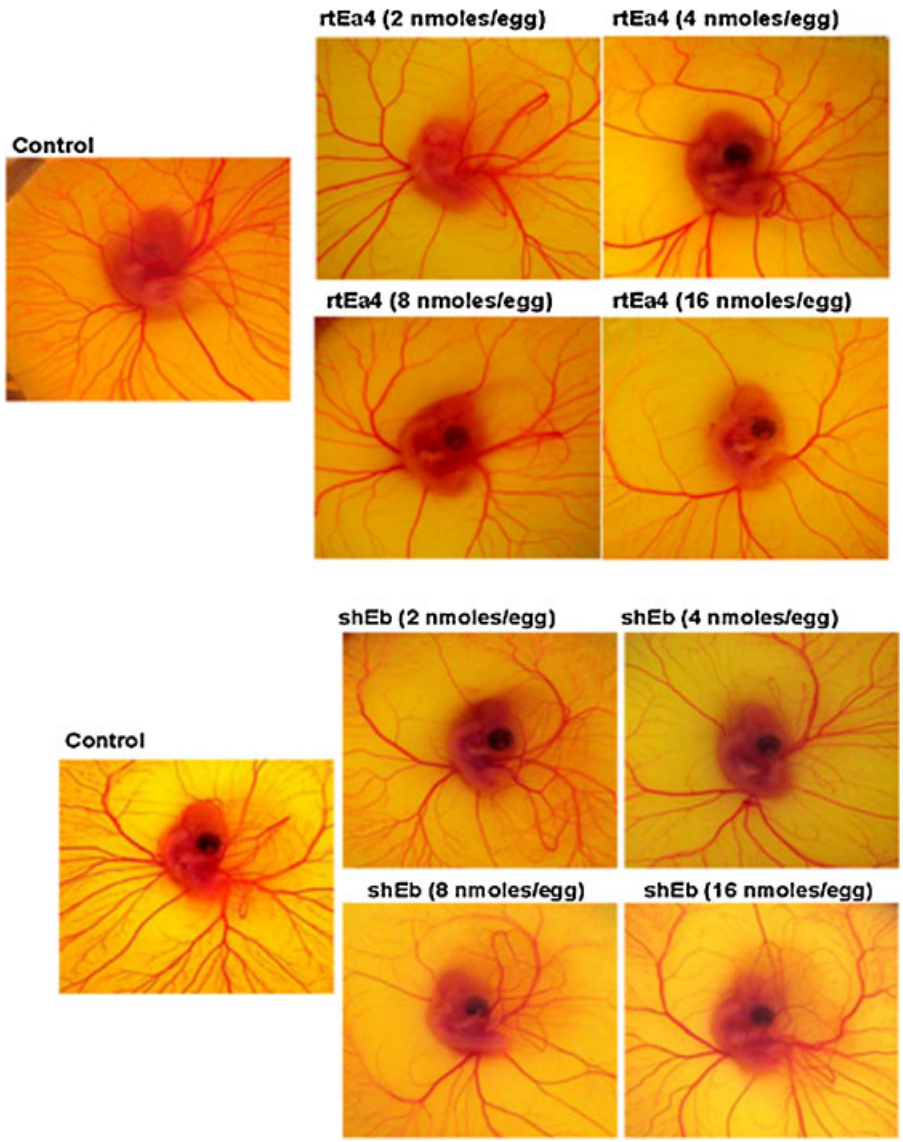

Fig. 4. Antiangiogenic effect of rtEa4-peptide on the CAM of chicken embryos. Three-day-old chicken embryos were treated with various doses (2–16 nmol) of rtEa4-peptide (A) or and synthetic hEb-peptide, and the embryos were photographed at day 5. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

classical assays for studying angiogenesis in vivo [Ribatti and Vacca, 1999], and we adopted this assay system to confirm whether rtEa4- or synthetic hEb-peptide possesses antiangiogenic activity. Results presented in Figure 5A showed that the antiangiogenic activity of a known antiangiogenic factor, endostatin, can be reproducibly assessed by the chicken CAM assay. Furthermore, both rtEa4-peptide and synthetic hEb-peptide at 2–16 nmol exerted a dose-dependent inhibition of angiogenesis on CAM (Figs. 4A,B and 5B). The doses of E-peptide that exhibited antiangiogenic activity in CAM are in the similar order of magnitudes with the doses that inhibited cancer cell growth in the chick embryos.

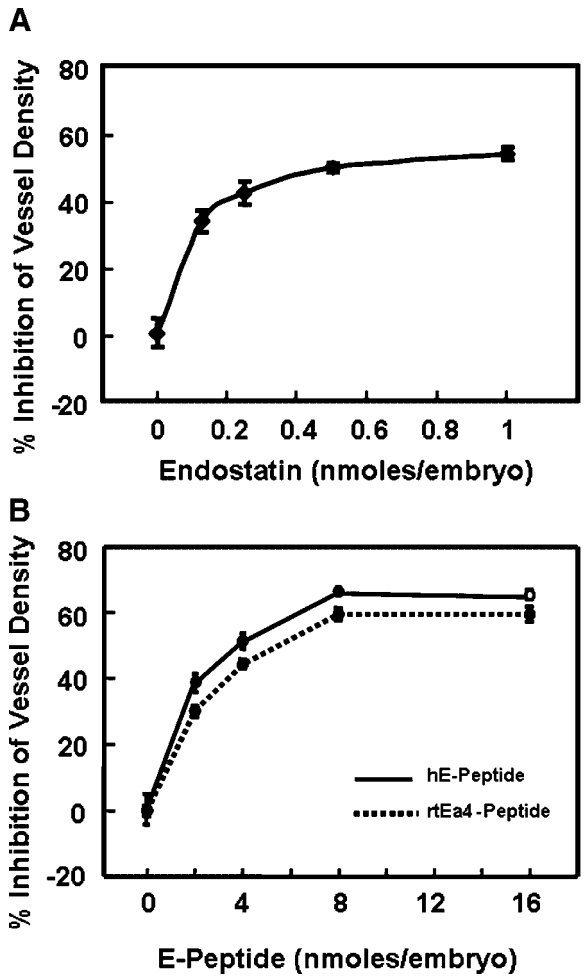

Fig. 5. Dose-dependent response of antiangiogenetic effect of endostatin, rtEa4- or hEb-peptide on the CAM of chicken embryos. Various amounts of endostatin, rtEa4-peptide or hEb-peptide were applied to the CAM of day 3 chicken embryos and the data were scored at day 5. Percent of inhibition of vessel density = [(vessel density of control—vessel density of treated embryos)/vessel density of control] × 100%. Each data point is the average of vessel density from 10 embryos ± standard error (SE). **A**: Antiangiogenic effect of endostatin; (**B**) antiangiogenic effect rtEa4- or hEb-peptide.

Regulation of Gene Expression in MDA-MB-231 Cells by rtEa4-Peptide

In our earlier studies, we have shown that introduction of a rtEa4-peptide gene into MDA-MB-231 cells or a hEb-peptide gene into human neuroblastoma cells (SK-N-F1) by transfection resulted in the transfectants losing malignant characteristics (i.e., colony formation in soft agar and invasion through matrigel membrane) [Chen et al., 2002; Kuo and Chen, 2002]. These results suggest that rtEa4-peptide or hEb-peptide may down regulate genes responsible for the malignancy of cancer cells. To confirm if rtEa4- or hEb-peptide inhibits the growth, metastasis, and angiogenesis of MDA-MB-231 cells by up- and/or down-regulation of genes related to these activities, we screened a human gene chips containing 9500 unique cDNA clones using cDNA prepared from mRNA of MDA-MB231 cells transfected with expression vectors with and without the secreted form of rtEa4-peptide gene. The reason for using RNA isolated from MDA-MB-231 cells transfected with the secreted form of rtEa4-peptide gene is to ensure that the resulting MDA-MB-231 cells are under constant exposure to rtEa4-peptide. Table III presents a partial list of genes that are up- or down-regulated by rtEa4-peptide. Furthermore, the mRNA levels of some of these genes in rtEa4-transfected and non-transfected MDA-MB-231 cells were assessed by the comparative real-time RT-PCR analysis. Results presented in Table IV are in good agreement with those in Table III. Among those genes analyzed, cysteine-rich angiogenic inducer gene may be related to tumor-induced angiogenesis, genes of urokinase and plasminogen activator inhibitor related to the invasive activity, genes of fibronectin 1 and laminin receptor related to attachment and anchorage-dependent cell growth, and genes of capase 3 and BCL 2 related to apoptosis. Although we did not pick up up-regulation of caspase 3 gene from microarray screening because the gene is not included in the array, results of preliminary studies showed that rtEa4- or hEb-peptide induced apoptosis in MDA-MB-231 cells by TUNEL assay (Chen and Chen, in preparation). Recently we have reported that down regulation of the expression of urokinase and plasmanogen activator

TABLE III. A Partial List of Genes Up- or Down-Regulated by rtEa4-Peptide in MDA-MB-231 Cells

Name of the genes	Ratio[a]	Acession #
Up-regulated genes		
TYPO3 protein tyrosine kinas	1.3	AA564121
Fibronectin 1	1.2	R42093
Cytochrome c-1	1.0	AA037369
DEAD/H (Asdp-Glu-Ala-Asp/His) box polypeptide 9 (RNA helicase)	1.1	AA028972
DEAD/H (Asdp-Glu-Ala-Asp/His) box polypeptide 5 (RNA helicase, 68 kDa)	0.7	H18448
Tumor rejection antigen (gp96) 1	0.6	AA027981
Tissue inhibitor metalloproteinase 1 (erythroid potentiating activity)	0.6	AA059307
Heat shock 90 kDa protein 1, beta	0.6	AA055974
Heat shock 90 kDa protein 1, alpha	0.5	N20012
Heat shock 70 kDa protein 10	0.6	N26743
Phospholipase C, gama 2 (phosphatidyleinositol-specific)	0.6	H57180
Laminin receptor 1 (67 kDa)	0.4	W46382
RAN, member RAS oncogene family	0.4	R60931
Mitogene-activated protein kinase 2	0.4	N71990
Down-regulated genes		
Tumor-associated calcium signal transducer 2	−1.6	AA029700
EGF-containing fibulin-like extracellular matrix protein	−1.5	R27614
BCL2	−1.3	N38908
Cysteine-rich angiogenic inducer 61	−1.4	W48667
Prion protein (p27–30)	−1.0	H15255
Transglutaminase 2 (C polypeptide)	−1.2	H11775
Serum-inducible kinase	−0.9	H52648
Plasminogen activator, urokinase	−0.9	R74194
Plasminogen activator inhibitor, type 1	−0.9	R21222
Gelsolin (amyloidosis, finish type)	−0.9	H06524
Thrombospondin 1	−0.8	AA187188
Keratin 7	−0.8	H02522
Oncogene TC21	−0.8	N23355
BRB7, member of RAS oncogene family	−0.7	AA084368
BCL-like 1	−0.4	H40035
Oncogene TC21	−0.7	R79785
Vav 1 oncogene	−0.4	T65770
Insulin-like growth factor binding protein 3	−0.3	AA135554
Insulin-like growth factor binding protein 7	−1.0	N92373

[a]Ratio = log [color signal intensity of rtEa4-peptide treated cells/color signal intensity of control cells]. When ratio is "+", up-regulation; when ratio is "−", down regulation.

inhibitor genes in MDA-MB-231 cells by rtEa4-peptide resulted in loss of the invasive activity of the cells [Siri et al., 2006]. Furthermore, we have also shown that introduction of a transgene encoding a secreted form of rtEa4-peptide or hEb-peptide into embryos of medaka or zebrafish resulted in severe defects of vasculature development [Chun and Chen, 2004; Chun et al., 2006]. Together, these results support the notion that rtEa4- or synthetic hEb-peptide possesses antiangiogenic activity capable of inhibiting tumor-induced angiogenesis and

TABLE IV. Comparative Real-Time RT-PCR Analysis of Genes Up- or Down-Regulated by rtEa4-Peptide

Name of genes	Relative level of expression[a] [$2^{-(S\Delta CT-C\Delta CT)}$]
Plasminogen activator, urokinase (uPA)	0.52 ± 0.10
Plasminogen activator inhibitor type 1 (PAI 1)	0.42 ± 0.04
BCL 2	0.19 ± 0.03
Cysteine-rich angiogenesis inducer 61	0.71 ± 0.12
Tumor-associated Ca^{++} signal transducer 2	0.22 ± 0.07
TYPO3 protein tyrosine kinase	3.02 ± 0.80
Tumor rejection antigen (Gp96)	4.96 ± 0.26
Heat shock 90 kDa protein 1α	3.40 ± 0.35
Heat shock 70 kDa protein 10	2.92 ± 0.39
Caspase 3	4.58 ± 0.35
Fibronectin 1	2.32 ± 0.31
Laminin receptor 1	1.41 ± 0.11

[a]Relative level of expression = $2^{-(S\Delta CT-C\Delta CT)}$, where SΔCT is the difference between the CT number of the sample (MDA-MB-231 cells transfected with rtEa4-peptide) and the house keeping gene (β-actin) and CΔCT is the difference between the CT number of the control (MDA-MB-231 cells) and the house keeping gene (β-actin). $2^{-(S\Delta CT-C\Delta CT)} < 1$, down-regulated gene; $2^{-(S\Delta CT-C\Delta CT)} > 1$, up-regulated gene.

anti-metastatic activity capable of restricting the invasion of MDA-MB-231 cells on chicken CAM.

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. While it has been shown that Ea4-peptide of trout pro-IGF-I possessed mitogenic activity in non-transformed cells [Tian et al., 1999], we have also shown the same peptide exerted inhibitory activities on anchorage-independent growth and invasion in established human cancer cells in vitro [Chen et al., 2002; Kuo and Chen, 2002]. In this study, we have further shown that rtEa4-peptide exerted inhibitory activities on cancer cell growth, metastasis and cancer-induced angiogenesis in developing chicken embryos. Furthermore, we have also found that both rtEa4-peptide and hEb-peptide share similar nature of biological activities (i.e., existence of functional conservation), though both molecules only share 40% of identity in their primary structure [Chen et al., 2002; Kuo and Chen, 2002, 2003]. 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 increasing evidence indicating that the C-peptide of pro-insulin elicits functions different from the mature insulin [Ido et al., 1997; Johansson et al., 1992a,b; Wahren et al., 2000]. Although the physiological relevance of these E-peptide activities remains unclear, Previous published results from our laboratory have indicated the presence of a high affinity binding component specific for rtEa4- or hEb-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 are inhibited by signal transduction inhibitors involving MAP kinase/PI3-kinase cascades [Kuo and Chen, 2002, 2003]. Overall, these new findings highlight the existence of a conserved mechanism(s) of E-peptide action and 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. Unarguably, more studies are required to address the biological roles of the pro-IGF-I E-peptides and further dissect the complexity of the IGF system, our novel discoveries point out the importance of questioning the previously established concepts of IGF actions.

The hallmark characteristics of solid tumors are anchorage-independent growth, metastasis and tumor-induced angiogenesis. An ideal approach for human cancer therapy will be controlling all of these three activities simultaneously with one agent. Since we have demonstrated that rainbow trout Ea4-peptide or human Eb-peptide of pro-IGF-I inhibited anchorage-Independent growth, invasion and tumor-induced angiogenesis of several well-established human cancer cell lines in vivo and in vitro, these peptides might have the potential to be developed into an ideal therapeutic agent for human cancer treatment.

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