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Functional Studies on a Novel Engineered Peptide Derived from C-Terminal of Human Endostatin

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ABSTRACT

Endostatin, the C-terminal fragment of collagen XVIII, is known as an endogenous inhibitor of angiogenesis and is currently used as an anticancer drug. Endostatin fragments can be used as alternatives for full-length molecule, among which a peptide derived from the C-terminal fragment of protein, including residues 123-184 was shown to effectively inhibit angiogenesis and tumor growth. The aim of current study was to design a shortened peptide of the fragment 123-184. For this purpose, the presumably non-functional segments were deleted, including β-hairpin segment, comprising residues 145-163 and C-terminus of the protein, including residues 175-184. The designed 30-amino acid peptide that encompasses random coils in 123-184 (referred to as C-peptide) was synthesized and characterized. C-peptide inhibited the proliferation of the Human Umbilical Vein Endothelial Cells (HUVECs) with an IC50 value of 0.35 μM. Administration of C-peptide caused the regression of 4T1 murine mammary carcinoma tumor growth, considerable reduced tumor cell proliferation (Ki67 expression) and angiogenesis (CD31 and CD34 expression), and the induction of apoptosis (increased TUNEL staining). These results confirm that the random coils of the C-terminal domain of endostatin are implicated in its antiangiogenic and antitumor properties.

Keywords: Endostatin, Peptide design, Angiogenesis, Tumor growth, Cell proliferation

INTRODUCTION

Tumor growth depends on the nutrients and oxygen. Angiogenesis, the formation of new microvessel from the pre-established ones, is required for tumor growth and metastasis [1]. Angiogenesis is controlled by positive and negative regulators, *i.e.* pro and anti-angiogenic factors [2,3]. Pro-angiogenic factors are generally growth factors such as vascular endothelial growth factors (VEFG) and basic fibroblast growth factors (bFGF), which induce different aspects of endothelial cell biology, including proliferation and migration and tubulogenesis [4]. In contrast, anti-angiogenic factors that are predominantly derived from the extra cellular matrix inhibit angiogenesis and concomitantly promote apoptosis [5].

Endostatin, a 184 amino acids (20-kDa) C-terminal proteolytic fragment of collagen XVIII, was shown to interfere with multiple angiogenesis signaling pathways (targets around 12% of all regulatory genes of the human genome) and inhibit blood vessel formation and, consequently, tumor cell growth in more than 65 different tumor types [6-8]. Currently, the recombinant endostatin, known as Endostar, is used as an anti-cancer drug. Full-length endostatin can be replaced by short peptide fragments, among which two well-known fragments, including a 27-amino acid zinc-binding fragment, corresponding to the N-terminal domain of the molecule, and the C-terminal fragment, corresponding to the residues 134-187, mimic the antitumor and anti-angiogenic properties of the entire molecule [9,1].

The aim of present study was to investigate the anti-angiogenic activity of an engineered peptide derived from the C-terminal fragment (residues 123-178) of human endostatin. Specifically, we have designed a peptide corresponding to sequence 123 to 171 devoid of the beta structured component (145 to 163) in order to study the

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effect of this deletion on the activity of this peptide. Our results show this peptide exhibit potent anti-angiogeinc activity *in vivo* and *in vitro* even at the very low concentration.

MATERIALS AND METHODS

Peptide, Chemical and Reagent

The peptide was synthesized and purified by high-performance liquid chromatography to a purity of 90%, analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), and confirmed by electrospray ionization mass spectrometry (ESI-MS) analysis (Shine Gene Biotechnologies, Inc., Shanghai, China). Anti-CD31 (Ab32457), anti-CD34 (Ab81289), anti-Ki-67 (Ab15580), TUNEL assays were performed using an in situ Cell Death Detection Kit POD (Roche Diagnostic GmbH, Germany).

In Vivo Antitumor Efficacy Evaluation

The animal study was performed under a protocol that approved by the Institutional Animal Care and Use Committee (IACUC) of Tehran University of Medical Sciences Tumor cells (4T1; 1 × 106 cells/500 µl or 1 × 105 cells/50 µl) were injected subcutaneously into the right flanks of mice (n = 3-5). To generate the metastatic model, 4T1 tumor models were sterilized, excised from the breast cancer-bearing BALB/c mice, cut into pieces of < 0.3 cm^3, and subcutaneously implanted into the animals' right flanks under ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia [11,12]. When the tumor volume reached (~400 mm^3), tumor bearing BALB/c mice were randomly divided to two groups (n = 6 mice/group). The treatment groups received 5 mg/kg i.p of the C-peptide daily, whereas the control group received the equal volume of PBS for two weeks. To monitor the growth of the tumor, length and width of the tumors were measured every other day with digital caliper (Mitutoyo, Japan), and the tumor volume was calculated by using the formula: (Volume = 0.52 × length × width2) 45.

Cell Proliferation Assay

The effects of C-peptide on the proliferation of HUVEC cells were quantified after 24, 48 and 72 h by 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, Missouri, USA) assays as described previously with some modifications [11]. The absorbance was measured at 570 nm with background subtraction of 630 nm using an ELISA reader (Space Fax 2100, Awareness, USA). Briefly, 2 × 10^3 HUVECs were added to each well of a plate in DMEM media containing 5% FBS and incubated overnight at 37 °C. Cells were then transferred to FBS free medium (Sigma, St. Louis, Missouri, USA) at 37 °C, 5% CO_2. Cells were incubated with varying concentrations of C-peptide (0.15-1.23 µM) for comparison with untreated control for 48 h under the same conditions.

Immunohistochemistry

Excised tumor tissues for immunohistochemical (IHC) analysis were fixed in formalin (4%), embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E) [14]. IHC was followed by heat-induced epitope retrieval in a buffer at pH 9.0. IHC staining for CD31 and CD34 (to assign MVD) [15]. Ki67 (to determine the percentage of Ki67-positive cells) was performed on formalin-fixed paraffin embedded sections. Staining for cell death with TUNEL (terminal deoxynucleotidyl transferase nick-end labeling) was performed, followed by enzymatic development in diaminobenzidine (DAB) (Invitrogen, Carlsbad, California, USA) detection and counter-staining in hemotoxyline. Images were acquired by microscopy (Olympus BX-51, Japan). Numbers of positive cells were determined by analyzing five random tissue samples under scale bars 100 and 20 µm, with quantification using ImageJ.

Statistical Analysis

The Prism software (version 6.00 for Windows, GraphPad Software, La Jolla, California, USA; www.graphpad.com) was used for data analysis, for the generation of graphs, and for statistical analysis. Data were provided as mean ± SEM. One-way ANOVA followed by Tukey's post-hoc test was used to statistical significance for multiple comparisons and was used for therapeutic efficacy in affecting tumor growth). The immunohistochemical data were analyzed by student t-test. $P < 0.05$ was interpreted as a statistically significant.

RESULT

Peptide Design

The C-terminal fragment of endostatin, including residues 123 to 184, was shown to inhibit the cell proliferation and migration of endothelial cells and tumor growth even more potently than the full-length endostatin [9]. The present study aimed at shortening this fragment, particularly by maintaining its antitumor activity. Importantly, other peptide fragments that reported as alternatives for full-length endostatin, including fragments corresponding to residues 1-27 [16], 6-49 [17], 60-70 [18], and 180-199 [19], occur in the loop structure. Therefore, the β-hairpin segment, including residues 145-163, and the short β-strand 172-175, that are unlikely to be implicated in the antiangiogenic and antitumor activities of this fragment were altogether deleted. In addition, the residues 175 to 184 was deleted that has also been previously considered to be nonfunctional [20]. The sequence of the designed 30-amino acids peptide (referred to as C-peptide) was 2HN-SDPNGRRLTESYCETWRTEAPSSCHHAYIV-COOH.

C-Peptide Inhibited the Proliferation of Endothelial Cells *in Vitro*

To study the anti-endothelial effect of the peptide, we evaluated the proliferation of human umbilical vein endothelial cells (HUVECs) by MTT assay. As shown in Fig.1 proliferation is inhibited in C-peptide treated cells vs. untreated control. At 0.18 μM, the proliferation of HUVECs was significantly inhibited ($P < 0.05$) and maximal inhibition of proliferation achieved at the concentration 1.47 μM ($P < 0.0001$). The half inhibitory concentration (IC_{50}) values of C-peptide was 0.35 μM.

C-peptide Led to the Regression of Mammary Carcinoma Tumor Growth

To investigate whether C-peptide has anti-tumor efficacy, the peptide administrated into BALB/C mice, bearing mammary tumor cell line 4T1 by daily intraperitoneal (i.p.) injection at a concentration of 5 mg/kg. Peptide treatment was started at day 14 after tumor implantation. Mice were randomly divided in two groups (n = 6 mice in each group). The emerging data show that C-peptide resulted in the significant tumor growth regression compared to PBS-treated controls. The average size of C-peptide treated tumors at the end of experiment, was 591 ± 54 mm^3 *versus* 1635 ± 532 mm^3 for controls (64% reduction, $P < 0.05$, Fig. 2).

Immunohistochemical Analyses

Since angiogenesis is crucial for tumor growth, we then estimated the influence of C-peptide on tumor angiogenesis. To this end, mammary carcinoma tumor-bearing BALB/C mice were examined by IHC for the endothelial cell markers CD31 and CD34 followed by calculation of microvascular density (MVD). To determine MVD tumor tissue, paraffin sections were immunohistochemically stained with anti-CD31 and anti-CD34 antibodies. As shown in Fig.3a, peptide-treated tumors had considerably less CD31-positive structures rather than untreated controls. The MVD in the peptide-treated mice (1.56 ± 0.53; $P < 0.0001$) was significantly lower than that of PBS-treated tumors (16.72 ± 1.31; $P < 0.0001$). Similar to CD31, peptide treated tumors also displayed reduced numbers of CD34-positive structures with the MVD of (2.25 ± 0.39; $P < 0.0001$ as compared to the controls with the MVD of (19.82 ± 0.57; $P < 0.0001$) (Fig. 3a).

To determine whether the breast tumor growth is associated with reduced cell proliferation within tumor tissue, paraffin sections from tumors extracted on day 14 after treatments of tumors were stained with anti-Ki67 antibody, a well-known tumor cell proliferative index. Tumor nuclei of untreated mice were almost all positive for Ki67, in contrast to the few positive nuclei in peptide-treated tumors (Fig. 3b). Quantitative analysis revealed significant ($P < 0.0001$), approximately X-fold reduction of Ki67-positive nuclei in breast tumors administered with 5 mg/kg/day C-peptide (3.02 ± 1.03, n = 6) compared with control mice (28.60 ± 0.83; n = 6; Fig. 3b). Results of Ki-67 staining suggest reduced cell proliferation within breast tumors 14 days after treatments by C-peptide.

Considering that anti-vascular therapy renders cancer cells mores sensitive to hypoxia induced apoptosis, we hypothesized that the defective tumor growth in peptide-treated mice should also be associated with the promotion of apoptosis within the tumor tissue. To test this hypothesis, breast tumors extracted on day 14 after peptide administration subjected to TUNEL staining (indicative of

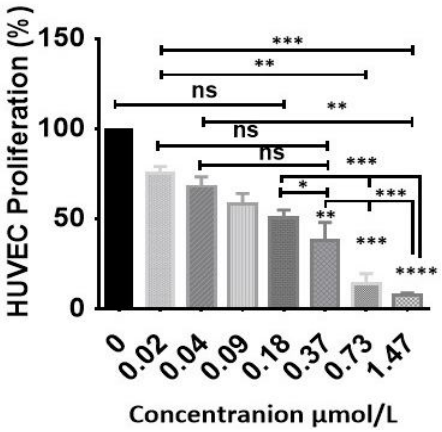

Fig. 1. Inhibition of HUVEC proliferation with C-peptide at the concentration of 0.02-1.47 µM comparing with untreated control. Analysis was performed using Prism 6, One-way ANOVA; ± SEM, n = 6.

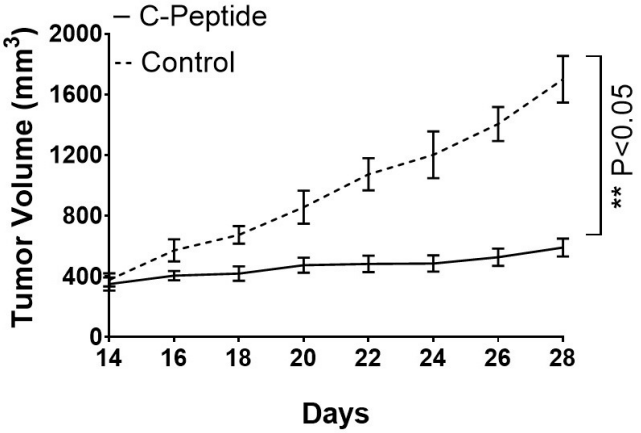

Fig. 2. Inhibition of tumor growth by C-peptide in BALB/C mice with 4T1 cell line implantation. Tumor growth inhibition; lines, mean tumor volume for each group; bars, ± SEM. Significant inhibition of tumor growth occurred in animals treated with C-peptide when compared with control. In one-way repeated measures ANOVA statistical analysis, $^{**}P < 0.05$.

late apoptosis) [21-23]. Compared with the percentage of TUNEL-positive cells in the tumors from mice in the control group (6.251 ± 0.388), the percentages of TUNEL-positive cells in the tumors from mice in treatment group were increased significantly (5 mg/kg/day, 37.36 ± 0.685; $P < 0.001$; Fig. 3c).

DISCUSSIONS

Endostar, a recombinant human endostatin, resulted in significant therapeutic effect in treating late stage non-small-cell lung carcinoma and was approved by the State Food and Drug Administration of China (CFDA) [24]. However, recombinant endostatin faces to limitations for clinical cancer treatment; it is inherently unstable and insoluble, and its production is costly [25]. These limitations may be overcome by application of short fragments instead of full-length endostatin [26]. The peptide fragment corresponding to the residues 134-178 was shown to mimic the antiangiogenic antitumor properties of human endostatin [9]. In the present study, we have shown that a shortened fragment of segment 123-188, composed of

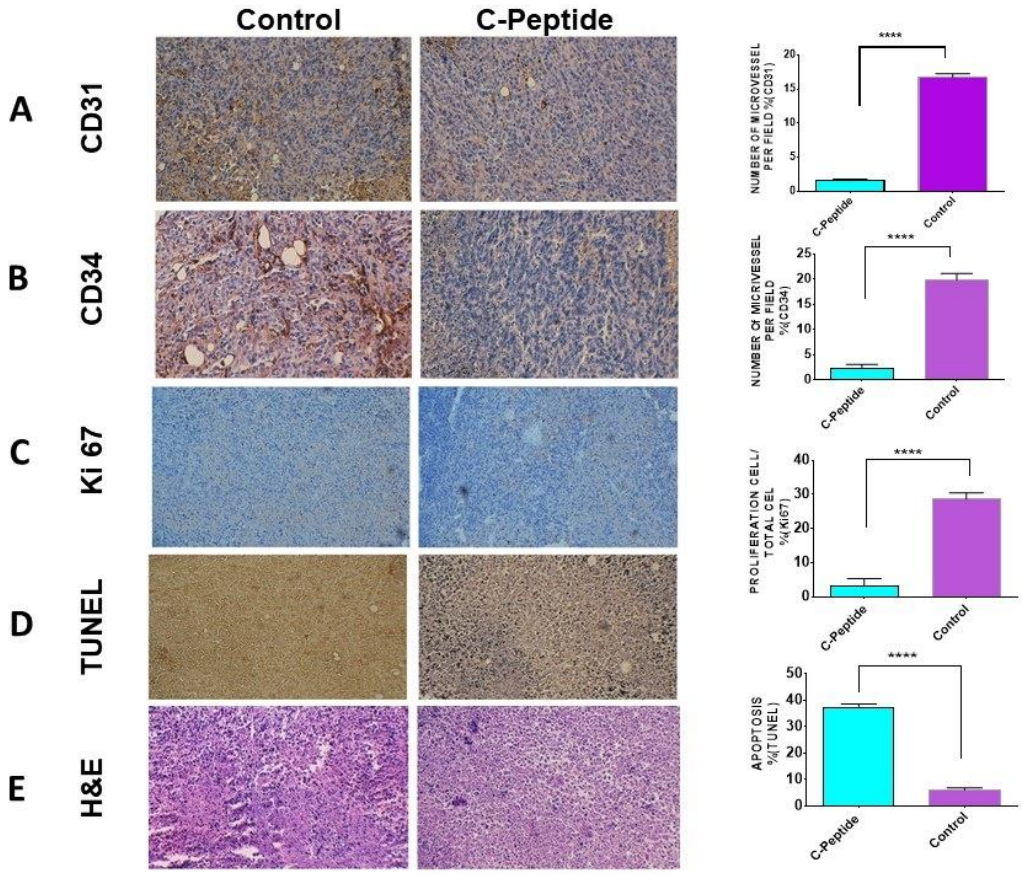

Fig. 3. IHC images indicating the extensive antiangiogenic, antiproliferative, and apoptosis-inducing effects of C-peptide. A) CD31 and B) CD34 showed reduced sprouting in the treatment *vs*. PBS control group, C) Ki67 and H and E. Decreasing proliferation index (Ki67) as indicated by brown spots in the C-Peptide treated *vs*. PBS control, D) TUNEL staining level (apoptosis factors) that were induced by C-peptide treatment. Data were analyzed by ImageJ, and visualized as columns bars using Prism 6. Results shown are representative or mean ± SEM (n = 6); ****$P \leq 0.0001$ *vs*. PBS control. Data are presented as number of vessels per field, percentage of positive proliferative cells per total cells, and percentage of induction of apoptosis relative to PBS control. E) hematoxylin and eosin (H&E) staining (scale bar, 100 μm), for groups treated with 5 mg/kg C-Peptide and the PBS control.

amino acids 123-164 and 164-171, referred to as C-peptide, could inhibit the proliferation of HUVECs and cells and growth of 4T1 mammary carcinoma tumors in BALB/C mice. In addition, immunohistochemical analyses indicated that the expression of angiogenesis-related markers, including CD31 and CD34 were reduced, the level of tumor cell proliferative index Ki67 was decreased. In addition, the staining of TUNEL in tumors treated by C-peptide compared to PBS-treated controls, indicating that the administration of peptide led to the promotion of apoptosis in tumor tissues.

The rationale behind shortening of the C-terminal segment of endostatin was removal of three β-strands. We speculated that the role of these β-structures are purely structural. Our results suggested that removal of these structures was unable to impair the antiangiogenic and antitumor activities of endostatin peptide, implying that these regions are not implicated in the biological activities

of the peptide fragment and full-length endostatin as well. Previous investigations demonstrated that the disulfide bond Cys135-Cys165 is critical for the activity of this fragment [27]. Furthermore, three critical Arg residues, including Arg128, Arg129 and Arg139, were identified as critical for the activity of this fragment [28,29]. Arginine residues that predominantly clustered on the protein surface play significant role in the binding to the receptors like integrin and consequently in its function [8]. Particularly Faye *et al.* reported that Arg 139 is essential for binding to transglutaminase-2 in ECM [30]. Furthermore, it is shown that mutants lacking disulfide bond of Cys135-Cys165 lost nearly their tertiary structure, native, stabilities and function [31]. Notably, both factors implicated in the antiangiogenic and antitumor activities of this fragment, including three Arg residues and disulfide bond are present in C-peptide, confirming that these factors are required for the activity of this fragment.

Peptides are attractive for treatment of cancer due to advantages over protein drugs, such as low cost of synthesis, low side-effects, good penetration into tumor tissues and biocompatibility. In the present study, we indicated that the 30-amino acid fragment derived from the C-terminal domain of human endostatin effectively inhibited growth of a metastatic breast cancer model and has the potential for clinical investigations. The results of current study, in accordance with previous investigations [9,10], confirmed that the loop regions play a dominant role in the biological activity of human endostatin, whereas the C-terminal domain β-strands are not implicated in the function of protein and merely have a structural role.

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CONFLICT OF INTREST

All authors declare that they have no conflicts of interest.

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