Thymosin β10 Inhibits Cell Migration and Capillary-Like Tube Formation of Human Coronary Artery Endothelial Cells

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Thymosin β10 is a cytoplasm G-actin sequestering protein whose functions are largely unknown. To determine the direct effects of exogenous thymosin β10 on angiogenic potentials as endothelial cell migration and capillary-like tube formation, human coronary artery endothelial cells (HCAECs) were incubated with increasing doses of thymosin β10 (25–100 ng/ml). By using a modified Boyden chamber assay, thymosin β10 inhibited cell migration in a dose- and time-dependent manner with the maximal effect being a 36% reduction at 100 ng/ml as compared to controls (*P* < 0.01). In addition, thymosin β10 (100 ng/ml) significantly inhibited the capillary-like tube-formation of HCAECs on Matrigel, showing a 21% reduction of the total tube length as compared to negative controls (*P* < 0.01). Furthermore, by using real time PCR analysis, thymosin β10 significantly decreased mRNA levels of vascular endothelial growth factor (VEGF), VEGF receptor-1 (VEGFR-1) and integrin αV after 24 h treatment in HCAECs. By contrast, thymosin β4 significantly increased HCAEC migration. These results indicate that thymosin β10, but not thymosin β4, have direct inhibitive effects on endothelial migration and tube formation that might be mediated via downregulation of VEGF, VEGFR-1 and integrin αV in HCAECs. This study suggests a potential therapeutic application of thymosin β10 to the diseases with excessive angiogenesis such as cancer. Cell Motil. Cytoskeleton 63:222–230, 2006. © 2006 Wiley-Liss, Inc.

Key words: thymosin β10; thymosin β4 cell migration; tube formation; angiogenesis

INTRODUCTION

Thymosins comprise a family of small proteins originally isolated from calf thymus [Low and Goldstein, 1978], and are divided into three classes (α, β, and γ) based on their isoelectric point. The β-thymosins are structurally related and highly conserved acidic polypeptides. Initially, β thymosins were suggested to have roles in regulating the immune response. Recently, the high expression of β thymosins has been found in a number of other tissues and cells, suggesting many other functions [Horecker and Morgan, 1984; Huff et al., 2001]. The most abundant β thymosins in mammalian cells are thymosin β4 and thymosin β10. Both proteins have been shown to bind to and sequester G-actin [Safer et al., 1991; Yu et al., 1993], thereby modulating cell mobility [Yu et al., 1994;
Sun et al., 1996]. Previous reports have indicated a variety of human tumors overexpress thymosin β10 such as renal and medullary thyroid carcinomas, melanomas [Santelli et al., 1999], and pancreatic [Aldinger et al., 2005] and gastric carcinomas [Oien et al., 2003]. However, thymosin β10 is also reported to decrease in ovarian carcinoma tissues and cell lines. Studies also showed that the overexpression of thymosin β10 with adeno virus in ovarian cancer cell lines decreased cell growth and accelerated apoptosis [Hall, 1995]. Regardless of these investigations, the exact roles of thymosin β10 are not clear.

Angiogenesis, the formation of new blood vessels from the pre-existing vasculature, plays an essential role in tumor growth [Folkman and Shing, 1992; Risau, 1997]. Thus, antiangiogenesis therapies have become very promising in the treatment of cancer [Folkman, 1995]. Excessive angiogenesis is involved in many diseases such as cancer, psoriasis, arthritis, retinopathy, diabetes, atherosclerosis, and infectious diseases [Carmeliet, 2003]. Vascular endothelial growth factor (VEGF) is a major regulator of endothelial cell proliferation, migration, and angiogenesis. Matrix metalloproteinases (MMPs) and integrins provide permissive effects for endothelial cell migration by breaking down the capillary basement membrane, thereby playing an essential role in cell migration and angiogenesis. Thymosin β4 has been known to enhance endothelial cell proliferation, migration, and angiogenesis via its actin binding site [Lin and Morrison-Bogorad, 1990; Grant et al., 1995, 1999; Malinda et al., 1997; Anadon et al., 2001; Sosne et al., 2002; Cha et al., 2003; Philp et al., 2003, 2004; Bock-Marquette et al., 2004]. However, thymosin β4 and thymosin β10 showed distinct patterns of expression in several tissues [Lin and Morrison-Bogorad, 1990] and played different roles during rodent development [Anadon et al., 2001]. The role of thymosin β10 in angiogenesis is largely unknown.

In order to investigate the direct effect of thymosin β10 on angiogenesis, we treated human coronary artery endothelial cells (HCAECs) with synthetic human thymosin β10 and investigated the endothelial functions as migration and capillary-like tube formation in vitro. We found that thymosin β10 inhibited HCAEC migration and tube formation on Matrigel. This study may provide evidence for the potential application of thymosin β10 as an antiangiogenic therapy for the angiogenesis-associated disease such as cancer.

MATERIALS AND METHODS

Chemicals and Reagents

Synthetic human thymosin β10 and thymosin β4 were purchased from ALPCO Diagnostics (Windham, NH). Calcein-AM was from Calbiochem (San Diego, CA). Trizol reagent was from Invitrogen (Carlsbad, CA). Human recombinant VEGF was obtained from Sigma-Aldrich Co. (St. Louis, MO).

Cell Culture

HCAECs were purchased from Cambrex Bio Science Walkersville (Walkersville, MD). The cells were routinely cultured in Endothelial Basal Medium-2 (EBM-2) with growth factors and antibiotics (EGM-2-MV BulletKit, Cambrex Bio Science Walkersville) supplemented with 10% fetal bovine serum (FCS, Gibco, Invitrogen Corporation, Grand Island, NY) at 37°C in humidified air and CO₂ (19:1). Prior to each experiment, HCAECs were placed in the EBM-2 medium with 1% FCS, without addition of growth factors, for 16 h (serum starvation). HCAECs were used between passages 3 and 7.

Cell Migration Assay

Cell migration was measured using a modified Boyden chamber assay, following a modified Endothelial Cell Migration protocol provided by BD BioCoat Angiogenesis System (Bedford, MA). Briefly, serum-starved cells were trypsin-harvested in the starvation medium. Cell suspensions (250 µl, 5 × 10⁵ cells/well) with different doses of thymosin β10 (25–100 ng/ml) were added to the transwell insert (3-µm pore size, Costar, Cambridge, MA). Then, 750 µl of starvation medium was added to the lower chamber and incubated for a certain period of time. Each treatment was repeated in 4 independent transwells. After 4–24 h incubation at 37°C in a 5% CO₂ atmosphere, the chambers were incubated in Hank’s Balanced Salt Solution (HBSS) with 50 nM Calcein-AM, a fluorescence dye, for an additional 90 min to label living cells. The cells were fixed in 2% paraformaldehyde for 5 min and washed in PBS. The nonmigrating cells in the upper chamber were scraped off using blunted forceps and swabs and washed with PBS. The fluorescence from the cells migrated to the lower chamber and was measured using a fluorescence microplate reader (FLx800, Bio-Tek Instruments, Winooski, Vermont) from the bottom at 485/535 nm wavelength. Each plate was read at four different spots of each transwell. The migrated cells were represented by the ratio of fluorescence as compared to the control. The procedure was also used for determining the effect of thymosin β4 and VEGF (positive control) on cell migration. The migrated cells in the filters were also observed under a fluorescence microscope (Olympus; Tokyo, Japan).

Wound Healing Assay

The wound healing assay was performed according to a previous report [Ettenson and Gotlieb, 1992]. After
HCAECs were grown to confluence in six-well plates, a scratch was made with a sterile cell scraper. The starting point was marked with a marker pen at the bottom of the plate. Cells were washed with basal medium twice and were incubated with or without thymosin β10 (100 ng/ml) or VEGF (20 ng/ml) in basal medium and the cells were incubated for 16 h. Photos were taken using an inverted phase contrast microscope (Olympus, Tokyo, Japan).

Capillary-Like Tube Formation Assay

The formation of capillary-like structures by HCAECs on Matrigel (BD Biosciences, Bedford, MA) was studied as previously described [Nagata et al., 2003]. Twenty-four-well culture plates were coated with Matrigel according to the manufacturer’s instructions. HCAECs were pretreated with or without thymosin β10 (100 ng/ml) or VEGF (20 ng/ml) in the starvation medium for 24 h. Then, trypsin-harvested HCAECs were seeded onto the coated plates at 5 × 10³ cells per well in the fresh assay medium and incubated at 37°C for 18 h. Tube formation was observed using an inverted phase contrast microscope (Olympus, Tokyo, Japan). Images were captured at a magnification of 100× with a digital microscope camera system. The degree of the tube formation was quantified by measuring the length of tubes in three randomly chosen fields from each well using an Image-Pro Plus software (Media Cybernetics, San Diego, CA) and was calculated against untreated groups. Each experiment was repeated three times.

RNA Isolation and Real Time RT-PCR

Serum-starved HCAECs were treated with thymosin β10 (50 and 100 ng/ml) for 24 h and total RNA was isolated using Trizol reagent, then treated with TURBO™ DNase (RNase-free) (Ambion, Austin, TX) to eliminate potential genomic DNA contamination. One microgram of total RNA was reverse-transcribed in 20 μl of reaction solution using the iScript™ cDNA Synthesis Kit. Human VEGF, VEGFR-1, VEGFR-2, MMPs, and integrins as well as β-actin primers were designed using Beacon Designer software (Table I). Each pair of primers was selected to yield a single amplicon, which was verified by dissociation curves and analysis in agarose gels. The real-time PCR reactions were performed with the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories) in 96-well reaction plates. Reaction volumes were 25 μl, containing 2 μl cDNA and 100 μM of each pair of primers and iQ™ SYBR Green Supermix (Bio-Rad). Thermal cycling conditions included pre-incubation 95°C for 3 min followed by 40 PCR cycles at 95°C for 20 s and 60°C for 1 min. All reactions were run in triplicate. The iCycler software was used to analyze the calibration curve by plotting the threshold cycle (Ct) versus the logarithm of the number of copies for each calibrator. The quality and quantities of samples were normalized based on that of the housekeeping gene β-actin as [2^(Ct-β-actin-Ct gene)].

Statistical Analysis

Data are presented as mean ± SEM as compared to the negative control. Statistical significance was determined by a Student’s t test (two tailed). A value of P < 0.05 was considered significant.

RESULTS

Thymosin β10 Inhibits Endothelial Cell Migration in the Boyden Chamber Model

In order to investigate the effect of thymosin β10 on HCAEC migration, cells were treated with increasing concentrations of thymosin β10 (25–100 ng/ml) in the upper chamber of a modified Boyden chamber. The cells migrated through the polystyrene-membrane with 3-μm size pores and were stained with Calcein-AM, a fluorescence dye, and measured with a fluorescence reader. As shown in Fig. 1, thymosin β10 significantly decreased HCAEC migration in a dose-dependent manner and the maximum effect was at 100 ng/ml with a 36% reduction.
of HCAEC migration as compared to the medium-alone control (Fig. 1A, $P < 0.01$). To confirm the above finding, we performed a time course study at 4, 8, 12, and 24 h and observed a detectable inhibitory effect of thymosin $\beta 10$ (100 ng/ml) on cell migration as early as 4 h after cell seeding and the most significant effect was observed at 24 h (Fig. 1B). In order to exclude the possible cytotoxic effect of thymosin $\beta 10$ on HCAECs during the migration assay, cell viability was evaluated by MTS assay. After 24 h of treatment with increasing concentrations (25–100 ng/mL) of thymosin $\beta 10$, no cell viability change was observed in the treated sample (data not shown). These data indicate that thymosin $\beta 10$-induced inhibition of HCAEC migration was not caused by its cytotoxic effect. In a separate experiment, thymosin $\beta 4$ was compared with thymosin $\beta 10$ on cell migration with the Boyden chamber. As shown in Fig. 2, thymosin $\beta 4$ significantly increased HCAEC migration, whereas thymosin $\beta 10$ significantly inhibited HCAEC migration as compared to controls (Fig. 2A, $P < 0.05$). The cells that had migrated to the lower chamber were also visualized under fluorescence microscope (Fig. 2B).

**Thymosin $\beta 10$ Inhibits Endothelial Cell Migration in the Wound Healing Model**

To further test the effect on endothelial cell migration, a wound healing assay was used to show the cell morphology and behavior after exogenous thymosin $\beta 10$ (100 ng/ml) treatment. As shown in Fig. 3, thymosin $\beta 10$ substantially inhibited the cell migration during the wound healing process, whereas VEGF (20 ng/ml) increased the cell migration as compared to the control.

**Thymosin $\beta 10$ Inhibits Capillary-Like Tube Formation in Vitro**

To investigate whether thymosin $\beta 10$ could affect the angiogenic property of endothelial cells, we performed a capillary-like tube formation assay on Matrigel, a more commonly used method for in vitro angiogenesis. HCAECs were pretreated with or without thymosin $\beta 10$ (100 ng/ml) or VEGF (20 ng/ml) for 24 h and then seeded $5 \times 10^4$ cells/well on Matrigel in the 24-well plate with fresh medium. The capillary-like tube was developed and photos were taken after an 18 h incubation. Treatment with 100 ng/ml of thymosin $\beta 10$ inhibited the capillary-like tube formation as compared to the negative controls in the plain medium at 18 h (Fig. 4A). Quantitative analyses revealed that thymosin $\beta 10$ significantly reduced the total length of capillary-like tubes by 21% at 18 h as compared to negative controls, whereas VEGF significantly increased it by 38% as compared to the negative controls (Fig. 4B, $P < 0.01$). These results suggest that thymosin $\beta 10$ inhibits capillary-like tube formation of HCAECs.

**Thymosin $\beta 10$ Decreases the Expression of VEGF, VEGFR-1, and Integrin aV in HCAECs**

VEGF, mediated through 2 major receptors such as VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), is a major regulator of endothelial cell proliferation, migration, and angiogenesis [Braunwald, 1997]. MMPs and integrins play an essential role in cell migration and angiogenesis. Expression of VEGF, VEGFR-1 and VEGFR-2, several MMPs and integrins in HCAECs before and after thymosin $\beta 10$ treatment was evaluated by real time PCR. As shown in Fig. 5, the cells treated with thymosin $\beta 10$ (100 ng/ml) for 24 h showed significant reductions of the
mRNA levels of VEGF, VEGFR-1, and integrin αV in HCAECs by 36%, 31%, and 33%, respectively, as compared to that in control cells ($P < 0.05$). However, no changes were observed in the mRNA expression of VEGFR-2, MMPs, and other integrins (data not shown). Thus, we demonstrate that thymosin β10 downregulates several angiogenic factors including VEGF, VEGFR-1, and integrin αV in HCAECs.

Fig. 2. The effects of thymosin β10 and thymosin β4 on HCAEC migration. Serum-starved HCAECs were seeded onto the transwell plate with thymosin β10 (100 ng/ml), thymosins β4 (100 ng/ml), VEGF (20 ng/ml), or basal medium as a control. After 6 h incubation, HCAEC migrated to the lower chamber were stained with calcein-AM. The cells migrated to the lower chambers were measured by a fluorescence plate reader. A: The quantitation of the HCAECs migrated to the lower chamber after treatments. Results are expressed as percentage of the fluorescence on treated versus untreated groups. Each bar represents the mean ± SEM. *$P < 0.05$ and **$P < 0.01$ as compared to untreated cells, $n = 4$ per group. B: The representative photomicrographs of the migrated cells to the lower chambers after calcein-AM staining [a, the control; b, thymosin β10 (100 ng/ml); c, thymosins β4 (100 ng/ml); and d, VEGF (20 ng/ml)]. Scale bar is 100 μm.
DISCUSSION

In cultured HCAECs, thymosin β10 significantly inhibited endothelial cell migration in a dose- and time-dependent manner. In addition, thymosin β10 directly inhibited the capillary-like tube formation of endothelial cells on Matrigel and reduced the expression of VEGF, VEGFR-1, and integrin αV in HCAECs. These data indicate that thymosin β10 have a directly inhibitive effect on angiogenesis in vitro.

The effect of thymosin β10 on angiogenesis was first observed by Koutrafouri et al. [2001] using a chick chorioallantoic membrane model, and they showed that overexpression of thymosine β10 using an adenovirus vector inhibited angiogenesis. More recently, Lee et al. [2005] demonstrated that overexpression of thymosin β10 inhibited VEGF-induced angiogenesis in vitro and inhibited tumor growth as well as angiogenesis in a mouse orthotopic tumor model. These data can only represent the effect of intracellular thymosin β10 on angiogenesis because adenovirus vector overexpresses thymosin β10 in the cell cytoplasm and thymosin β10 lacks secretion signal peptides. However, in our present study, we treated vascular endothelial cells with increasing doses of exogenous thymosin β10 and the direct or extracellular effect of thymosin β10 on angiogenesis was observed. Thymosin β10 might carry out its effects via its relevant receptors or ligands, which are currently unknown. We observed that exogenous thymosin β10 has a direct inhibitive effect on vascular endothelial cell migration and capillary-like tube formation.

Endothelial cell proliferation and migration are initial steps critical to the angiogenic process. Angiogenesis involves the migration of endothelial cells and their organization into a network of tube-like structures [Carmeliet, 2003]. In our study, quantitation of the cell migration is achieved by post-labeling cells with fluorescent dye and measuring the fluorescence of the migrated cells with a fluorescent plate reader. The fluorescent signal can be directly correlated to the cell number. It eliminates tedious manual cell counting and increases throughput, accuracy, and productivity by automating the endothelial cell migration assay. Our data showed that thymosin β10 significantly inhibited endothelial cell migration in a dose- and time-dependent manner. Cells treated with thymosin β10 significantly decreased endothelial migration by 36% as compared to controls ($P < 0.01$). Since the cell viability was not affected, the inhibitive effect of thymosin β10 on cell migration may not be caused by its possible cytotoxic effect. Lee et al showed that overexpression of thymosin β10 in human umbilical vein endothelial cells (HUVEC) with adenovirus vector significantly inhibited VEGF-induced HUVEC migration as compared to the control [Lee et al., 2005]. Our result provides the evidence, for the first time, that exogenous thymosin β10 has a direct inhibitive effect on endothelial cell migration. It indicates that thymosin β10 inhibits endothelial

Fig. 3. Effect of thymosin β10 on HCAEC migration in the wound healing model. Confluent HCAECs were wounded by a scratch injury line made with a sterile cell scraper. Cells were then treated with or without thymosin β10 (100 ng/ml) or VEGF (20 ng/ml). Photos were taken at 16 h. Dotted lines delimit the initially wounded regions. A, the control; B, thymosin β10 (100 ng/ml); and C, VEGF (20 ng/ml). Scale bar is 200 μm.
cell migration, which is the key event of the angiogenic process.

All β-thymosin family peptides highly conserve the actin binding motif (LKKTETQ) [Huff et al., 2001], which is essential for their angiogenic activity [Philp et al., 2003]. Thymosin β4 has been demonstrated to enhance cell migration and angiogenesis via its effect on actin binding. Here our study showed a distinct effect of thymosin β4 and thymosin β10 on HCAEC migration. Thymosin β4 increased HCAEC migration, while thymosin β10 inhibited HCAEC migration. These have also been demonstrated in the other independent study [Koutrafouri et al., 2001]. Despite the conserved motif in their structure, thymosin β4 and thymosin β10 showed distinct patterns of expression in several tissues [Lin and Morrison-Bogorad, 1990] and played different roles during rodent development [Anadon et al., 2001]. Moreover, thymosin β10 mRNA levels were very low in the cardiovascular system of early mouse embryos, in contrast to thymosin β4 mRNA levels [Carpintero et al., 1996]. Angiogenesis actively occurs in early development and is commonly controlled by the balance between angiogenic and anti-angiogenic factors. These literature findings further suggest that thymosin β4 and thymosin β10 act on vessel development in a complementary way in vivo and this may also be extended to the angiogenesis process. This hy-

Fig. 4. Effect of thymosin β10 on capillary-like tube formation of HCAECs. Serum-starved HCAECs were treated with or without thymosin β10 (100 ng/ml) or VEGF (20 ng/ml) for 24 h before they were seeded onto Matrigel with fresh medium. Capillary-like tube formation was observed and photos were taken at 18 h after seeding. The total length of capillary-like tubes was measured and normalized with controls. A: Typical photos of HCAECs capillary-like tube formation on Matrigel after treatment at 18 h. a, control, b, thymosin β10, and c, VEGF. B: Tube length measurement. The length of tubes was measured using an Image-Pro Plus software and was calculated against the untreated (control) groups. **P < 0.01 versus control, n = 3 per group.

Fig. 5. Effect of thymosin β10 on mRNA levels of several angiogenic factors. Serum-starved HCAECs were treated with thymosin β10 (50 and 100 ng/ml) for 24 h. The cDNA was synthesized from total mRNA of the cells by reverse transcription. The mRNA levels of VEGF, VEGFR1, and integrin αv were analyzed by real-time PCR and were normalized based on that of the housekeeping gene β-actin as 2^(-ΔΔCt β-actin gene). Values are expressed as a fold change as compared to the control, n = 4. Data represent as mean ± SEM.
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hothesis is also supported by our observation that thymosin β10 reduces VEGF receptors, MMPs, and integrins, whereas thymosin β4 increases expression of VEGF [Cha et al., 2003].

Capillary-like tube formation on Matrigel is a widely used in vitro angiogenesis assay. Matrigel polymerizes to produce biologically active matrix materials resembling the mammalian cellular basement membrane which is effective for the attachment and differentiation of many kinds of cells including vascular endothelial cells. Therefore, Matrigel provides a physiologically relevant environment for studies of cell morphology, biochemical function, migration or invasion, and gene expression [Nicosia and Ottinetti, 1990]. Our study shows that thymosin β10 inhibits or delays capillary-like tube formation as compared to controls.

VEGF is a well-known angiogenic factor. It promotes endothelial cell proliferation and migration via its two main receptors (flt-1 and flt-2). Integrins provide a permissive effect for endothelial cell migration via breaking down the capillary basement membrane. Therefore, integrins play an essential role in cell migration and angiogenesis. Lee et al showed that overexpression of thymosin β10 by adenovirus vector inhibited VEGF expression in endothelial cells [Lee et al., 2005]. Our data showed that exogenous thymosin β10 significantly reduced not only VEGF but also VEGFR-1 and integrin αV expression in HCAECs. These data suggest that thymosin β10 inhibits the possible autocrine effect of VEGF in endothelial cells and thus, has a direct anti-angiogenic effect.

In summary, the results of the present study show that exogenous thymosin β10 exerts a direct effect on vascular endothelial cells by inhibiting cell migration, tube formation, and angiogenesis. This study provides further evidence for the potential antiangiogenic role of thymosin β10, which may have therapeutic values for cancer and other angiogenesis-associated diseases.

REFERENCES


