Matrigel induces thymosin β4 gene in differentiating endothelial cells

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SUMMARY

We performed differential cDNA hybridization using RNA from endothelial cells cultured for 4 hours on either plastic or basement membrane matrix (Matrigel), and identified early genes induced during the morphological differentiation into capillary-like tubes. The mRNA for one clone, thymosin β4, was increased 5-fold. Immunostaining localized thymosin β4 in vivo in both growing and mature vessels as well as in other tissues. Endothelial cells transfected with thymosin β4 showed an increased rate of attachment and spreading on matrix components, and an accelerated rate of tube formation on Matrigel. An antisense oligo to thymosin β4 inhibited tube formation on Matrigel. The results suggest that thymosin β4 is induced and likely involved in differentiating endothelial cells. Thymosin β4 may play a role in vessel formation in vivo.

Key words: neovascularization, endothelium, matrix, differentiation, Matrigel, subtraction library, thymosin beta 4, basement membrane

INTRODUCTION

The maintenance and integrity of the blood vessel wall primarily depends on the stability of the endothelial cells and associated vascular cells. Although both small and large blood vessels are quiescent in the adult, they can readily form new vessels in response to changes such as injury or tumor growth (Folkman, 1992; Folkman and Hanahan, 1991; Folkman and Ingber, 1992; Folkman and Shing, 1992; Paku and Paweletz, 1991). The molecular events which occur in stable vessels or during new blood vessel formation (angiogenesis) are still unclear. Several in vivo and in vitro models have been developed that mimic at least some aspects of the angiogenic process, and these models provide insight into the steps and initiating factors involved in stimulating endothelial cells to form and maintain structurally intact vessels (Ingber et al., 1987; Jaffe et al., 1973; Kramer and Fuh, 1985; Madri and Pratt, 1986; Nicosia et al., 1984). For example, Maciag and coworkers (Maciag, 1990) have shown that when confluent, cultured endothelial cells are treated with phorbol esters approximately 50% of the cells form tube networks. Using this model of endothelial cell differentiation with subtractive hybridization, a novel protein, EDG-1, was identified and shown to increase during tube formation (Hla and Maciag, 1990a,b; Maciag, 1990).

Large and small blood vessels are composed of an inner endothelial cell layer, which is in direct contact with an associated basement membrane. A major factor regulating endothelial cell behavior is the basement membrane, which provides many signals and maintains the endothelium in a differentiated state in vivo (Ingber and Folkman, 1989; Kramer and Fuh, 1985; Kubota et al., 1988; Madri et al., 1980) A basement membrane substratum, Matrigel, has been used in vitro with endothelial cells and found to induce rapid formation of a capillary-like tube network (Grant et al., 1992; Kubota et al., 1988). When the cells are seeded onto Matrigel, they spread, attach and migrate across the surface over a 2 hour period, and then between 4-6 hours the cells align and form lateral associations which progress to the formation of tubes over the next 10 hours (Grant et al., 1990, 1991). During this morphologic differentiation, cell division is greatly reduced. This process is blocked by cycloheximide suggesting that protein synthesis is important (Grant et al., 1991). While this model does not mimic all the events that occur during vessel formation, it provides a powerful tool for the examination of certain molecular and biochemical events that transpire during endothelial cell morphogenesis and differentiation. In this study, we have investigated the early genes which are induced during the endothelial cell differentiation on Matrigel.

Using a differential cDNA cloning technique performed with the RNA from endothelial cells cultured for 4 hours on either plastic or Matrigel, we have identified several genes which are induced by Matrigel. One of these genes encoded a small polypeptide (4.9 kDa) first identified and isolated from the calf thymus, called thymosin β4 (Badamchian et al., 1988; Gomez-Marquez et al., 1989; Low and Goldstein, 1984, 1985; Low et al., 1990). Thymosin β4 contains 43 amino acids and exhibits important activities in the regulation and differentiation of thymus-dependent lymphocytes (Low and Goldstein, 1984, 1985). For example, previous studies have demonstrated
that thymosin \( \beta 4 \) is effective in partially or fully reconstituting
immune functions in thymic-deprived or immunodeprived
animals (Low and Goldstein, 1981). The sequence of this
protein is well conserved since it is over 95% homologous in
human, mouse, rat and cow (Hall, 1991; Low and Goldstein,
1985). In addition, this protein is ubiquitously expressed in
many cells and tissues (Condon and Hall, 1992; Lin and
Morrison-Bogorad, 1990). Thymosin \( \beta 4 \) is linked to the differen-
tiation of not only hematopoietic cells but also to cells in
developing human embryonic kidney (Hall, 1991). Thymosin
\( \beta 4 \) is also increased in the serum of patients over 60, in
newborns, and in patients suffering from AIDS, hepatitis, and
inflammatory bowel disease (Naylor et al., 1986). This small
polypeptide is differentially expressed in the developing brain
tissues during embryogenesis of the rat and is present in lower
amounts in embryonic lung, kidney, spleen, adrenal glands,
heart and liver (Lin and Morrison-Bogorad, 1990). Thymosin
\( \beta 4 \) expression is also directly associated with cell cycle regu-
lation (Schöbitz et al., 1991b). Nothing is known about the
expression or the regulation of thymosin \( \beta 4 \) in endothelial
cells. In this report, we provide evidence that this polypeptide
is involved in the regulation of endothelial cell differentiation.

**MATERIALS AND METHODS**

**Cells**

Human umbilical vein endothelial cells (HUVEC) were isolated from
freshly delivered cords (Grant et al., 1991). The cells were grown and
passaged (2 to 7 times) in Medium 199 (Gibco Inc.) containing 20% calf serum (HyClone), 200 ng/ml ECGS (Collaborative Res. Inc.
Bedford MA), 5 ng/ml sodium heparin (Sigma), 100 \( \mu \)g/ml penicillin-streptomycin (Gibco, BRL, Gaithersburg, MD), fungizone (2.7 \( \mu \)g/ml amphotericin B), and 25 \( \mu \)g/ml gentamicin. Matrigel (at 15 mg/ml), collagen IV and laminin are routinely prepared in the laboratory
(Timpl et al., 1979; Kleinman et al., 1986). Cells were grown to con-
fluency on either plastic or glass coverslips for light microscopy. Light
micrographs were taken with a Zeiss IM microscope equipped with
either Hoffman modulation contrast or phase contrast.

**Preparation of RNA from HUVEC on Matrigel and plastic**

Matrigel at 15 mg/ml (Kleinman et al., 1986), was used to coat 40
150-mm Nunc plates (approximately 1 ml of Matrigel per plate, a
thickness of approximately 0.3 mm) and was allowed to polymerize
at 37°C for a minimum of 30 minutes. One hundred and sixty control
plates lacking Matrigel were incubated at the same time at 37°C
with 5 ml of complete Medium 199. Trypsin-released passage four
HUVEC were plated at a density of 1 million cells per 150 mm plate
(30% confluence) and incubated at 37°C, with 5% \( \mathrm{CO}_{2} \)95% air for 4
hours, at which time the cells on Matrigel were organizing into
networks (see Fig. 1). The medium from all plates was aspirated and
then the plates were washed with Dulbecco’s phosphate-buffered saline (DPBS). The cells were lysed with 4 M guanidine isothio-
cyanate, 25 mM sodium acetate, and 100 mM \( \beta \)-mercaptoethanol, and
removed after either gentle rocking (cells on Matrigel) or scraping
(cells on plastic). Total cellular RNA was obtained by centrifugation
over a CsCl2 cushion (Sambrook et al., 1989). Five milligrams of total
RNA were obtained from the cells grown on plastic for 4 hours. The
mRNA was isolated from total RNA of cells incubated on Matrigel
by the use of an oligo dT Sepharose 4B column (Poly-Quick Kit,
Stratagene, La Jolla, CA). A cDNA probe was generated from this
Matrigel-induced mRNA using Moloney murine leukemia (M-
MuLV) reverse transcriptase (Boehringer Mannheim) for screening
the library.

Library construction and identification of Matrigel induced genes

A Matrigel-induced cDNA library was constructed using the Uni ZAP
XR unidirectional cDNA synthesis Kit (Stratagene, La Jolla, CA)
from the purified mRNA (as prepared above) and packed (Gigapack
Plus 7, Stratagene, La Jolla, CA) as per the manufacturer’s pro-
cedures. Following amplification, the cDNA library was screened by
subtractive hybridization using XL1 Blue cells (Stratagene, La Jolla,
CA). Double lifts of the plaques were transferred to nitrocellulose
membranes (Schleicher and Schuell, Keene, NH) which were subse-
quently denatured, neutralized, rinsed and crosslinked. For cDNA
screening, poly(A) RNA was isolated from HUVEC that were
cultured on either plastic or Matrigel for 4 hours. Briefly, differential
plaque hybridization was performed using \( \text{P} \)-labeled probes synthe-
sized by reverse transcriptase reaction from mRNA obtained from
cells cultured on Matrigel and plastic. Excess total RNA from
HUVEC grown on plastic was included with the Matrigel-induced
drome during hybridization to compete out common genes. The dif-
ferential hybridization was carried out using 5 mg of total RNA from
HUVEC on plastic with 0.5 \( \mu \)g of labeled Matrigel-induced cDNA,
inubcated overnight at 65°C in 5x Denhardt’s solution (0.1% (w/v)
Ficoll, 1.0% (w/v) polyvinylpyrrolidone, 0.1% bovine serum
albumen), 6x SSC (0.9 M NaCl, 0.09 M sodium citrate), 0.5% SDS,
and 15 \( \mu \)g/\( \mu \)l salmon sperm DNA. The filters were washed with 2x
SSC + 0.1% SDS twice, then 0.5x SSC + 0.5% SDS three times at 65°C.
After exposure to Kodak XAR film, positive plaques were iden-
tified and selected for further screening. Approximately 10^6 plaques
were examined. Identified induced clones were rescreened three
times. Several clones were found to be induced on Matrigel and these
inserts were rescued using R408 helper phage and XL1-Blue cells
(Stratagene, La Jolla, CA).

Selected colonies were then streaked onto new LB/amp plates for
further investigation and sequencing. Seventeen clones were identi-
fied. Plasmid DNA was purified using a Qiagen (Chatsworth, CA)
plasmid preparation kit. The double stranded cDNA inserts were
sequenced from the T3 or T7 primer site in Bluescript with Sequenase
Version 1.0 T7 DNA polymerase kit (United States Biochemical,
Cleveland, Ohio). In some cases, the sequencing reaction was done
using Applied Biosystems Inc. AmpliTaqR DNA polymerase and a
6% sequencing gel was then run on the automated sequencer. In each
case, the sequence of the insert of a particular clone was compared
(using the VAX, Fasta search; Pearson and Lipman, 1988) to known
sequences in the database.

**Northern blot analysis**

HUVEC, at 70% confluence, were lifted and replated in fresh medium
on 150 mm Nunc tissue culture plates or on Matrigel-coated plates.
The plates were incubated for 1, 2, 4, 6, or 20 hours at 37°C. Total
RNA was isolated as described above (Sambrook et al., 1989). A total
of 10 \( \mu \)g of RNA was loaded per lane in a formaldehyde-1% agarose
gel, electrophoresed, and blotted onto Nytran (Schleicher and Schuell,
Keene, NH). The integrity and equal loading of each sample were
confirmed by staining the gel with ethidium bromide.

The cDNA inserts from the subtracted clones were gel purified after
electrophoresis using a QIAEX kit for nucleic acid extraction from
gels (QIAGEN Inc., Chatsworth, CA), then 25-50 ng of the purified
cDNA was labeled with \( \text{P} \)-dCTP using a random prime kit
(Boehringer Mannheim, Indianapolis, IN) and used to probe the
filters. The Nytran filters with RNA from HUVEC were preincubated
in prehybridization buffer (2x SSC, 2x Denhardt’s, 0.5% SDS, and
1 mg/ml salmon sperm DNA) for 2 hours and then incubated with the
denatured labeled probe overnight. The following day, the filters
were washed with 2x SSC + 0.1% SDS, followed by 1x SSC + 0.1% SDS,
and then 0.1x SSC + 0.1% SDS at 20°C or 37°C. The filters were then
wrapped in plastic and exposed to Kodak XAR film. The relative inte-
grated density of RNA expression was done in the following way. The
images on X-ray films were transferred to a Power Macintosh 7100/66
using a Nikon video camera connected to a Quickcapture frame grabber board. The bands were then quantitated using the NIH Image 1.57 software and the accompanying gel plotting macro.

**Immunohistochemistry**

HUVEC were plated on Matrigel-coated (15 mg/ml, at a thickness of 0.3-0.5 mm) LabTek multiwell slides, and incubated at 37°C for 18-20 hours. The slides were processed for immunohistochemistry by aspirating the media, washing with warm medium 199 and fixing for 15 minutes with 4% formaldehyde in DPBS + 5% sucrose. Before immunostaining, the slides were washed with DPBS and the cells were treated with 0.4% Triton X-100 in PBS for 5 minutes. The slides were incubated in normal goat serum for 10 minutes at room temperature to block nonspecific binding then incubated with a rabbit anti-human thymosin β4 antiserum (1:200; Low and Goldstein, 1985; Naylor et al., 1984, 1986) at 37°C for 1 hour. In some cases, 20 U of Rhodamine phalloidin, a stain for F-actin (Molecular Probes, Inc., Eugene, OR), was premixed with the thymosin β4 antiserum before incubation with the cells on the slides. Following the incubation of the primary antibody, the cells on Matrigel were washed three times in DPBS (5 minutes each) and then incubated for 30 minutes at 37°C with a goat anti-rabbit antibody (2 µg/ml) linked to Bodipy (Molecular Probes, Inc., Eugene, OR). The slides were then washed 3 times with DPBS and a coverslip was mounted on top with 2.5% N-propyl gallate (US Biochemicals). Since the Matrigel itself absorbs the probes non-specifically, the background staining on the surface of the section was high. A Carl Zeiss LSM 310 upright confocal microscope/IBM system was used to focus on the specific cellular staining.

Tissue from an in vivo angiogenesis assay was obtained from a murine-Matrigel subcutaneous implant (Grant et al., 1993; Kibbey et al., 1992; Passaniti et al., 1992) containing 50 ng/ml basic fibroblast growth factor. The excised sample contained surrounding epidermis, the dermis and associated musculature from the abdominal wall. The tissue was fixed with 4% formaldehyde, washed with DPBS, dehydrated and embedded in paraffin. Sections were made (4 µm) and mounted on sialinated glass slides. Before immunohistochemical staining of the sections, the slides were deparaffinized, rehydrated, and rinsed several times in DPBS. After a 10 minute incubation of the tissue sections with goat serum, the sections were stained with thymosin β4 antibody as outlined above for HUVEC on Matrigel, except a Hoechst stain for the nucleus was added to the secondary goat anti-rabbit IgG antibody bound to Texas Red (Molecular Probes, Inc., Eugene, OR). Photographs were taken with an Olympus AH-2 microscope equipped for epifluorescence.

**Transfection and expression of the thymosin β4 cDNA in HUVEC**

The thymosin β4 cDNA insert was excised using XhoI and EcoRI

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**Fig. 1.** Differentiation of HUVEC on Matrigel over time. Photographs show HUVEC cultured on plastic (A) or Matrigel (B,C,D) for two, four and twenty hours. Bar, 100 µm.

**Fig. 2.** Northern blot of thymosin β4 expression in HUVEC on plastic and on Matrigel. RNA was purified from cells cultured on plastic or Matrigel and run on a 1% agarose gel. After transfer to Nytran (inset shows ethidium bromide (EtBr) stained gel), a [32P]dCTP labeled thymosin β4 probe was used to detect expression (see inset). The integrated density for each time point is indicated on the graph.
restriction enzymes, and subcloned by ligation into the pcDNA 1 eukaryotic expression vector, containing a CMV promotor (InVitrogen, San Diego, CA). After a plasmid MagicTMminiprep (Promega Corp., Madison, WI), 10 μg of the plasmid, possessing the thymosin β4 insert, was used to transiently transfect passage 4-6 HUVEC cells. Briefly, 1.1x10⁶ cells were synchronized by the addition of 2 M thymidine (Sigma; 174 μl per 10 ml of media) for 16-18 hours. When the cells began to divide again, the transfection was done by electroporation at 170 mV and a capacitance of 960 μF with a Gene Pulser (Bio-Rad Laboratories, Melville, NY) (Giordano et al., 1991; Goldstein et al., 1989). As a control, HUVEC were mock transfected with the pcDNA vector alone without the insert. Following the transfection, the cells were plated in normal HUVEC medium containing 0.005 M sodium butyrate (Sigma Biochemicals, St Louis MO) for 14-16 hours. Butyrate treatment alone had no effect on either endogenous thymosin β4 expression or tube formation as evidenced by the mock transfection control. Cells were incubated at 37°C for 24 and 48 hours after electroporation and immunostained with rabbit anti-human thymosin β4 protein alone or double-labeled with mouse antihuman vWF (monoclonal antibody from Dakopratt, Carpinteria, CA) using both rhodamine and fluorescein-labeled secondary antibodies as outlined above.

**Attachment assay**

Three 24-well Costar tissue culture plates were either uncoated or coated with 5 μg/well of laminin or 10 μg/well of collagen IV in 200 μl of medium 199 for 1 hour at 37°C. In some experiments, different concentrations of laminin (2, 5, 10, 15, 20 μg) were used to coat the culture plates. The wells were aspirated and 500 μl of medium 199 with 3% BSA was added to each well and the plates were incubated at 37°C for 1 hour. HUVEC, untransfected, mock transfected, and thymosin β4 transfected HUVEC were lifted after 24 hours using 0.25% trypsin/EDTA (Gibco BRL, Gaithersburg MD) and diluted to 150,000 cells per ml in medium 199 plus 3% BSA.

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**Fig. 3.** Immunohistochemical staining for thymosin β4. (A) Hoffman modulation contrast photograph of HUVEC tube formation on Matrigel (18 hours). (B) Tubes on Matrigel stained for thymosin β4 (green) and F-actin (red); micrograph was taken on a Zeiss confocal microscope Arrow, shows staining intracellularly with actin fibres in the periphery. (C) Masson Trichrome staining of a histologic section of a Matrigel plug containing bFGF showing blood vessels forming in the matrix; red blood cells are present in the lumen. (D) Immunostaining of a similar section, at a lower magnification, of Matrigel-bFGF-containing plug using a thymosin β4 antibody and Hoechst stain to identify the nuclei. Arrows indicate single cells which do not appear stained for thymosin β4 and are not aligned with other cells. (E) Rhodamine and FITC staining for thymosin β4 in a muscular artery (Ar), vein (Vn) and arteriole (Arl) in the same tissue section containing the plug. (F) Positive thymosin β4 staining of capillaries between skeletal muscle cells (SMC). Note the preferential staining of the intima of the large muscular artery (arrows). Bars: (A to C), 10 μm; (D to F), 100 μm.
BSA. In the attachment assay, the cells (75,000) were added to the well and were incubated for 30 minutes at 37°C and then each well was washed once with DPBS. The attached cells were fixed and stained with the DiffQuick system (Baxter Scientific) and examined with an Olympus inverted microscope. For quantitation of cell attachment, the Optomax image analysis program attached to the Olympus microscope was used (Grant et al., 1992). Each condition was carried out in six replicates, ten random fields of view were quantitated per well, and the experiment was repeated at least three times for each condition.

**Formation of capillary-like structures on Matrigel**

Four-well Nunc plates (16 mm well) were coated with 250 μl of Matrigel and 40,000 mock transfected or thymosin β4 transfected HUVEC were added to each well (Grant et al., 1990). The plates were incubated for 24 hours, and, during this time, pictures were taken at 2, 4, and 6 hours.

**Effect of sense and antisense oligos on capillary-like tube formation on Matrigel**

Two 15mer phosphorothioated oligonucleotide sequences were prepared by SyntheCell corporation (Rockville, MD): sense, 5’-TCTGACAAACCCGGGAT-3’, and antisense, 5’-ATCGGGTTTGTCA-3’, which correspond to the first 15 nucleotides at the 5’ initiation site of thymosin β4 mRNA. The oligos were purified (verified on an electrophoretic gel), dried, and rehydrated before use. The Matrigel-coated plates containing HUVEC were incubated at 37°C for 18 hours in the presence of the oligos. The cells were fixed and stained (DiffQuick), and tube area was measured on the Optomax image analyzer. Data are expressed as the percentage tube area in experimental wells compared to control.

**RESULTS**

**Identification of cDNA clones following incubation on Matrigel**

HUVEC cultured on plastic display a cobblestone morphology, whereas on Matrigel the cells attach within two hours and by four hours migration and cell-cell association result in reorganization and initiation of tube morphogenesis (Fig. 1). Differential hybridization of mRNA from endothelial cells on plastic vs Matrigel at 4 hours resulted in 17 clones which were found to range in size from 500 to 3,000 basepairs. One clone was identical in nucleotide sequence to human thymosin beta-4 (not shown). Northern analysis of thymosin β4 was determined in HUVEC on both plastic and Matrigel (Fig. 2). The thymosin β4 cDNA clone isolated from endothelial cells recognized a message of 800 bp; slightly larger than found in thymocytes due to an extended 3’ region. Cells cultured on plastic do not significantly change their expression of thymosin β4 over a 6 hour period, whereas cells cultured on Matrigel show a 5-fold increase at four through six hours (Fig. 2). This demonstrates that on Matrigel there is an increase in the synthesis of thymosin β4 mRNA.

**Distribution of thymosin β4 in capillary-like structures in vitro and in vessels in vivo**

The incubation of HUVEC on Matrigel results in the elongation and reorganization of the cells into tube-like structures (Fig. 3A). This network of tubes was attached firmly to

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**Fig. 4.** Immunofluorescence of HUVEC transfected with either control vector (A) or thymosin β4-containing vector at 24 hours (B,C,D), or thymosin β4 at 48 hours (E) after transfection. All panels are stained using a rabbit anti-human antibody to thymosin β4. Arrows in B indicate increased intracellular staining in positive transfected cells. (D) A positively stained dividing cell (arrowhead). (E) Thymosin β4 staining (arrow) appearing in granules 48 hours after transfection. Bar, 10 μm.
the Matrigel via regions of flattened endothelial cells. Cells which were immunostained with thymosin β4 antibody, and viewed by confocal microscopy demonstrated positive immunofluorescent staining in most of the cells comprising the tube structures (Fig. 3B). Actin colocalization (red fluorochrome) showed some filaments running within the axis of the tube in the periphery of the cell cytoplasm. Staining for actin in the flattened junctional area appeared somewhat more diffuse.

When bFGF-supplemented-Matrigel (bFGF is added to increase angiogenesis in the Matrigel in vivo angiogenesis model) is implanted subcutaneously in mice, many regions of the implants contain distinct blood vessels (Passaniti et al., 1992) (Fig. 3C). Previously, Passaniti et al. (1992) and Kibbey et al. (1992) have shown, by using antibodies to factor VIII, that the vessels in the Matrigel contain endothelial cells and are true capillaries. When these paraffin sections were immunostained with an antibody to thymosin β4, numerous cells within the Matrigel were stained (Fig. 3D). In particular, cells organized into linear vessel-like structures were all strongly stained whereas single cells did not appear to be stained (Fig. 3D, arrows). Other areas of the tissue sections were also examined and staining was found in the basal layer of the epithelium of the skin as well as in some cells of the connective tissue (not shown). Large and medium size vessels in the dermis adjacent to the Matrigel plugs were also positively stained (Fig. 3E). Specifically, the arterial endothelium and the first layer of smooth muscle cells were stained most prominently. In the skeletal muscle of the mouse abdomen, staining for thymosin β4 was found primarily in the capillaries situated between the skeletal muscle fibers (Fig. 3F). These data indicate that thymosin β4 is present in forming vessels in the in vitro and in vivo angiogenesis assays as well as in mature vessels.

Expression of transfected thymosin β4 in HUVECs

Thymosin β4 full length cDNA was transfected into endothelial cells and the presence of the protein was detected by FITC-immunocytochemistry. Most control cells, either untransfected or mock transfected with the vector alone, showed low thymosin β4 staining (Fig. 4A) with approximately 10% of the cells stained strongly positively. HUVEC transfected with the thymosin β4 cDNA were strongly stained within 24 hours, with 50-60% of the cells showing strong positive staining (Fig. 4B). At this time, the fluorescence was primarily diffuse and cytoplasmic, although in some cases the staining appeared brighter in perinuclear regions (Fig. 4C and D). Staining for thymosin β4 was also present in cells undergoing mitosis (Fig. 4D). When the HUVEC were examined 48 hours after transfection, the staining was redistributed to granule-like structures located at the periphery of the cytoplasm (Fig. 4E). Foreskin fibroblasts were also transfected with the thymosin β4 clone and demonstrated a similar cytoplasmic distribution as the endothelial cells at 24 and 48 hours (data not shown).

Behavior of thymosin β4 transfected cells

We next examined the morphology of thymosin β4-transfected HUVEC plated on plastic for 30 minutes. Like normal cells, the mock transfected cells (Fig. 5A) were not fully adherent nor did they all spread within 30 minutes (cells usually take about 60 minutes to attach and spread). In contrast, transfected cells became rapidly adherent and were almost completely spread within 30 minutes (Fig. 5B). In attachment assays normal HUVEC showed a dose-dependent increase in attach-
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Table 1. The effect of thymosin β4 sense and antisense oligos on tube formation in vitro

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<th>Amount (µg)</th>
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<th>Antisense (%)</th>
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The effect of thymosin β4 sense and antisense oligos added to HUVEC during tube morphogenesis on Matrigel. The data are expressed as percentage tube area/control; the average of three wells represents each data point. The experiment was done twice.

Fig. 6. The effect of thymosin β4 on tube formation. Time course of capillary-like tube formation of mock (MT) or thymosin β4 (T) transfected HUVEC. The left panels are mock transfected cells photographed on Matrigel at 2, 4 and 6 hours, and the right panels contain micrographs of thymosin β4 transfected cells at the same time periods. This experiment was repeated three times Bar, 100 µm.

Fig. 7. Western blots of HUVEC whole cell lysate cultured on laminin, in the presence of either sense or antisense 15mer oligos, or no oligos at all (Control). Blots were probed with antibody to thymosin β4 or normal rabbit antiserum.
DISCUSSION

The formation of capillary-like tubes from human umbilical vein endothelial cells cultured on Matrigel is a complex process which involves several steps and the biosynthesis of numerous gene products. We have shown previously (Grant et al., 1991) that the addition of cycloheximide or actinomycin D to this assay system inhibits the differentiation of the cells into tubes. Here we have tried to further the understanding of this process by determining the identity of some of the early genes by subtractive cDNA cloning. One of the clones, thymosin β4, previously not known to be associated with endothelial cells, nor known to be involved in their cell regulation, was found by northern analysis to increase at early times (over a 6 hour period) in cells on Matrigel. Using an antibody to human thymosin β4, we found this protein was also abundant in cells forming tubes on Matrigel or in vessels in the in vivo murine angiogenesis assay (Passaniti et al., 1992; Kibbey et al., 1992). In situ, thymosin β4 was found to be present in both large and small vessels in mouse tissue. Transfection of this gene and its subsequent increased expression in cultured HUVEC demonstrated that the protein is associated with more rapid cell attachment, spreading, and formation of tubes on Matrigel. All these processes are essential for tube formation. Antisense oligos to the thymosin β4 mRNA inhibited tube formation on Matrigel, again indicating its importance in the process. Although the cells attached more rapidly at very early time points, no effect on total cell adhesion to either laminin or Matrigel, again indicating its importance in the process. There are inherent toxicity problems with the antisense/sense probes that make it difficult to clearly interpret the data. These data, however, when examined in total, suggest that thymosin β4 is expressed and is biologically active in differentiating endothelial cells.

Although thymosin β4 was not previously shown to be involved in blood vessel formation and stabilization, it may play an important role in normal vessel development, differentiation, and maintenance. There is strong evidence that thymosin β4, besides being a thymic hormone, is also a differentiation factor for many cell types (Low and Goldstein, 1984; Schöbitz et al., 1991b; Shimamura et al., 1990). Thymosin β4 was first shown to be associated with the maturation of developing T-lymphocytes in the thymus (Low et al., 1990; Schöbitz et al., 1991a; Talaev et al., 1991). More recently this molecule has been shown to be associated with the development of B-lymphocytes, platelets, and macrophages, and thus appears to be considered a marker for hematopoietic cell differentiation (Gomez-Marquez et al., 1989; Hall, 1991; Lin and Morrison-Bogorad, 1990). Thymosin β4 is also expressed in human tumors, the developing human kidney (Hall, 1991), and is present at high levels in the fetal cortex and cerebellum of the developing rat brain (Lin and Morrison-Bogorad, 1990). We find that thymosin β4 may be associated with the morphogenesis of HUVEC into tubes, and is present in vessels in vivo. Since this is the first study that illustrates that the expression of thymosin β4 is involved in endothelial cell differentiation, further mechanistic investigations are needed to establish if thymosin β4 induces differentiation in endothelial cells or is a product of the differentiation process.

It is an interesting fact that cultured endothelial cells plated on Matrigel stop proliferating (Kubota et al., 1988), whereas, the proliferation of several tumor cell types is accelerated by Matrigel (Fridman et al., 1991). This difference in the two cell types frequently correlates with the presence of thymosin β4. For example, in ROS osteosarcoma cells, thymosin β4 is expressed more in cells possessing the slow growing, differentiated osteoblast-like phenotype (17/2.8 cells), than the more undifferentiated cells (25/1 cells) (Atkinson et al., 1990) exhibiting the proliferative phenotype. An induction of the thymosin β4 mRNA has been shown during differentiation in various phases of the cell cycle of lymphoid cell lines (Schöbitz et al., 1991a). In these studies, thymosin β4 mRNA content increased rapidly 1 hour after ConA stimulation of lymphocytes; thereafter, the mRNA levels continued to increase, plateaued at about 48 hours, and then returned to the initial values once the cells were quiescent again (Schöbitz et al., 1991b). It appears that thymosin β4 is modulated with cell proliferation changes, with the levels rising in rapidly proliferating cells. Thus, the stimulation of the lymphocyte proliferation by ConA results in an increase in thymosin β4 and with subsequent decrease the proliferative rate of the cells. Thymosin β4 appears to suppress cell proliferation.

HUVEC plated on Matrigel are also very sensitive to cytochalasin D (Grant et al., 1991), a compound that interrupts the organization of the cytoskeleton. The reorganization of actin filaments into fiber bundles is essential for tube formation. Recent investigations have shown that thymosin β4 is an actin monomer sequestering protein (Cassimeris et al., 1992; Safer, 1992; Safer et al., 1991; Sanders et al., 1992; Vancompernel et al., 1992; Yu et al., 1993). Thymosin β4, like other actin monomer sequestering proteins such as profilin, destrin, actinobindin, and coflin, has the ability to inhibit the polymerization of purified actin subunits, and has been recently shown to induce depolymerization of actin filaments in cultured epithelial cells and in fibroblasts (Safer, 1992). More recently, the role of thymosin β4 in actin regulation has been reevaluated. It now appears that thymosin β4 can also stimulate the polymerization of actin monomers in the presence of low amounts of profilin (Pantalone and Carlier, 1993). The polymerization and depolymerization of actin is important in cell locomotion and cell spreading (Safer et al., 1991; Stossel, 1983), and now in tube formation. We showed that the over-expression of thymosin β4 could promote accelerated HUVEC attachment, spreading, and tube formation. Based on indirect evidence, if thymosin can bind to monomeric actin in this endothelial cell system, it may slow down migration of the cells across Matrigel and preferentially promote organization and stabilization of the tube network on Matrigel. If profilin is present in endothelial cells during the over-expression of thymosin β4, actin filament polymerization may increase and provide additional filaments. Thus, thymosin β4’s actin sequestering ability may help to promote the formation of vessels. Since cell-cell interactions are also important in tube formation, and actin assembly is required for this process, thymosin β4 may also be enhancing cell cell organization. We now have preliminary results that indicate that the addition of thymosin β4 protein to HUVEC on glass slides actually results in an increase as well as a rearrangement of the actin stress fibers (D. S. Grant, unpublished). Therefore, the exact mechanism of action of thymosin β4 on HUVEC cytoskeleton and the resulting biological response may be very complex.
The use of this subtractive cDNA cloning has provided information regarding the gene products induced during tube formation on Matrigel. We have demonstrated that thymosin β4 is biologically active in tube formation and possibly in angiogenesis in vivo. Thymosin β4 may not be acting alone in vessel formation but is nevertheless essential. The mechanism of thymosin β4 action may be on cell cycle, and/or hormonal, and/or on actin regulation in the differentiating endothelial cells, but that has not yet been directly shown. Alternatively, thymosin β4 may have additional biological actions yet to be defined.

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REFERENCES


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