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Identification of novel anti-angiogenic factors by in silico functional gene screening method

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Abstract

Angiogenesis, the formation of new blood vessels out of pre-existing capillaries, occurs in a variety of pathophysiological conditions, and is regulated by a balance of angiogenic activators and inhibitors. To identify novel angiogenic factors, we developed a gene screening method by combining the prediction analysis of transcription factor (TF) binding site and the chromosomal localization analysis. First, we analyzed the promoter sequences from known angiogenesis-related factors using the MATINSPECTOR program in TRANSFAC database. Interestingly, we found that the binding site of LMO2 complex is highly conserved in the promoter regions of these factors. Second, we analyzed chromosome loci based on the hypothesis that angiogenesis-related factors might be co-localized in a specific chromosomal band. We found that angiogenesis-related factors are localized in specific 14 chromosomal bands including 5q31 and 19q13 using AngioDB and LocusLink database mining. From these two approaches, we identified 32 novel candidates that have the LMO2 complex binding site in their promoter and are located on one of 14 chromosomal bands. Among them, human recombinant troponin T and spectrin markedly inhibited the neovascular-

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ization in vivo and in vitro. Collectively, we suggest that the combination of the prediction analysis of TF binding site and the chromosomal localization analysis might be a useful strategy for gene screening of angiogenesis.
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Keywords: Angiogenesis; Transcription factor; Chromosomal localization; Gene screening

1. Introduction

Angiogenesis, new capillaries sprouting from pre-existing vessels, occurs in a variety of physiological and pathological conditions including embryonic development, wound healing, and tumor growth. Angiogenesis is regulated by a balance of angiogenic activators and inhibitors, which act as molecular basis for understanding angiogenic process and therapeutic targets in clinical fields (Folkman and Klagsbrun, 1987; Yancopoulos et al., 1998; Lee et al., 2001). Therefore, a number of molecules participating in angiogenesis have been identified by conventional experimental methods (Cuevas and Asin-Cardiel, 2000). However, it is expected that more novel protein factors will be involved in the complex pathways of angiogenesis.

To identify novel angiogenesis-related factors that regulate the spatial and temporal blood vessel networks, much of the research in this field focused on gene screening methods. However, current gene screening methods have been limited by the laborious and time-consuming experimental methodology. To overcome these limitations, in silico (or virtual) analysis has been recently developed (Leung et al., 2001). It provides an idea of the approximate result and prevents unnecessary experiments. For instance, Bortoluzzi and Danieli (1999) reported that in silico analysis of tissue-specific transcription patterns was developed using biological tools and databases publicly available in the World Wide Web. Furthermore, the increasing number of biological information from whole human genome available on the public database promoted in silico screening of genome locus and gene expression profiles.

In this study, we developed an in silico gene screening method combining of two strategies to screen and identify novel angiogenic factors. The first strategy is the prediction analysis of the conserved transcription factor (TF) binding site in promoter regions of the known angiogenesis-related factors of the angiogenesis database (AngioDB; Sohn et al., 2002) using the MATLNSPECTOR program in TRANSFAC database (Quandt et al., 1995). The second one is based on the hypothesis that genes involved in angiogenic process might be co-localized on the specific chromosomal band(s). It was recently reported that genes with similar functions tend to occur in adjacent positions along the chromosomes in the *Saccharomyces cerevisiae* genome (Cohen et al., 2000).

Remarkably, we found that the binding site of LMO2 complex exists in the promoter of angiogenesis-related factors as the highest frequency. The LMO2, DNA-binding protein complex, is composed of the TAL1, E47, GATA-1 and Ldb1/NLI proteins, and regulates distinct phases of angiogenesis and hematopoiesis (Yamada et al., 2000). Therefore, we targeted LMO2 complex-binding site for in silico screening of novel factors involved in angiogenesis. Furthermore, we identified 14 chromosomal bands including 5q31 and 19q13 through mining information from AngioDB and LocusLink databases. Thereafter, we found that two novel angiogenic factors identified by in silico screening method, troponin T and spectrin, have anti-angiogenic activities both in vivo and in vitro angiogenesis assays. Our results are the first one describing an in silico screening method to identify novel anti-angiogenic factors.

2. Materials and methods

2.1. Materials and cell culture

Human recombinant troponin T and spectrin were purchased from Sigma Corp. (St. Louis, MO). Human umbilical vein endothelial cells (HUVECs) were provided by primary cell culture.

The HUVECs (passage 6–8) were plated onto 0.3% gelatin-coated culture flasks and grown in M199 (Life Technologies, New York) containing heat-inactivated 20% fetal bovine serum (FBS) (Life Technologies), 3 ng ml^{-1} bFGF, 100 μg ml^{-1} heparin, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37 °C incubator in a humidified atmosphere containing 5% CO_2 (Kim et al., 2001).

2.2. Tube formation assay

250 μl Matrigel (10 mg ml^{-1}) was pipetted into 24-well culture plates and polymerized for 30 min at 37 °C. HUVECs (1×10^5 cells) were seeded on the surface of Matrigel. Then, troponin T or spectrin was added and incubated for 16 or 24 h, respectively. Morphological changes of cells were observed under a microscope and captured through a phase-contrast microscope and photographed.

2.3. Wounding migration assay

HUVECs were plated onto 0.3% gelatin-coated 60 mm culture dishes. At 90% confluency, the endothelial cell monolayers were wounded 2 mm in width with a sterilized razor blade and marked a reference line. Plates were rinsed with serum-free medium to remove cellular debris. Fresh medium with 20% serum and 1 mM thymidine (to inhibit cell proliferation) was then added. HUVECs were allowed to migrate for 16 h and rinsed with serum-free medium, followed by fixing with absolute methanol and staining with Giemsa. Migration was quantitated with counting the number of cells that moved beyond the reference line. And photographs were taken through an inverted microscope ($\times 40$). This experiment was repeated three times. These data was displayed as percent of control condition. All the control experiments were carried out in the same media containing drug-free vehicle. Data were expressed as mean $\pm$ standard error of the mean (S.E.M.) and were analyzed using one way analysis of variance and Student's t-test for individual comparisons. P values less than 0.05 are considered to be statistically significant.

2.4. In vivo chick chorioallantoic membrane (CAM) assay

The fertilized chicken eggs used in this study were kept in humidified incubator at 37 °C. After 3-day incubation, about 3 ml of albumin were aspirated from eggs with an 18-gauge hypodermic needle through the small hole drilled at the narrow end of the eggs. At the stage of 3-day-old chick embryo, the shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. At the stage of 4.5-day-old chick embryo, a sample-loaded thermanox-coverslip was applied to the CAM surface, and then the chick embryo was returned to the incubator. Two days later, an appropriate volume of a 10% fat emulsion (Intralipose 10%) was injected using a 33-gauge needle into a 6.5-day-old embryo chorioallantois, and observed under a microscope. At least 20 eggs were used for each dose of sample.

3. Results

3.1. Resources for angiogenesis-related factors in AngioDB

The information for the known angiogenesis-related factors in human was obtained from the angiogenesis database (AngioDB; Sohn et al., 2002). AngioDB is a hand-curated secondary database, provides a signaling network of angiogenesis-related biomolecules in human. The 309 records of AngioDB consisted of 12 fields and provided a list of angiogenesis-related factors classified by three categories as angiogenic activators, angiogenic signaling molecules and angiogenic inhibitors. The database has an external link to National Center for Biotechnology Information (NCBI) databases. AngioDB is available through the internet at http://angiodb.snu.ac.kr/.

3.2. Conservation of LMO2 complex binding site in the angiogenesis-related factors

To search TF binding sites in promoter sequences of the known angiogenesis-related factors,

Table 1
Prediction results for the angiogenesis-related factors having LMO2 complex binding site in their promoters

Category[a]	Data set	Promoter reported factors[b]	Predicted factors[c]
Activator	75	30	23
Inhibitor	43	17	15
Signaling proteins	38	18	10

[a] Category indicates the functional three groups of AngioDB.
[b] These factors indicate that promoter sequences were reported in GenBank DB.
[c] These factors indicate that the LMO2 complex binding site is contained in the promoter.

we firstly extracted the promoter sequences of angiogenesis-related factors from GenBank DB (Benson et al., 2000). Among 156 factors in AngioDB, we extracted 65 promoter sequences reported in GenBank DB, and analyzed TF binding sites of these 65 sequences using the MATINSPECTOR ver 2.2 in TRANSFAC DB ver 4.0 (http://transfac.gbf.de/TRANSFAC/index.html; Wingender et al., 2001) (Table 1). The TRANSFAC database provides vast amounts of information about genomic binding sites of eukaryotic TFs and the binding proteins. MATINSPECTOR is a program that utilizes a library of precompiled weight matrices for TF binding sites to scan potential binding sequences of the corresponding factors (Misener and Krawetz, 1999). This program determines an individual score for each sequence segment with all matrices selected for the analysis, and reports all matches that score equal or above a user-defined threshold. Since default similarity value (core similarity, 0.75; matrix similarity, 0.85) of MATINSPECTOR program produced high number of false positives, we used vertebrate matrix and selected strict threshold values (core similarity, 1.0; matrix similarity, 0.9) for efficient analysis of promoter sequences.

As a result, we found that the LMO2 complex binding site is highly conserved in 48 of 65 promoter sequences (Table 1). LMO2-mediated transcription complex has been reported to reg-

Table 2
Chromosomal distribution of angiogenesis-related factors on human genome

Group size[a]	Number of groups	Chromosomal bands	Number of genes[b]	Description[c]
Singlet	62			
Pair	21			
Triplet	9	4q12	18	KDR, SCYB5, platelet factor 4
		6q23	19	IGF2R, OCT1, plasminogen
		11p15	140	Adrenomedullin, IGF2, TNNI
		11q13	156	TEM1, VEGFB, FGF3
		11q23	68	ATM, apolipoprotein A4, ETS1
		12q13	155	ERBB3, SP1, NAB2
		14q24	52	HIF-Iα, PGF, FOS
		16p13	101	CREBBP, UBE21, TPSB2
		19p13	244	ICAM1, CREB-H, TCF3
Quadruplet	3	1q21	74	ARNT, integrin α10, apolipoprotein A2, ephrin-A1
		4q21	21	Platelet factor 4, IL8, SCYB10, heparanase
		22q13	98	TIMP3, PDGF-β, EP300, ECGF1
Pentadruplet	1	19q13	79	PLAUR, TGFBl, CGB, TNNI3, hyaluronan synthase l
Hexadruplet	1	5q31	188	Integrin α, FGF1, EGR1, IL4, PDGFR-β, SPARC

[a] Each group indicated the number of co-localized genes in one chromosomal band.
[b] Total gene number in each band.
[c] Description of the angiogenesis-related factors located in each chromosomal band.

Table 3
Functional grouping list for 32 novel candidates of angiogenesis-related factors

Gene ontology[a] (number of candidates)	Description
Cell adhesion (2)	Neural cell adhesion molecule 1
	Lutheran blood group
Cell proliferation (4)	Interleukin 6 receptor
	Cyclin-dependent kinase 2,4
	Somatostatin receptor 5
Cell shape and cell size control (5)	Loricrin
	Spectrin alpha, beta
	Periplakin
	Troponin T
Metabolism (2)	Heme oxygenase 1
	Tryptophan hydroxylase
Signal transduction (3)	Adenylate cyclase 9
	Anti-mullerian hormone type 2 receptor
	Chorionic gonadotropin beta polypeptide
Transcription factor (2)	α-globin transcription factor CP2
	CCAAT/enhancer binding protein α
Transporter (4)	Aquaporin 2, 5
	Uncoupling protein 2, 3
Not classified (10)	Coagulation factor 5
	Iodothyronine deiodinase type 2
	Ems 1 sequence (cortactin)
	GTPase regulator associated with the focal adhesion kinase pp125(FAK)RAF
	Histidine-rich calcium-binding protein
	Lactate dehydrogenase A
	LIM domain protein
	Myocilin
	Ribonuclease, Rnase A family 3
	Selenoprotein P

[a] Term of gene ontology™ consortium.

ulate angiogenesis and target a new anti-angiogenesis drug (Yamada et al., 2000, 2002). Thus, we targeted LMO2 complex binding site for mining of novel angiogenesis-related factors from human genome.

3.3. The chromosomal distribution of the known angiogenesis-related factors on human genome

To determine the target chromosomal bands, we analyzed the chromosomal distribution of the angiogenesis-related factors of AngioDB using LocusLink DB (http://www.ncbi.nlm.nih.gov/LocusLink/; Pruitt and Maglott, 2001). We hypothesized that angiogenesis-related factors tend to occur in adjacent loci along the chromosomes in human genome. Accordingly, we selected 14 chromosomal bands, such as 4q12, 5p31 and 19q13, at which more than three angiogenesis-related factors are co-localized (Table 2).

3.4. Selection of novel candidates

To screen genes that have a LMO2 complex binding site in their promoter sequences, we extracted the known promoter sequences of 1413 genes in 14 chromosomal bands selected from GenBank DB. The 316 out of 1413 promoter sequences were used to search LMO2 complex binding site by MATINSPECTOR program. Thereafter, we identified 32 novel candidates that have the LMO2 complex binding site in their promoter region and are located on one of 14 chromosomal bands.

And then we classified these 32 candidates by term of Gene Ontology™ (http://www.geneontology.org) (Table 3). The terms were related with each step of angiogenic processes. Among 32 candidates, we selected two candidates that related with "cell shape and cell size control" and "cytoskeletal" terms of Gene Ontology™. Capillary endothelial cells can be switched between growth and differentiation by altering cell–extracellular matrix interactions and thereby, modulating cell shape. Moreover, proliferation of endothelial cell is tightly regulated by endothelial cell shape and cytoskeletal structure (Ingber et al., 1995). Therefore, we selected two candidates related with cell shape and cytoskeletal structure as novel angiogenesis-related factors.

The first candidate is troponin T that is localized on 19q13, and has 12 LMO2 complex binding sites in promoter region (Fig. 1). The second one is erythrocytic spectrin which includes alpha and beta isoforms localized on 1q21 and 14q24, respectively. These spectrin isoforms have two (alpha) or seven (beta) LMO2 complex binding sites in their promoter region, respectively (Fig. 1).

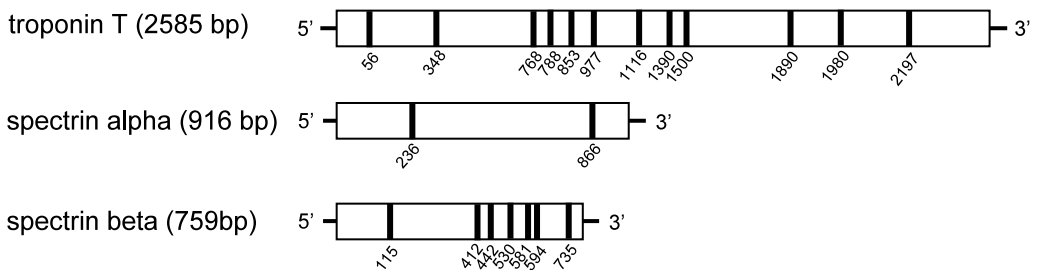


Fig. 1. Alignment of predicted LMO2 complex binding sites in the promoter regions of selected candidates. These prediction results were performed with the MATINSPECTOR ver. 2.2 in TRANSFAC DB ver 4.0. Site labels indicate predicted LMO2 complex binding sites in the 5′→3′ order as shown.

3.5. Troponin T and spectrin inhibit the tube formation and migration of HUVEC

We investigated the effect of troponin T and spectrin on each step of angiogenesis using in vitro angiogenesis assays. We firstly examined the effect of troponin T and spectrin on the HUVEC viability at several concentrations of candidates (from 10 to 200 ng ml^{-1}). These candidates did not show any cytotoxic effect on the HUVECs (data not shown). To determine the effect of candidates on HUVEC tubular differentiation, we did a tube formation assay (Fig. 2). The morphological differentiation of HUVECs was normally stimulated when HUVECs were placed on Matrigel-coated plate. However, in the treatment of troponin T or spectrin, HUVECs on Matrigel failed to form vessel-like structure. In addition, these proteins strongly inhibited the migration of HUVECs from the edge of the wound into the open area (Fig. 3A). The inhibitory activity of each candidate on the migration of HUVECs was about 43% (troponin T) and 36% (spectrin) compared with that of control (Fig. 3B).

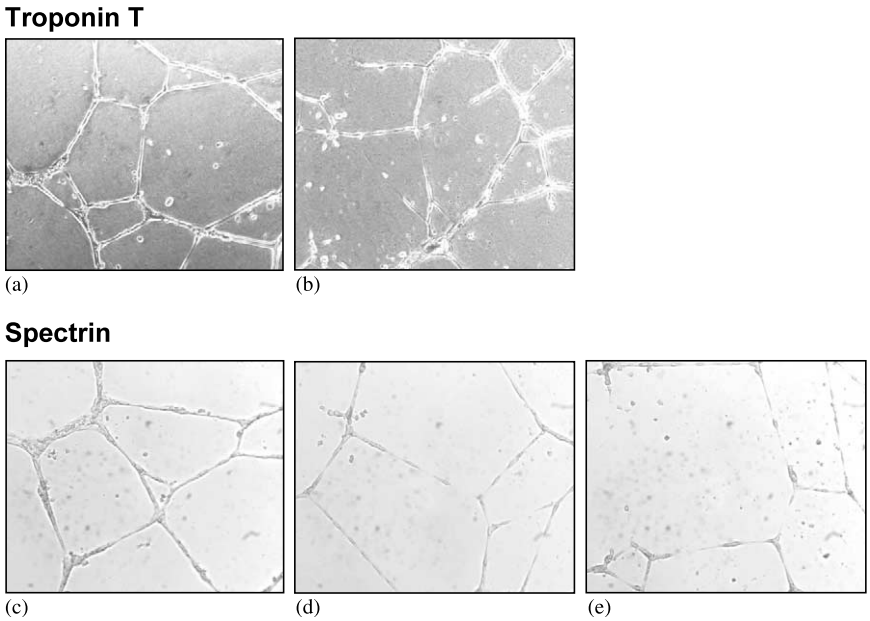


Fig. 2. Inhibitory effects of troponin T and spectrin on the tube formation of HUVECs. HUVECs were seeded on Matrigel and incubated with M199 containing 20% FBS (control; a, c), M199 with (b) troponin T (100 ng ml^{-1}) for 16 h, (d) spectrin (100 ng ml^{-1}) or (e) spectrin (200 ng ml^{-1}) for 24 h, respectively. The formation of tubular structures was detected by phase-contrast microscopy.

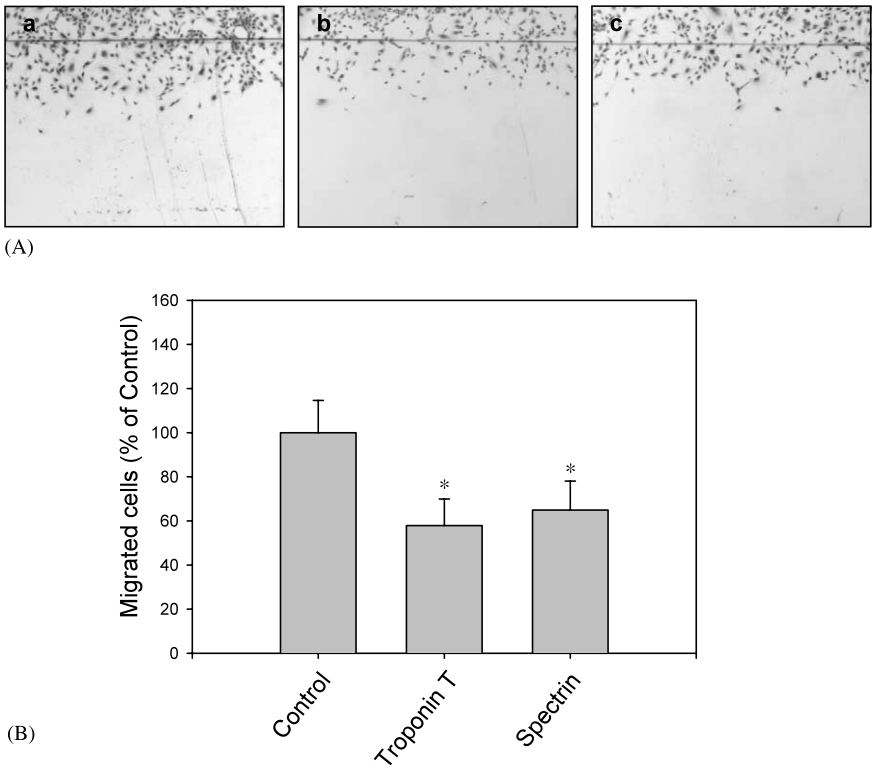


Fig. 3. Inhibitory effects of troponin T and spectrin on the migration of HUVECs. (A) Confluent HUVECs monolayer was wounded and incubated with (a) control, (b) troponin T (100 ng ml^{-1}) or (c) spectrin (100 ng ml^{-1}) for 16 h, respectively. (B) Migration was measured as described in Section 2. Results were expressed as the percentage of control ± S.E.M. *, $P < 0.05$ compared with control.

Therefore, our results showed that anti-angiogenic activity of troponin T and spectrin could be derived by inhibiting tube formation and migration of endothelial cells.

3.6. Troponin T and spectrin inhibit angiogenesis in vivo

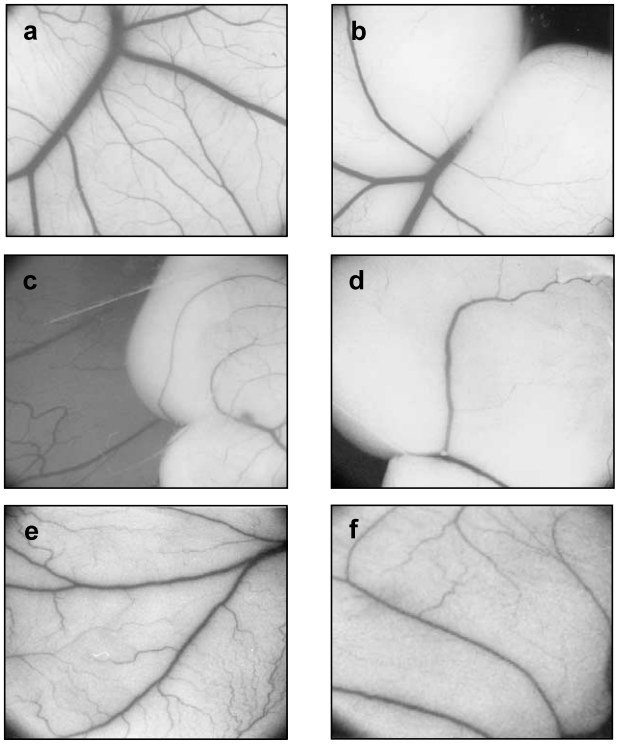
We performed in vivo CAM assay to confirm the anti-angiogenic activity of troponin T and spectrin. As shown in Fig. 4A, application of them showed wide avascular zones reflecting anti-angiogenic activity that was similar to all trans-retinoic acid (RA), a positive control. In addition, troponin T and spectrin strongly inhibited chick embryonic angiogenesis in a dose-dependent manner and ID_{50} values were shown in 150 ng per egg (troponin T) and 220 ng per egg (spectrin), respectively (Fig. 4B).

4. Discussion

The elucidation of complete genome sequences raised the possibility of in silico deduction of gene function on a genome-wide basis. Application of in silico method was expanded to gene screening, metabolic modeling and drug discovery fields. Recently, it was reported that novel endothelial-specific genes were identified by in silico subtraction of libraries from dbEST collection (Huminiecki and Bicknell, 2000), and in silico screening of tumor-specific expressed sequences were performed by computer-based differential display (Baranova et al., 2001).

In this study, we developed a new gene screening method for identification of novel angiogenesis-related factors. This method was constructed by combination of the prediction analysis of TF binding site and the chromosomal locus analysis.

The first strategy was based on the fact that TF binding sites and specific TFs are required for the identification of vascular-specific genes (Oettgen, 2001). Especially, targeted disruption of the zinc finger TF LMO2, the major member of the LMO2 complex, showed that LMO2 is essential for angiogenic remodeling of the existing capillary network. This LMO2 complex is specifically



(A)

	Dose (ng/egg)	Inhibitor activity (%) (inhibited/total)
Control	0	8 (4/50)
Retinoic acid	1000	87 (48/55)
Troponin T	50	28 (5/18)
	200	61 (14/23)
	500	75 (15/20)
Spectrin	50	24 (7/29)
	200	48 (10/21)
	500	72 (18/25)

(B)

Fig. 4. Anti-angiogenic effects of troponin T and spectrin on the chick CAMs. (A) Thermanox coverslips were treated with troponin T or spectrin on the CAMs surface of 4.5-day-old-chick embryo. After 2 days, intralipose was injected into chorioallantois and the chick embryo was observed under a microscope. (a) Control CAMs treated with blank coverslips showed no anti-angiogenic response. Positive responses were seen in the CAMs implanted with coverslips loaded with (b) RA (1 μg per eggs), (c) troponin T (200 ng per eggs), (d) troponin T (500 ng per eggs), (e) spectrin (200 ng per eggs), (f) spectrin (500 ng per eggs), respectively. (B) Dose dependent inhibitory effects of troponin T and spectrin.Fig. 4 (*Continued*)

needed for angiogenesis (Yamada et al., 2000). However, angiogenesis-specific genes containing LMO2 complex binding site were not reported yet. Therefore, we applied LMO2 complex binding site to in silico angiogenesis-related gene screening method.

The second strategy for in silico screening of angiogenesis-related factors is that genes with similar functions were located in adjacent positions on the chromosome. We, therefore, hypothesized that this regional coexpression might be required for the efficient regulation of gene expression in huge human genome. Genes with similar function are localized on adjacent chromosomal positions (Cohen et al., 2000). In addition, genes involved in the same process display correlated expression on the same chromosome in the *S. cerevisiae* genome.

For in silico angiogenesis-related gene screening, we established two criteria. First, promoter regions of candidate factors contain conserved LMO2 complex binding site. Second, novel angiogenesis-related factors are located on specific chromosomal bands, where more than three known angiogenesis-related factors were localized adjacently. Using this gene screening approach, we found 32 candidates as novel angiogenesis-related factors including troponin T and spectrin (Table 3).

Troponin T, one subunit of the troponin complex, plays an important role in regulation of muscle contraction. Components of troponin are widely used as biochemical markers of various heart injuries (Bhayana and Henderson, 1995; Hamm and Katus, 1995). Troponin I, one of troponin isoform, changes the morphology of capillary endothelial cells into flat, elongated, and refractile-appearing form, inhibits the growth of endothelial cells and increases adhesion with extracellular matrix (Braunhut and Palomares, 1991; Moses et al., 1999). Spectrin forms a submembrane cytoskeleton network with actin and a number of other accessory proteins (Viel and Branton, 1996). In addition, spectrin may play the dynamic and critical functions in development and human disease including spherocytosis, elliptocytosis (Dubreuil and Grushko, 1998). However, a role for troponin T and spectrin in angiogenesis has not been recognized. Our data showed a new role of troponin T and spectrin as anti-angiogenic factor (Figs. 2–4). Further studies are in progress to elucidate the detailed roles and exact mechanisms of these factors.

We, therefore, proposed that a novel in silico method for the screening of angiogenesis-related factors will be helpful to make the angiogenesis-related gene profiles. Furthermore, these profiles provide much information about molecular mechanisms during angiogenesis, and apply the basic knowledge of angiogenic diseases for clinical and therapeutic fields. Moreover, this new in silico screening method can be applied to identify novel molecules involved in various biological processes such as cell cycle, apoptosis and differentiation.

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