Augmented Expression of the Tight Junction Protein Occludin in Brain Endothelial Cell Line TR-BBB by Rat Angiopoietin-1 Expressed in Baculovirus-Infected Sf Plus Insect Cells

Hisashi Iizasa,[1,2] Sun-Hye Bae,[1,3] Tomoko Asashima,[1] Tomohide Kitano,[1] Noriko Matsunaga,[1] Tetsuya Terasaki,[2,4,5] Young-Sook Kang,[3] and Emi Nakashima[1,2,6]

Received June 6, 2002; accepted July 11, 2002

KEY WORDS: angiopoietin-1; tight junction; occludin; permeability; blood-brain barrier.

INTRODUCTION

The blood-brain barrier (BBB), which has extremely low drug permeability, is composed of brain endothelial cells linked together by tight junctions (TJs). Occludin plays a key role in TJ formation and BBB maturation. In epithelial cells, occludin is induced by several cytokines and hormones, such as interleukin-15 (IL-15) and prolactin, but the physiologic regulator of the expression of occludin in the BBB is not known (1,2). Angiopoietin-1 (Ang1) was recently identified as a ligand of the tyrosine kinase receptor Tie-2, and is expressed in mural cells (3). Ang1 gene-disrupted mice exhibit hemorrhage from capillaries (4), whereas mice transgenic for Ang1 have leakage-resistant blood vessels (5). These findings indicate that Ang1 may control capillary permeability *in vivo*. However, the mechanism of regulation of capillary permeability by Ang1 remains unclear. The rat is a useful animal model to study the BBB and TJ, but the human or mouse recombinant protein has generally been used in rat studies on Ang1 (6). In addition, a recombinant Ang1 system capable of providing large amounts of protein is not yet available.

[1] Department of Pharmaceutics, Kyoritsu College of Pharmacy, 1-5-30 Shiba-koen, Minato-ku, Tokyo 105-8512, Japan.
[2] CREST, Japan Science and Technology Corporation (JST) Japan.
[3] School of Pharmacy, Sookmyung Women's University, Chungpa-Dong 2ka 53-12, Yongsan-ku, Seoul 140-742, Korea.
[4] Department of Molecular Biopharmacy and Genetics, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan.
[5] New Industry Creation Hatchery Center, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan.
[6] To whom correspondence should be addressed at Department of Pharmaceutics, Kyoritsu College of Pharmacy, 1-5-30 Shiba-koen, Minato-ku, Tokyo 105-8512, Japan. (e-mail: nakashima-em@kyoritsu-ph.ac.jp)

ABBREVIATIONS: Ang1, angiopoietin-1; BBB, blood-brain barrier; cDNA, complementary DNA; GLA, γ-linolenic acid; IL-15, interleukin-15; mRNA, messenger RNA; PCR, polymerase chain reaction; S*f* (+), S*f* plus; SFM, Sf900II serum-free media; TJ, tight junction.

Here we describe the molecular cloning of rat Ang1, and its expression in baculovirus-infected S*f* plus (S*f* (+)) cells. The recombinant rat Ang1 induced expression of occludin in the conditionally immortalized brain capillary endothelial cell line TR-BBB13 *in vitro*. We suggest that Ang1 regulates the capillary permeability in the BBB by promoting the formation of TJ.

MATERIALS AND METHODS

Molecular Cloning of Rat Ang1

The reverse transcription-polymerase chain reaction (PCR) approach was used to clone the rat Ang1 homologue. Total RNA was isolated from rat placenta using ISOGEN reagent (Nippon Gene). To obtain the complementary DNA (cDNA) sequence of rat Ang1, messenger RNA (mRNA) was isolated using a PolyA Tract mRNA Isolation System IV (Promega). The corresponding single-strand cDNA was generated by using oligo$(dT)_{15}$ and ReverTra Ace (Toyobo). After 30 cycles of PCR amplification with an annealing temperature of 59°C, one primer pair (sense primer, 5′-AGGCTCCACGCTGAACGGT-3′; antisense primer, 5′-CTGCTGGAACGGAGACAATG-3′) generated a 2.3-kb DNA fragment from rat placenta cDNA. DNA sequencing revealed that this fragment showed a high homology to mouse Ang1. All animal experiments complied with the standards set out in the guidelines of Kyoritsu College of Pharmacy.

Generation of Baculovirus Recombinants for Rat Ang1 Gene

S*f* (+) (Gibco-BRL) insect cells were propagated in S*f*900II-serum-free medium (SFM) (Gibco-BRL) at 28°C (7). Competent *E. coli* DH10BAC cells, containing bacmid (baculovirus shuttle vector plasmid) and helper plasmid, were used to generate recombinant bacmids according to the manufacturer's (BAC-TO-BAC Baculovirus Expression System, Gibco-BRL) instructions. The 1.4-kb rat Ang1 was excised from the respective plasmids by the use of appropriate restriction enzymes. Insertion of the fragments into the pFASTBAC1 donor plasmid (Gibco-BRL) yielded 1.4-kb recombinant rat Ang1. The pFASTBAC1 recombinant was transformed into DH10BAC cells, and the gene of interest was transferred into bacmid through lacZ gene disruption. White colonies were obtained from LB plates containing ampicillin, kanamycin, gentamycin, tetracycline, bluo-gal, and IPTG. S*f* (+) cells were transfected with recombinant bacmid using CELLFECTIN reagent (Gibco-BRL). A mixture of recombinant bacmid and CELLFECTIN diluted in SFM without antibiotics was laid over the washed S*f* (+) cells in 6-well plates. The cells were incubated for 5 h at 28°C, rinsed, and incubated for another 72 h. Media were harvested and centrifuged, and the virus-containing supernatant was titrated. The infective titers of recombinant baculovirus stocks that were grown in suspension culture of S*f* (+) cells, were compared.

Detection of Recombinant Rat Ang1 or Occludin with Western Blotting

Conditionally immortalized rat brain capillary TR-BBB13 cells were treated with 50-μM γ-linolenic acid (GLA) and 300 ng/ml of rat Ang1 for 48 h (8,9). Cells were also treated with 100 μl of SFM as a negative control. Cells were

0724-8741/02/1100-1757/0 © 2002 Plenum Publishing Corporation

pelleted and lysed in buffer containing 1% SDS, 10 mM Tris-HCl, 1 mM EDTA, 10% glycerin and 200 U/ml Trasylol. Protein concentrations were measured using BCA and a plate reader. Samples (7 μg/ml) or supernatant from recombinant baculovirus-infected S*f* (+) cells were applied to a 10% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose membrane and blocked in 10% skimmed milk for 60 min, then probed with goat anti-Ang1 antibody (1:100) (Santa Cruz) or goat anti-occludin antibody (1:100) (Santa Cruz) and a horseradish peroxidase-conjugated anti-goat IgG (1:80,000) (Sigma). Protein bands were analyzed with Quantity One Software.

RESULTS AND DISCUSSION

Cloning and Structure of Rat Ang1

We cloned rat Ang1 cDNA (GenBank Accession No. AB080023), which contained a single open reading frame of 1,494 bp encoding a 498-amino-acid protein (Fig. 1). The predicted rat Ang1 amino acid sequence contains 36 potential N-glycosylation sites. Rat Ang1 gene is 95% identical to mouse Ang1 gene, though it lacks the 269th glycine in comparison with human and mouse Ang1 (Fig. 1). However, this site is not located in the fibronectin-like domain or coiled-coil domain that are reported to play key roles in the bioactivity (10).

Expression of Recombinant Rat Ang1

Ang1 has a characteristic protein structure with a coiled-coil domain in the NH_2-terminal portion and fibronectin-like domain in the COOH-terminal portion (3). We used S*f* (+) insect cells to produce recombinant rat Ang1. A major band of 65 kDa was detected mainly in the culture supernatant by western blotting (Fig. 2). It is reported that 75-kDa recombinant human Ang1 is produced by COS7 cells in culture supernatant (11). Because Ang1 is known to be a glycosylated

```
   1 AGGCTCCACGCTGAACGGTTACACAGAGAGGAAACAATAAATCTAAGCTACTATTGCAAT
  61 AAATATCTCAAGTTTTAACGAAGGAAACTATCATTACAGTAATTTTTTTAAGTAACGCGT
 121 TTTATAACAAAGCTAACAAATGGCTAGTTTTCTGTGGATCTTCTTCAAATGCTTTCTTTA
 181 ACGGGGAGAGCGTCAAACAACCAGTTTTACCTGAAATAAAGAACTAGTTTAAAGGTCAGA
 241 AGAGAGGAGCAAGCTTTGCAGGAGGCACGGAAGGCGAGTGCTGGCAGTACAATGACAGTT
                                                      M  T  V    3

 301 TTCCTTTCCTTTGCATTCTTCGCTGCCATTCTGACTCACATAGGGTGCAGCAACCAGCGC
     F  L  S  F  A  F  F  A  A  I  L  T  H  I  G  C  S  N  Q  R   23

 361 CGGAGTCCAGAAAACGGAGGGAGAAGATATAACCGAATTCAACATGGGCAATGTGCCTAC
     R  S  P  E  N  G  G  R  R  Y  N  R  I  Q  H  G  Q  C  A  Y   43

 421 ACTTTCATTCTTCCAGAACACGACGGGAACTGCCGTGAGAGTGCGACAGAGCAGTACAAC
     T  F  I  L  P  E  H  D  G  N  C  R  E  S  A  T  E  Q  Y  N   63

 481 ACCAACGCTCTGCAAAGGGATGCTCCACACGTGGAGACGGATTTCTCTTCCCAGAAACTT
     T  N  A  L  Q  R  D  A  P  H  V  E  T  D  F  S  S  Q  K  L   83

 541 CAGCATCTGGAGCATGTGATGGAAAATTATACTCAGTGGCTGCAAAAACTTGAGAATTAC
     Q  H  L  E  H  V  M  E  N  Y  T  Q  W  L  Q  K  L  E  N  Y  103

 601 ATTGTGGAAAATATGAAGTCGGAGATGGCCCAGATACAACAGAATGCGGTTCAAAACCAC
     I  V  E  N  M  K  S  E  M  A  Q  I  Q  Q  N  A  V  Q  N  H  123

 661 ACGGCCACCATGCTGGAGATAGGAACCAGCCTCTTGTCTCAGACTGCAGAGCAGACCCGA
     T  A  T  M  L  E  I  G  T  S  L  L  S  Q  T  A  E  Q  T  R  143

 721 AAGCTCACAGATGTGGAGACCCAGGTACTAAATCAAACATCCCGTCTTGAAATCCAACTG
     K  L  T  D  V  E  T  Q  V  L  N  Q  T  S  R  L  E  I  Q  L  163

 781 CTGGAGAATTCATTATCAACATACGAGCTAGAGAAACAGCTTCTCCAACAGACAAATGAA
     L  E  N  S  L  S  T  Y  E  L  E  K  Q  L  L  Q  Q  T  N  E  183

 841 ATTCTGAAGATTCAGGAAAAAAACAGTTTATTAGAGCATAAAATCCTAGAAATGGAGGGA
     I  L  K  I  Q  E  K  N  S  L  L  E  H  K  I  L  E  M  E  G  203

 901 AAACACAAGGAAGAGCTGGACACCTTGAAGGAGGAGAAAGAAAACCTTCAAGGCTTGGTT
     K  H  K  E  E  L  D  T  L  K  E  E  K  E  N  L  Q  G  L  V  223

 961 ACTCGTCAGACATTCATCATCCAAGAATTGGAGAAGCAACTTAGCAGAGCTACCAGCAAC
     T  R  Q  T  F  I  I  Q  E  L  E  K  Q  L  S  R  A  T  S  N  243

1021 AACAGTGTTCTGCAGAAGCAACAACTGGAGCTCATGGACACAGTCCATAACCTTGTCAGC
     N  S  V  L  Q  K  Q  Q  L  E  L  M  D  T  V  H  N  L  V  S  263

1081 CTTTGCACAAAAGAAGTTTTGCTAAAGGGAGGAAAAAGAGAAGAAGAGAAACCATTTCGA
     L  C  T  K  E  V  L  L  K  G  G  K  R  E  E  E  K  P  F  R  283

1141 GACTGTGCAGATGTATATCAAGCTGGTTTTAATAAGAGTGGAATCTACACTATTTATTTT
     D  C  A  D  V  Y  Q  A  G  F  N  K  S  G  I  Y  T  I  Y  F  303

1201 AATAATATGCCAGAACCCAAAAAGGTATTTTGCAATATGGATGTGAATGAAGGAGGATGG
     N  N  M  P  E  P  K  K  V  F  C  N  M  D  V  N  E  G  G  W  323

1261 ACAGTAATACAACACCGTGAGGATGGAAGCCTAGATTTCCAGAGGGGCTGGAAGGAGTAT
     T  V  I  Q  H  R  E  D  G  S  L  D  F  Q  R  G  W  K  E  Y  343

1321 AAAATGGGTTTTGGGAATCCCTCTGGTGAATATTGGCTTGGGAACGAGTTCATTTTTGCA
     K  M  G  F  G  N  P  S  G  E  Y  W  L  G  N  E  F  I  F  A  363

1381 ATAACCAGTCAGAGGCAGTACATGCTGAGGATCGAGCTGATGGACTGGGAAGGGAACCGA
     I  T  S  Q  R  Q  Y  M  L  R  I  E  L  M  D  W  E  G  N  R  383

1441 GCCTACTCACAGTACGACAGATTCCACATAGGAAACCAGAAGCAGAACTACAGGTTATAT
     A  Y  S  Q  Y  D  R  F  H  I  G  N  Q  K  Q  N  Y  R  L  Y  403

1501 TTAAAGGGTCACACGGGGACAGCAGGCAAACAGAGCAGCTTGATCTTACATGGTGCTGAT
     L  K  G  H  T  G  T  A  G  K  Q  S  S  L  I  L  H  G  A  D  423

1561 TTCAGCACAAAGGACGCTGATAACGACAACTGTATGTGCAAATGCGCCCTTATGCTAACA
     F  S  T  K  D  A  D  N  D  N  C  M  C  K  C  A  L  M  L  T  443

1621 GGAGGTTGGTGGTTTGATGCCTGTGGCCCTTCCAATCTAAACGGAATGTTCTACACTGCA
     G  G  W  W  F  D  A  C  G  P  S  N  L  N  G  M  F  Y  T  A  463

1681 GGGCAAAACCATGGAAAACTGAATGGGATAAAGTGGCACTACTTCAAAGGACCCAGTTAC
     G  Q  N  H  G  K  L  N  G  I  K  W  H  Y  F  K  G  P  S  Y  483

1741 TCCTTACGTTCCACAACCATGATGATCCGGCCCTTGGACTTCTGAAGGCGCTATGCCTAG
     S  L  R  S  T  T  M  M  I  R  P  L  D  F  *                 497

1801 TATTAGAAACCTGAAATAAATCTGGGGATGTTCCCGAATGAGAAGCTATCTGGAAGCTTC
1861 CGAAACAACCCAGCATTGTCTCCGTTCCAGC
```

Fig. 1. Sequence of rat Ang1 cDNA and the predicted primary amino acid sequence. Putative N-linked glycosylation sites are boxed. The underline shows the coiled-coil domain. The shaded box indicates the fibronectin-like domain.

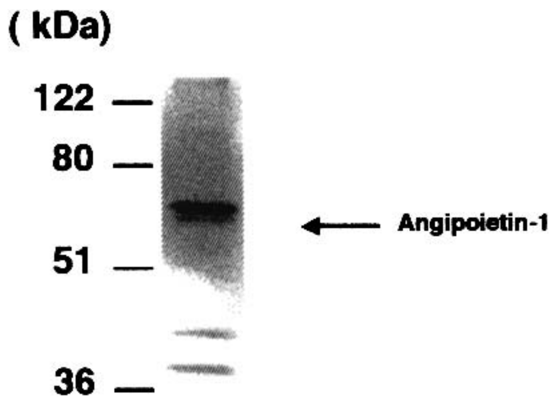

Fig. 2. Western blot of recombinant rat angiopoietin-1. The culture supernatant of Sf (+) insect cells with recombinant bacmid DNA and that of the cells infected with recombinant baculovirus stock were harvested at 72 h after transfection and at 72 h after infection, 3 times. Each sample was separated by SDS-polyacrylamide (10%) electrophoresis and detection was done by Western blot analysis with anti-Ang1 antibody.

protein that is secreted from the pericytes and vascular smooth muscle cells around endothelial cells, the reason for the size difference may be glycosylation (3). For example, Matsuura *et al.* reported that expressed envelope proteins were glycoproteins of 24 to 35 kDa in insect cells and glycoprotein of 35 kDa in mammalian cells (12). As shown in Fig. 2, we also detected glycoprotein of 62 to 65 kDa by Ang1-specific western blotting (Fig. 2).

The Expression of Occludin by Rat Ang1

Occludin is a transmembrane protein of approximate 65 kDa, and its extracellular domain binds to another occludin molecule on an adjacent cell to form a TJ. Treatment with rat Ang1 induced a 3-fold increase in the expression of occludin in TR-BBB13 in comparison with the SFM control (Fig. 3). GLA is known to be a potential inducer of occludin, and many phosphorylated upper bands can be seen in our system (9,13) (Fig. 3a, middle lane). This result is consistent with a previous report (13). Treatment of Ang1 also induced occludin phosphorylation in TR-BBB, as indicated by the upper bands (Fig. 3a, left band). This is the first report that Ang1 induces expression and phosphorylation of occludin in brain endothelial cells. Indeed, IL-15 and prolactin are reported to induce expression of occludin, but these proteins produced under abnormal conditions, such as inflammation and gestation (1,2). Ang1 is constitutively expressed in mural cells and induces angiogenesis (3,5). Ang1 also regulates expression of VE-cadherin and inhibits endothelial cell permeability (14). This result strongly suggests that Ang1 may regulate capillary permeability through modulating the expression of occludin in the brain. Recently, we established a conditionally immortalized bone marrow derived endothelial cell line, TR-BME (15). Brain capillaries show strong TJ formation in comparison with that in peripheral vessels. Using this cell line in comparison with TR-BBB, we intend to examine the role of Ang1 in TJ formation. In conclusion, our results show that Ang1 induces expression of the tight junction protein occludin in brain capillaries derived from endothelial cells. This finding suggests that transient expression of Ang1 by gene therapy may be a potential treatment to rescue brain edema.

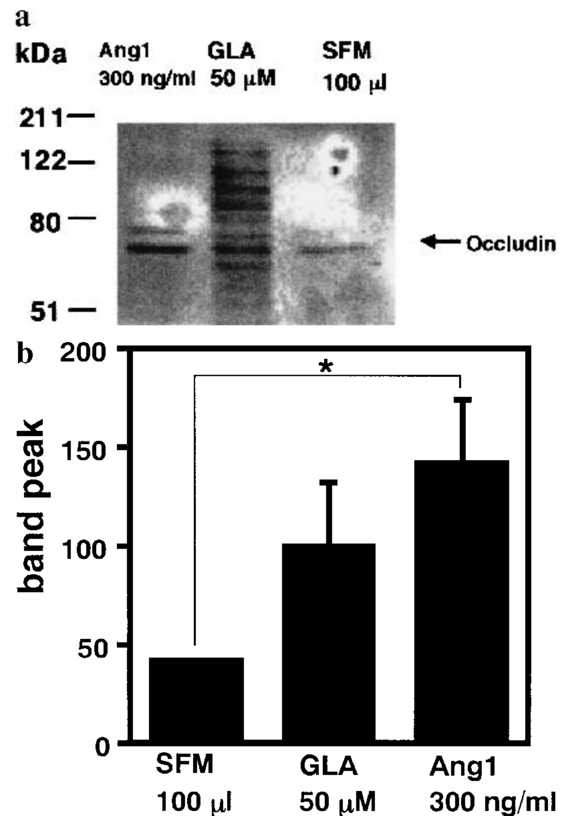

Fig. 3. Ang1 regulates the expression of occludin, as determined by Western blotting. TR-BBB13 was treated with Ang1 for 48 h and protein was extracted. Occludin was probed with rabbit anti-occludin antibody and visualized by means of enhanced chemiluminescence (a). The occludin band density in scanned images of the gel was calculated (b). Serum-free medium (SFM) was used as a control, with γ-linolenic acid (GLA) as a positive control. The gel shows the results of three independent experiments. All data were corrected based on the density of the SFM-treated control and the significance of differences was evaluated by means of the *t* test. Each bar shows the mean +/− SD of data ($n = 3$). * $p < 0.01$ compared to the control.

ACKNOWLEDGMENTS

The authors thank Ms. Y. Okubo and Ms. A. Matsushima for their technical assistance. This study was supported, in part, by the Private School Promotion Foundation, a Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Uehara Memorial Foundation.

REFERENCES

1. R. Nishiyama, T. Sakaguchi, T. Kinugasa, X. Gu, R. P. MacDermott, D. K. Podolsky, and H. C. Reinecker. Interleukin-2 receptor beta subunit-dependent and -independent regulation of intestinal epithelial tight junctions. *J. Biol. Chem.* **276**:35571–35580 (2001).
2. K. Stelwagen, H. A. McFadden, and J. Demmer. Prolactin, alone or in combination with glucocorticoids, enhances tight junction formation and expression of the tight junction protein occludin in mammary cells. *Mol. Cell Endocrinol.* **156**:55–61 (1999).
3. S. Davis, T. H. Aldrich, P. F. Jones, A. Acheson, D. L. Compton, V. Jain, T. E. Ryan, J. Bruno, C. Radziejewski, P. C. Maisonpi-

erre, and G. D. Yancopoulos. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87**:1161–1169 (1996).

4. C. Suri, P. F. Jones, S. Patan, S. Bartunkova, P. C. Maisonpierre, S. Davis, T. N. Sato, and G. D. Yancopoulos. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**:1171–1180 (1996).
5. G. Thurston, J. S. Rudge, E. Ioffe, H. Zhou, L. Ross, S. D. Croll, N. Glazer, J. Holash, D. M. McDonald, and G. D. Yancopoulos. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat. Med.* **6**:460–463 (2000).
6. J. D. Huber, K. A. Witt, S. Hom, R. D. Egleton, K. S. Mark, and T. P. Davis. Inflammatory pain alters blood-brain barrier permeability and tight junctional protein expression. *Am. J. Physiol. Heart Circ. Physiol.* **280**:H1241–H1248 (2001).
7. J. E. Bae and T. E. Toth. Cloning and kinetics of expression of Brucella abortus heat shock proteins by baculovirus recombinants. *Vet. Microbiol.* **75**:199–204 (2000).
8. K. I. Hosoya, T. Takashima, K. Tetsuka, T. Nagura, S. Ohtsuki, H. Takanaga, M. Ueda, N. Yanai, M. Obinata, and T. Terasaki. mRna expression and transport characterization of conditionally immortalized rat brain capillary endothelial cell lines; a new in vitro BBB model for drug targeting. *J. Drug Target.* **8**:357–370 (2000).
9. W. G. Jiang, R. P. Bryce, D. F. Horrobin, and R. E. Mansel. Regulation of tight junction permeability and occludin expression by polyunsaturated fatty acids. *Biochem. Biophys. Res. Commun.* **244**:414–420 (1998).
10. W. N. Procopio, P. I. Pelavin, W. M. Lee, and N. M. Yeilding. Angiopoietin-1 and -2 coiled coil domains mediate distinct homo-oligomerization patterns, but fibrinogen-like domains mediate ligand activity. *J. Biol. Chem.* **274**:30196–30201 (1999).
11. H. J. Kwak, J. N. So, S. J. Lee, I. Kim, and G. Y. Koh. Angiopoietin-1 is an apoptosis survival factor for endothelial cells. *FEBS Lett.* **448**:249–253 (1999).
12. Y. Matsuura, S. Harada, R. Suzuki, Y. Watanabe, Y. Inoue, I. Saito, and T. Miyamura. Expression of processed envelope protein of hepatitis C virus in mammalian and insect cells. *J. Virol.* **66**:1425–1431 (1992).
13. A. Sakakibara, M. Furuse, M. Saitou, Y. Ando-Akatsuka, and S. Tsukita. Possible involvement of phosphorylation of occludin in tight junction formation. *J. Cell Biol.* **137**:1393–1401 (1997).
14. J. R. Gamble, J. Drew, L. Trezise, A. Underwood, M. Parsons, L. Kasminkas, J. Rudge, G. Yancopoulos, and M. A. Vadas. Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. *Circ. Res.* **87**:603–607 (2000).
15. K. Hattori, M. Muta, M. Toi, H. Iizasa, M. Shinsei, T. Terasaki, M. Obinata, M. Ueda, and E. Nakashima. Establishment of bone marrow-derived endothelial cell lines from ts-SV40 T-antigen gene transgenic rats. *Pharm. Res.* **18**:9–15 (2001).