Molecular cloning and characterization of a novel angiopoietin family protein, angiopoietin-3

Injune Kim[a], Hee Jin Kwak[a], Ji Eun Ahn[a], June-No So[b], Mingzhu Liu[a], Keum Nim Koh[a], Gou Young Koh[a,*]

[a]*National Creative Research Initiatives Center for Cardiac Regeneration and Institute of Cardiovascular Research, Chonbuk National University School of Medicine, San 2-20, Keum-Am-Dong, Chonju 560-180, South Korea*
[b]*Department of Biotechnology, Woosuk University, Chonju, South Korea*

Received 8 December 1998

Abstract Using homology-based PCR, we have isolated cDNA encoding a novel member (491 amino acids) of the angiopoietin (Ang) family from human adult heart cDNA and have designated it angiopoietin-3 (Ang3). The NH_2-terminal and COOH-terminal portions of Ang-3 contain the characteristic coiled-coil domain and fibrinogen-like domain that are conserved in other known Angs. Ang3 has a highly hydrophobic region at the N-terminus (~21 amino acids) that is typical of a signal sequence for protein secretion. Ang3 mRNA is most abundant in adrenal gland, placenta, thyroid gland, heart and small intestine in human adult tissues. Additionally, Ang3 is a secretory protein, but is not a mitogen in endothelial cells.

Key words: Angiopoietin; Endothelial cell; Vasculogenesis

1. Introduction

The recent discovery of angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) has provided insight into the molecular and cellular mechanisms of blood vessel formation [1,2]. Ang1 and Ang2 share about 60% amino acid identity and bind with similar affinity to the endothelial cell tyrosine kinase receptor, Tie2 [1,2]. In vivo analysis by targeted gene inactivation revealed that Ang1 recruited and sustained periendothelial support cells [3], while Ang2 disrupted blood vessel formation in the developing embryo by antagonizing the effects of Ang1 on Tie2 [2]. Thus, Ang2 is a naturally occurring antagonist of Ang1 that competes for binding to Tie2 and blocks Ang1-induced Tie2 autophosphorylation [2]. However, ligands for Tie1, Tie2-related receptor, have not been identified.

Ang1 and Ang2 have a characteristic protein structure that contains a coiled-coil domain in the NH_2-terminal portion and a fibrinogen-like domain in the COOH-terminal portion [1,2]. Using homology-based PCR, we isolated cDNA encoding a novel member of the angiopoietin (Ang) family and have designated it angiopoietin-3 (Ang3). Our results demonstrate that Ang3 is a secretory protein and may be a ligand to the Tie1 or Tie2 receptor.

*Corresponding author. Fax: (82) (652) 270-4071.
E-mail: gykoh@moak.chonbuk.ac.kr

Abbreviations: PCR, polymerase chain reaction; CMV, cytomegalovirus; cDNA, complementary deoxyribonucleic acid

2. Materials and methods

2.1. Isolation of mouse and human Ang3

The partial cDNA of human Ang3 was amplified from the human adult hearts cDNA as a template by PCR for 30 cycles at an annealing temperature of 52°C using sense and antisense degenerate primers representing all possible codons corresponding to the amino acid sequences of human Ang1 and Ang2, GEYWLG and SNLNGM, respectively [1,2]. DNA of the expected size (~300 bp) was amplified. The amplified DNA was sequenced by cycle sequencing using AmpliCycle sequencing kit (Perkin Elmer). The novel amplified DNA was cloned into the pCR-Blunt vector (Invitrogen). To determine the entire coding region, the human adult heart cDNA was analyzed by the rapid amplification of cDNA ends (RACE) method (Clontech).

2.2. Northern blot analysis

^{32}P-labeled human Ang3 cDNA probe (nucleotides 1–714) was radio-labeled by the random priming method (Prime-a-Gene, Promega). Hybridizations were performed in human multiple tissue Northern blots (Clontech) according to the manufacturer's instructions.

2.3. Expression of Ang3 cDNA in NRK293 cells and detection of recombinant Ang3 protein by Western blotting analysis

Human Ang3 cDNA was inserted into CMV promoter driven mammalian cell expression vector, pcDNA3.1/Myc-His (Invitrogen), that has a DNA fragment (63 bp) encoding c-myc (EQKLISEEDL) and a 6×His tag (HHHHHH) at the 3′-terminus of coding region as an open reading frame (CMV-Ang3-M-H). The CMV-Ang3-M-H gene construct was transfected into NRK293 cells using Lipofectamine Plus (Gibco BRL) and incubated at 37. 5°C for 48 h in Dulbecco's Modified Eagle's Media with 2% fetal bovine serum under 5% CO_2-95% atmosphere. The culture supernatant and cell lysates of NRK293 cells transfected with the CMV-Ang3-M-H were purified by Ni-NTA Spin Column (Qiagen) and were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis system, and the proteins were electro-transferred to nitrocellulose membranes [4]. The nitrocellulose membranes were blocked by incubation in blocking buffer, incubated with anti-myc or anti-His antibody, washed, incubated with horseradish peroxidase conjugated secondary antibody, and signals were visualized by the ECL detection method according to the manufacturer's protocol (Amersham).

2.4. Mitogenic assay for Ang3 in endothelial cells

The human Ang3 cDNA and human $VEGF_{165}$ were inserted into the CMV promoter-driven mammalian cell expression vector, pcDNA3.1 (Invitrogen). The CMV-Ang3, CMV-$VEGF_{165}$ or pcDNA3.1 (mock) gene construct was transfected into NRK293 cells using Lipofectamine Plus (Gibco BRL). The cells were incubated at 37.5°C for 24 h in Dulbecco's modified Eagle's medium with 2% fetal bovine serum under 5% CO_2-95% atmosphere. Twenty-four hours after transfection, conditioned medium was collected from NRK293 cells. Mitogenic assays for Ang3 or $VEGF_{165}$ in human umbilical vein endothelial cells (HUVECs) were performed as previously described [5]. HUVECs between second and third passage were plated onto gelatinized 24-well plates (2×10^4 cells per well) in M-199 medium supplemented with 20% (v/v) fetal bovine serum and incubated for 24 h. Conditioned medium was diluted 10 times with the growth medium and cells were stimulated for 48 h. Fresh conditioned medium containing [^{3}H]thymidine (Amersham; 1 μCi/well) was added to the

PII: S0014-5793(99)00008-3

```
                                                  •
Ang3  MKTFTW-TLG VLFFLLVDTG HCRGGQFKIK KINQRRYPRA TDGKEEAKKC   49
Ang1  MTVFLS--FA FLAAILTHIG CSNQRRSPEN SG--RRYNRI QHGQ-----C   41
Ang2  MWQIVFFTL- -SCDLVLAAA YNNFRKSMDS IG--KKQYQV QHGS-----C   41
                          →
Ang3  AYTFLVPE-- ----QRITGP ICVNTKGQDA STIKDMITRM DLENLKDVLS   93
Ang1  AYTFILPEHD GNCRESTTDQ YNTNALQRDA PHVEPDFSSQ KLQHLEHVME   91
Ang2  SYTFLLPEMD -NCR-SSSSP YVSNAVQRDA P-LEYDDSVQ RLQVLENIME   88
                      Coiled-coil domain
Ang3  RQKREIDVLQ LV-V------ --DVDGNIV- N------E-- VKLL--RKE-   122
Ang1  NYTQWLQKLE NYIVENMKSE MAQIQQNAVQ NHTATMLEIG TSLLSQTAEQ   141
Ang2  NNTQWLMKLE NYIQDNMKKE MVEIQQNAVQ NQTAVMIEIG TNLLNQTAEQ   138
                      Coiled-coil domain
Ang3  SRNMNSRVTQ LYMQLLHEII RKRDNSLELS QLENKILNVT TEMLKMATRY   172
Ang1  TRKLTDVETQ VLNQTSRLEI QLLENSLSTY KLEKQLLQQT NEILKIHEKN   191
Ang2  TRKLTDVEAQ VLNQTTRLEL QLLEHSLSTN KLEKQILDQT SEINKLQDKN   188
                      Coiled-coil domain
Ang3  RELEVKYASL TDLVNNQSVM ITLLEE-QCL RIFSRQDTHV SPPLVQVVPQ   221
Ang1  SLLEHKILEM EG--KHKEEL DTLKEEKENL QGLVTRQTYI IQEL------   233
Ang2  SFLEKKVLAM ED--KHIIQL QSIKEEKDQL QVLVSKQNSI IEEL------   230
                              ←
Ang3  HIPNSQQYTP GLLGGNEIQR DPGYPRDLMP PPDLAT-SPT KSPFKIPPVT   270
Ang1  ----EKQLNR ATTNNSVLQK QQLELMDTVH --NLVN-LCT KEGVLLKGGK   276
Ang2  ----EKKIVT ATVNNSVLQK QQHDLMETVN --NLLTMMST SNSAKDPTVA   274
          →•      Fibrinogen-like domain  •
Ang3  FINEGPFKDC QQAKEAGHSV SGIYMIKPEN SNGPMQLWCE NSLDPGGWTV   320
Ang1  REEEKPFRDC ADVYQAGFNK SGIYTIYINN MPEPKKVFCN MDVNGGGWTV   326
Ang2  KEEQISFRDC AEVFKSGHTT NGIYTLTFPN STEEIKAYCD MEAGGGGWTI   324
                      Fibrinogen-like domain
Ang3  IQKRTDGSVN FFRNWENYKK GFGNIDGEYW LGLENIYMLS NQDNYKLLIE   370
Ang1  IQHREDGSLD FQRGWKEYKM GFGNPSGEYW LGNEFIFAIT SQRQYMLRIE   376
Ang2  IQRREDGSVD FQRTWKEYKV GFGNPSGEYW LGNEFVSQLT NQQRYVLKIH   374
                      Fibrinogen-like domain
Ang3  LEDWSDKKVY AEYSSFRLEP ESEFYRLRLG TYQGNAGD-S MMWHNGKQFT   419
Ang1  LMDWEGNRAY SQYDRFHIGN EKQNYRLYLK GHTGTAGKQS SLILHGADFS   426
Ang2  LKDWEGNEAY SLYEHFYLSS EELNYRIHLK GLTGTAGKIS SISQPGNDFS   424
                  •           •               ←
Ang3  TLDRDKDMYA GNCAHFHKGG WWYNACAHSN LNGVWYRGGH YRSKHQDGIF   469
Ang1  TKDADNDNCM CKCALMLTGG WWFDACGPSN LNGMFYTAGQ NHGK-LNGIK   475
Ang2  TKDGDNDKCI CKCSQMLTGG WWFDACGPSN LNGMYYPQRQ NTNK-FNGIK   473

Ang3  WAEYRGGSYS LRAVQMMIKP ID    491
Ang1  WHYFKGPSYS LRSTTMMIRP LDF   498
Ang2  WYYWKGSGYS LKATTMMIRP ADF   496
```

Fig. 1. Comparison of human Ang3 with other members of the Ang family. Alignment of the deduced amino acid sequences of human Ang3, human Ang1 [1], and human Ang2 [2] is shown. Residues that match the sequence of human Ang3 are shadowed. The asterisks above the human Ang3 sequence denote five cysteine residues that are conserved in other angiopoietin family members. The line below the human Ang3 sequence denotes a putative signal sequence for protein secretion [9]. The arrows delimit the coiled-coil and fibrinogen-like domains.

cells and stimulation was continued for another 24 h. Cells were washed with PBS and were lysed with 0.5 N sodium hydroxide, and incorporated radioactivity was determined by liquid scintillation counting. Each experiment was performed in triplicate.

3. Results and discussion

3.1. Cloning and analysis of human Ang3

Ang1 and Ang2 have characteristic protein structures that contain a coiled-coil domain in the NH_2-terminal portion and a fibrinogen-like domain in the COOH-terminal portion [1,2]. Using degenerative primers corresponding to the conserved amino acid sequences in fibrinogen-like domain, GEYWLG (Ang1, amino acids 353–358; Ang2, amino acids 351–356) and SNLNGM (Ang1, amino acids 455–460; Ang2, amino acids 453–458), a cDNA fragment encoding a novel Ang-related peptide was isolated from human adult heart cDNA. The nucleotide sequence of the entire coding region was obtained by the RACE method [6] using the human adult heart adapter-ligated cDNA as a template (accession number at GenBank nucleotide sequence database: AF107253). The nucleotide sequence of the coding region allowed elucidation of the complete amino acid sequence of a novel Ang (491 amino acids). We designated this human protein Ang3. Alignment of the deduced amino acid sequence of human Ang3 with those for Ang1 and Ang2 is shown in Fig. 1. This polypeptide was clearly a member of the angiopoietin family because the N-terminal and C-terminal portions of Ang3 contained the characteristic coiled-coil domain and fibrinogen-like domain that are found in other known Angs [1,2]. Five of the nine cysteines in Ang1 are conserved in Ang3 [1]. When gaps introduced into the amino acid sequences for the purpose of alignment are ignored for calculations, human Ang3 is 29% identical to human Ang1, and 26% identical to human Ang2. In the fibrinogen-like domains, the amino acid sequence of human Ang2 is 43% identical to human Ang1, and 41% identical to human Ang2, indicating that Ang3 is a novel angiopoietin family protein.

3.2. Expression of Ang3 mRNA in human adult tissues

The size of the main transcript was 3.0 kb, and a less abundant transcript of 4.0 kb was present in most of the human adult tissues where Ang3 mRNA was detected (Fig. 2). While both sizes of Ang3 mRNA transcripts were abundant in adrenal gland, placenta, thyroid gland, heart, skeletal muscle, and small intestine, they were less abundant in testis, ovary, colon, pancreas, kidney, and stomach. Thus, Ang3 mRNA is widely expressed in adult tissues and is expressed abundantly in

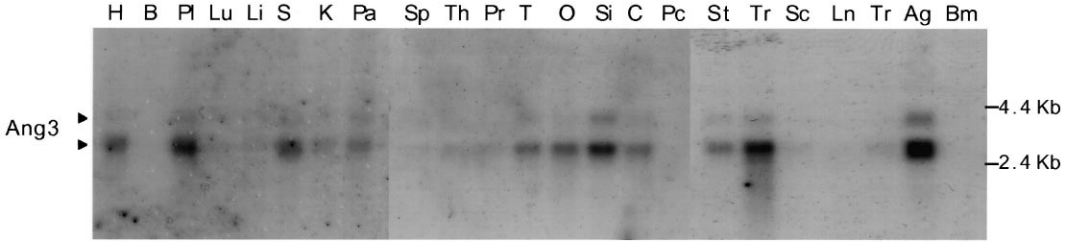

Fig. 2. Northern blot analyses for detection of Ang3 mRNA in human adult tissues. Each lane contains approximately 2 μg of purified polyadenylated RNA. Hybridization was performed with a ^{32}P-labeled human Ang3 probe. The sizes of RNA molecular size makers, in kb, for the Ang3 hybridization are shown to the right. Lanes H, B, Pl, Lu, Li, S, K, Pa, Sp, Th, Pr, T, O, Si, C, Pc, St, Tr, Sc, Ln, Tr, Ag and Bm indicate RNA from the adult heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocyte, stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland and bone marrow.

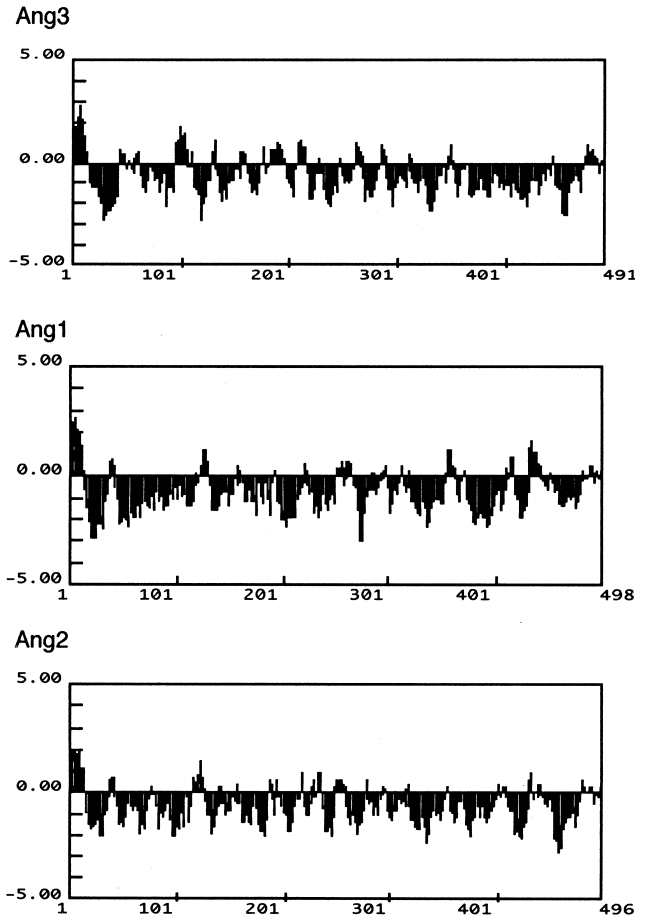

Fig. 3. The hydrophobicity profiles of human Ang3, Ang1 and Ang2. The profiles were generated according to Kyte and Doolittle [10] using a window of 10 residues.

highly vascularized adult tissues while Ang1 and Ang2 mRNAs are expressed in limited adult tissues [2]. These findings suggest that Ang3 is required for maintenance of the differentiated state of endothelial cells in most adult tissues [7,8].

3.3. *Ang3 is a secreted protein but not an endothelial cell mitogen*

The amino acid sequence of human Ang3 has a highly hydrophobic region at the N-terminus (~21 amino acids) that is typical of a signal sequence for protein secretion (Fig. 2). The signal sequence cleavage site was predicted to lie between amino acid positions 21 (C) and 22 (R) by the method of von Heijne [9]. Analysis and comparison of hydrophobicity profiles among Ang1, Ang2 and Ang3 were performed according to Kyte and Doolittle [10]. These results indicate that Ang3, like Ang1 and Ang2, mainly consists of hydrophilic amino acids (Fig. 3). To demonstrate that Ang3 is a secreted protein, NRK 293 cells were transfected with CMV promoter-driven mammalian cell expression vector containing human Ang3 cDNA with the 3′-terminal extension encoding c-myc and 6×His tags. To detect the Ang3 protein, both the culture supernatant and cell lysate were examined by Western blot analysis with anti-His tag antibody. A major band of ~66 kDa was detected in the culture supernatant, but not in the cell lysate (Fig. 4). The observed molecular mass of the

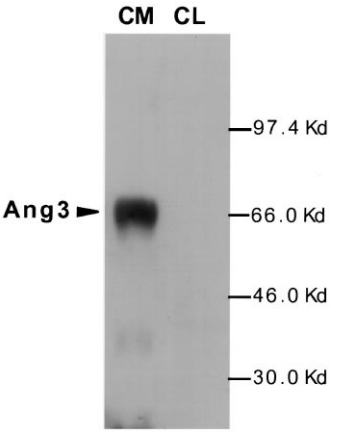

Fig. 4. Detection of human Ang3 protein from the culture medium and cell lysates of NRK 293 cells transfected with the CMV promoter-driven mammalian cell expression vector containing the human Ang3 cDNA with the 3′-terminal extension encoding c-myc and 6×His tags. The culture medium and cell extracts of the transfected NRK 293 cells were separated by SDS-polyacrylamide gel (10%) electrophoresis. Human Ang3 protein was detected by Western blotting analysis with anti-myc antibody (Invitrogen). CM, culture medium; CL, cell lysates. Rainbow molecular marker (Amersham) was used to estimate molecular mass.

major band was larger than the calculated molecular mass of recombinant Ang3 (~57 kDa). The amino acid sequence of Ang3 contained several potential glycosylation sites suggesting that the observed larger size of Ang3 may be produced as a glycoprotein. These results also indicate that human Ang3 is an efficiently secreted glycoprotein. Ang1 and Ang2 are both secreted glycoproteins with considerable sequence homology [1,2]. Both bind to the Tie2 receptor with similar affinity, but neither binds to the related receptor Tie1 [1,2]. Tie1 is remarkably similar in structure to Tie2 [11] and appears to control another aspect of vessel integrity. Knockout mice lacking Tie1 die between embryonic day 14.5 and birth of edema and hemorrhage, implicating that Tie1 controls fluid exchange across capillaries and hemodynamic stress resistance [12,13]. How-

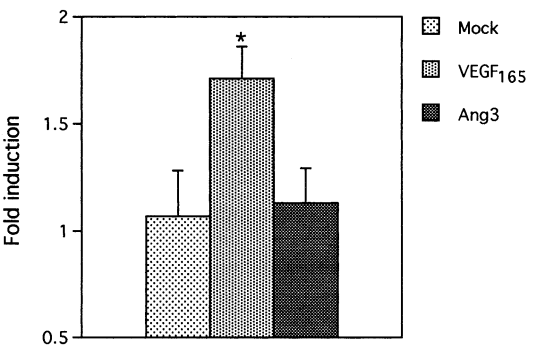

Fig. 5. The effect of Ang3 on [^{3}H]thymidine incorporation into endothelial cells. Conditioned medium from NRK 293 cells transfected with empty vector (mock) or expression vectors for $VEGF_{165}$ or Ang3 were diluted in growth medium, applied to HUVECs, and incorporation of [^{3}H]thymidine was measured. The detailed procedure is described in Section 2. The columns show the fold induction of [^{3}H]thymidine incorporation compared with basal activity induced by conditioned medium from mock-transfected cells. The bars show the mean ± S.D. of six independent experiments. Statistical analysis between the values of mock and the values of $VEGF_{165}$ or Ang3 were performed using Student's *t*-test ($^*P < 0.05$).

ever, ligands for Tie1 have not been identified. Ang3 is a possible ligand for Tie1 receptor.

The inability of Ang1 to stimulate endothelial cell proliferation was demonstrated [1]. We examined the ability of Ang3 to stimulate endothelial cell proliferation by analyzing the incorporation of [^{3}H]thymidine into HUVECs (Fig. 5). Conditioned medium from transfected NRK 293 cells expressing Ang3 did not alter in [^{3}H]thymidine incorporation into DNA of HUVECs, but in the positive controls, using conditioned medium from transfected NRK 293 cells expressing $VEGF_{165}$, incorporation increased approximately 1.7-fold. These results demonstrate that Ang3, like Ang1, is not an endothelial cell growth factor in vitro. Thus, Ang3, like Ang1, may have a vasculogenic effect rather than an angiogenic effect. Recent studies [14,15] indicate that transgenic overexpression of Ang1 or gene transfer of Ang1 increases vascularization in vivo.

In summary, using homology-based PCR, we have isolated cDNA encoding a novel member of the angiopoietin family and have designated it Ang3. The NH_2-terminal and COOH-terminal portions of Ang3 contain the characteristic coiled-coil domain and fibrinogen-like domain that are conserved in other known Angs. Ang3 mRNA is expressed in most tissues and is expressed abundantly in highly vascularized adult tissues. Ang3 is a secretory protein, but is not a mitogen in endothelial cells. Whether Ang3 binds to the known Tie1 or Tie2 receptor tyrosine kinases or to a novel and yet unidentified angiopoietin-3 receptor tyrosine kinase remains to be elucidated.

Acknowledgements: We thank So Young Kim and Jeong Jun Nah for excellent assistance, and Amy Frith-Terhune for help in the preparation of manuscript. This work was supported by the Creative Research Initiatives of the Korean Ministry of Science and Technology.

References

[1] Davis, S., Aldrich, T.H., Jones, P.F., Acheson, A., Compton, D.L., Jain, V., Ryan, T.E., Bruno, J., Radziejewski, C., Maisonpierre, P.C. and Yancopoulos, G.D. (1996) Cell 87, 1161–1169.
[2] Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N., Daly, T.J., Davis, S., Sato, T.N. and Yancopoulos, G.D. (1997) Science 277, 55–60.
[3] Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N. and Yancopoulos, G.D. (1996) Cell 87, 1171–1180.
[4] Koh, K.N., Kang, M.J., Terhune, A.F., Park, S.K., Kim, I., Lee, C.O. and Koh, G.Y. (1998) J. Mol. Cell. Cardiol. 30, 463–474.
[5] Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R.F., Alitalo, K. and Eriksson, U. (1996) Proc. Natl. Acad. Sci. USA 93, 2576–2581.
[6] Frohman, M.A., Dush, N.K. and Martin, G.R. (1988) Proc. Natl. Acad. Sci. USA 85, 8998–9002.
[7] Risau, W. (1995) FASEB J. 9, 926–933.
[8] Hanahan, D. (1997) Science 277, 48–50.
[9] von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690.
[10] Kyte, J. and Doolittle, R. (1982) J. Mol. Biol. 157, 105–132.
[11] Mustonen, T. and Alitalo, K. (1995) J. Cell. Biol. 129, 895–898.
[12] Sato, T.N., Tozawa, Y., Deutsch, U., Buchholz, K.W., Fujiwara, Y., Maguire, M.G., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995) Nature 376, 70–74.
[13] Puri, M.C., Rossant, J., Alitalo, K., Bernstein, A. and Partanen, J. (1995) EMBO J. 14, 5884.
[14] Suri, C., McClain, J., Thursyon, G., McDonald, D.M., Zhou, H., Oldmixon, E.H., Sato, T.N. and Yancopoulos, G.D. (1998) Science 282, 468–471.
[15] Shyu, K.G., Manor, O., Magner, M., Yancopoulos, G.D. and Isner, J.M. (1998) Circulation 98, 2081–2087.