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Genetic background influences therapeutic effectiveness of VEGF

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Abstract

Therapeutic angiogenesis has emerged as a promising therapy, but some patients are refractory to exogenous growth factors. In order to identify the genetic determinants of post-natal angiogenesis and physiological vessel formation, we investigated the genetic factors that affected ischemia-induced development of collaterals in mice. An ischemic hindlimb model was generated in C57BL/6, C3H/He, and BALB/c mice. Angiogenesis was markedly different among the mice as determined by the restoration of blood perfusion and capillary density of the ischemic muscle. Impaired collateral vessel formation in BALB/c mice was associated with reduced expression of vascular endothelial cell growth factor (VEGF). Intramuscular gene transfer of VEGF promoted collateral formation in C57BL/6J mice, but not in BALB/c mice. Ineffectiveness of VEGF in BALB/c mice was associated with impaired expression of VEGF receptor. Our findings suggest that genetic background may influence spontaneous collateral formation and therapeutic effectiveness of exogenous VEGF. Alternative strategies other than administration of VEGF alone might be needed to attain optimal angiogenesis in some patients.

Keywords: Angiogenesis; Strain; VEGF; Receptor; Collateral; Mouse

Angiogenesis is a physiological response to ischemia [1] and therapeutic angiogenesis has emerged as a promising therapy for ischemic disease [2]. However, there is clinical evidence to suggest that some patients, unlike healthy experimental animals, fail to develop spontaneous collateral circulation in response to tissue ischemia [3] and appear to be refractory to exogenous administration of growth factors [4,5]. Of clinical importance are recent reports that overdoses of vascular endothelial cell growth factor (VEGF) may cause fragile neovascularization which in turn would lead to deleterious complications such as bleeding and microvascular leakage [6–8]. Thus, it might be important to identify potential genetic determinants for post-natal angiogenesis to find an optimal therapeutic strategy for patients with ischemic disease.

Mice have been widely used to study the pathogenesis of human diseases including ischemia and to develop therapeutic strategies [9–11]. Furthermore, recent advances in gene-manipulating techniques have enabled us to produce various genetically modified mice to determine the role of specific molecules in a variety of biological phenomena including vascular remodeling [12]. Mouse genetics has been extensively characterized [13] and the mice full genome sequence is available [14]. Therefore, mice would be appropriate models to test whether the biological responses are genetically determined.

Here, we examined the development of collaterals in response to tissue ischemia in inbred mice. The results demonstrate that there is a marked heterogeneity in spontaneous angiogenesis and in the response to VEGF administration, suggesting that alternative strategies might be required to overcome unresponsiveness.

Materials and methods

Animals. Adult male 30- to 35-week-old inbred mice were purchased from SLC Japan (Shizuoka, Japan). All protocols involving experimental animals were in accordance with the local institutional

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guideline for animal care of the University of Tokyo and complied with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, revised 1985).

Mouse ischemic hindlimb model. Unilateral hindlimb ischemia was induced in 30- to 35-week-old male mice [9]. The animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The proximal portion of the femoral artery was ligated, followed by ligation of the distal portion of the saphenous artery. After that, the artery and all side branches were dissected and excised. The skin was closed with 5–0 surgical suture. Hindlimb blood perfusion was measured with a laser Doppler perfusion imager (LDPI) system (Moor Instruments, Devon, UK) as described elsewhere [15]. Excess hairs were removed from the limbs using depilatory cream before imaging and mice were placed on a heating plate at 40 °C to minimize the influence of the temperature. To avoid the influence of ambient light and temperature, the results are expressed as the ratio of perfusion in the right (ischemic) versus left (normal) limb.

Immunohistochemistry. Five weeks after surgery, mice were sacrificed by intraperitoneal injection of an overdose of pentobarbital. The whole limbs were fixed in methanol overnight. The femora were carefully removed and the ischemic thigh muscles were embedded in paraffin. Sections (5 μm) were de-paraffinized and incubated with a rat-monoclonal antibody against murine CD31 (clone MEC13.3, BD PharMingen, San Diego, CA) [9,15]. Antibody distribution was visualized using the avidin–biotin-complex technique and Vector Red chromogenic substrate (Vector Laboratories, Burlingame, CA), followed by counterstaining with hematoxylin. Capillaries were identified by positive staining for CD31 and morphology. Five different fields from each muscle were randomly selected and the number of capillaries was counted.

Reverse transcriptase-polymerase chain reaction. Total RNA was prepared from the ischemic muscle using RNAzol (Tel-Test, Friendswood, TX). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described elsewhere [16]. First strand cDNA was synthesized from 1 μg of total RNA, using random primer and MMLV-derived reverse transciptase (ReverTra Ace-α, TOYOBO, Osaka) and one-twentieth of the reaction mixture was used as template for PCR amplification. A set of primers, 5′-GCGGGCTGCCTCG CAGTC-3′ (sense) and 5′-TCACCGCCTTGGCTTGTCAC-3′ (antisense), yielded 715 bp ($VEGF_{188}$), 644 bp ($VEGF_{164}$), and 512 bp ($VEGF_{120}$) products.

Western blot analysis. Protein was extracted from the ischemic muscles. 100 μg of protein was separated on an 8% SDS–polyacrylamide gel and electroblotted onto a PVDF membrane (HyBond-P, Amersham Biosciences, Tokyo). The membrane was blocked with 5% milk in 0.1% Tween PBS (T-PBS) and incubated with polyclonal anti-murine Flt-1 antibody or monoclonal anti-murine KDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The blot was visualized as described [17].

Gene transfer of VEGF. Murine $VEGF_{164}$ cDNA was obtained by RT-PCR using RNA isolated from ischemic muscle of C57BL/6 mouse. The PCR product was sequenced and then subcloned into pcDNA3 expression vector. Fifty micrograms of naked plasmid in 25 μl PBS was injected in the right thigh muscles, followed by in vivo electroporation ($100\,V \times 50\,ms \times 6$ times) [18]. Three days after injection, unilateral hindlimb ischemia was induced and blood flow was monitored.

Statistics. All data are expressed as means ± SEM. Statistical comparisons among strains were performed by ANOVA followed by Student's t test. A p value of <0.05 was considered to be significant.

Results

Impaired collateral formation in BALB/c mice

In order to analyze spontaneous collateral development in response to ischemia, we excised femoral arteries of C57BL/6 ($n = 10$), C3H/He ($n = 10$), and BALB/c ($n = 10$) mice. Surgery induced severe ischemia in the right femoral artery in all mice. There was no significant difference in the degree of post-operative ischemia. Blood flow of the ischemic muscle increased gradually in C57BL/6 mice, while severe ischemia continued in BALB/c mice (Figs. 1A and B). C3H/He mice showed mild recovery of the blood flow (Fig. 1C). Anti-immunostaining on the ischemic muscle harvested at 5 weeks revealed increased capillary density in C57BL/6 mice (Fig. 2). The capillary density of BALB/c mice was

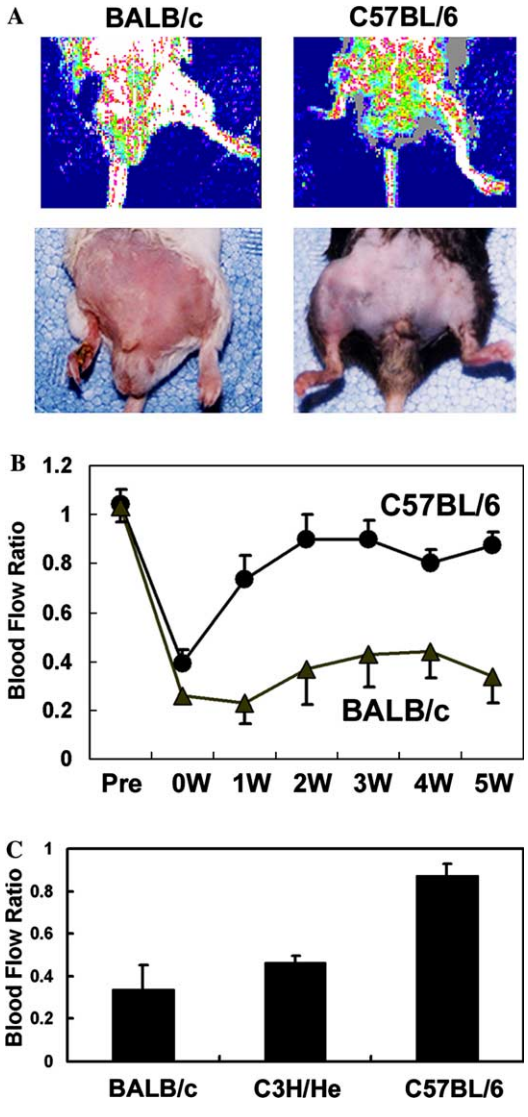

Fig. 1. Angiogenesis differed among the three mouse strains. Hindlimb ischemia was induced in BALB/c, C3H/He, and C57BL/6 mice. Blood flow of the ischemic hindlimb was monitored weekly by laser Doppler perfusion imaging (LDPI). (A) Representative blood flow imaging (top) and appearance (bottom) of BALB/c and C57BL/6 mice at 5 weeks. (B) Ratio of blood flow in the ischemic hindlimb to that in the non-ischemic hindlimb of BALB/c and C57BL/6 mice ($n = 10$ for each group). (C) The blood flow ratio in ischemic limb to that in non-ischemic limb evaluated at 5 weeks.

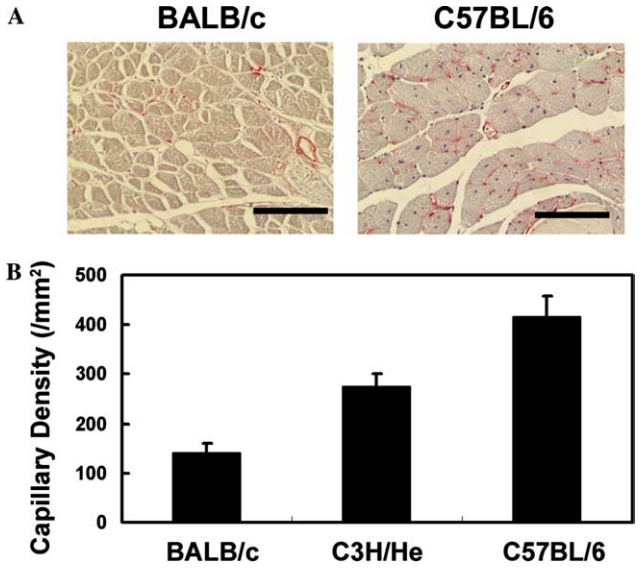

Fig. 2. Histological examination of angiogenesis (A) Paraffin-embedded sections were stained for CD31. Bar, 200 μm. (B) Capillary density of the ischemic hindlimb harvested at 5 weeks after surgery.

significantly lower than that of C57BL/6 or C3H/He mice. The histological evaluation of neovascularization corresponded to the physiological measurement of collateral flow.

Impaired VEGF expression in BALB/c mice

To identify the molecule that may be responsible for the diversity in blood flow recovery, we examined VEGF expression, which had been implicated for impaired angiogenesis in several murine models [11,19]. C57BL/6 mice showed the highest expression of VEGF already from the base line and BALB/c strain showed the lowest level of VEGF expression. We could detect three major isoforms of VEGF by RT-PCR [16]. The most abundantly expressed isoform was $VEGF_{165}$, which was reported to be the most active in vivo [16]. Rapid induction of all isoforms of VEGF expression by ischemia was observed in all three strains 6 h after surgery. These results suggest that reduced expression of VEGF may account for the

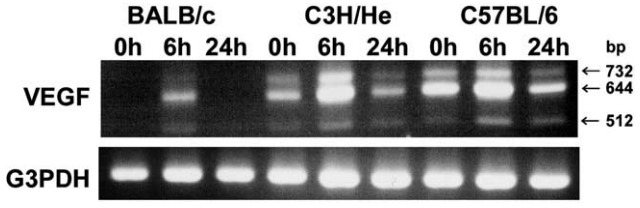

Fig. 3. RT-PCR analysis of VEGF expression. Three isoforms of VEGF transcripts ($VEGF_{188}$, 716 bp; $VEGF_{164}$, 644 bp; and $VEGF_{120}$, 512 bp) were demonstrated by RT-PCR using total RNA isolated from the ischemic muscle.

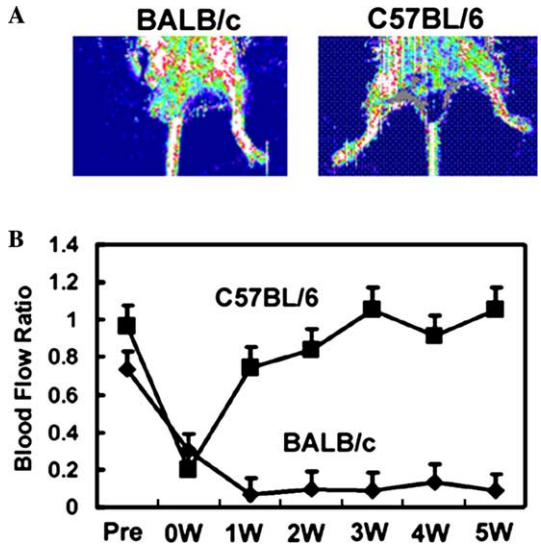

Fig. 4. Collateral development in response to VEGF gene transfer in C57BL/6 mice and in BALB/c mice. VEGF-pcDNA3 was injected into the hindlimb, followed by in vivo electroporation. After 3 days, hindlimb ischemia was induced and blood flow was monitored weekly. (A) Representative laser Doppler imaging at 5 weeks. (B) Ratio of blood flow in the ischemic hindlimb to that in the non-ischemic hindlimb of BALB/c and C57BL/6 mice, which had received VEGF gene.

impaired angiogenesis of BALB/c mice in response to ischemia (see Fig. 3).

Failure of exogenous VEGF to rescue impaired angiogenesis in BALB/c mice

In order to examine whether VEGF expression is a major determinant of ischemia-induced angiogenesis, we evaluated the effect of exogenous VEGF on the development of collaterals. VEGF gene was transferred to the right hindlimb 3 days prior to surgery. Although blood flow recovery tended to increase by VEGF gene transfer in C57BL/6 mice, administration of VEGF failed to rescue impaired angiogenesis in BALB/c mice (Fig. 4).

Induction of VEGF receptor was impaired in BALB/c strain

To study the molecular mechanism that might be responsible for genetic difference in responsiveness to exogenous VEGF, we analyzed the expression of VEGF receptors (Fig. 5). Relatively high amount of Flt-1 was expressed in non-ischemic muscle of C57BL/6 and C3H/He mice. BALB/c mice showed increased Flt-1 expression 24 h after surgery. In contrast, Flk-1 expression was not detected in any of the strains at baseline. Distinct induction of Flk-1 expression by ischemia was already observed at one day after surgery in C57BL/6 and C3H/He mice.

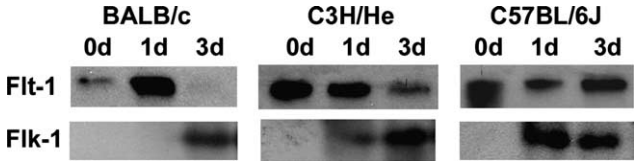

Fig. 5. Western blot analysis of Flt-1 and Flk-1. Western blot analysis was performed using extracts from ischemic muscles.

However, in BALB/c mice Flk-1 expression was not up-regulated in response to ischemia at one day after surgery.

Discussion

Our results indicate that angiogenesis is significantly influenced by genetic background in mice. VEGF expression in response to acute ischemia induced by femoral artery excision varied significantly among the three mouse strains. In BALB/c mice, impaired collateral development was associated with a reduction in VEGF expression. In C57BL/6J mice, VEGF expression level was the highest. These findings are consistent with the results of previous studies, which showed that VEGF played a critical role in both therapeutic and pathological angiogenesis [3,20–23].

Previously, it was demonstrated that exogenous VEGF can rescue impaired collateral formation in the ischemic hindlimb of Apo E−/− mice and aged mice [11,19]. Notably, these mice have the same genetic background of C57BL/6J. We found that exogenous VEGF alone did not compensate impaired angiogenesis in BALB/c mice, suggesting that there were other determinants of angiogenesis besides VEGF. It is known that VEGF binds to 2 major different receptors, namely Flt-1 and Flk-1/KDR, and functions not only as an angiogenic factor but also as a survival factor for endothelial cells [24]. In our study, BALB/c mice, in which autoamputation frequently occurred, showed impaired induction of Flk-1 compared with the other two strains. Baseline Flt-1 expression was also reduced in BALB/c mice. These findings suggest that impairment in expression of VEGF receptors might be, at least in part, responsible for retarded angiogenesis in BALB/c mice.

There is an individual variability in the degree of collateral formation among patients with myocardial or ischemia extremities [3]. The molecular basis of this heterogeneity is poorly understood. Our results indicate that the difference in the induction of VEGF receptors may be responsible for the variability of angiogenesis in response to tissue ischemia and suggest that an alternative strategy may be required to attain optimal therapeutic angiogenesis in some patients. Our study may also lead to a genetic linkage analysis to identify novel angiogenic factors that may influence the formation of collaterals.

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References

[1] J. Folkman, Clinical-applications of research on angiogenesis, N. Engl. J. Med. 333 (1995) 1757–1763.
[2] S. Freedman, J.M. Isner, Therapeutic angiogenesis for ischemic cardiovascular disease, J. Mol. Cell. Cardiol. 33 (2001) 379–393.
[3] A. Schultz, L. Lavie, I. Hochberg, R. Beyar, T. Stone, K. Skorecki, P. Lavie, A. Roguin, A.P. Levy, Interindividual heterogeneity in the hypoxic regulation of VEGF: significance for the development of the coronary artery collateral circulation, Circulation 100 (1999) 547–552.
[4] I. Baumgartner, A. Pieczek, O. Manor, R. Blair, M. Kearney, K. Walsh, J.M. Isner, Constitutive expression of phVEGF$_{165}$ following intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia, Circulation 97 (1998) 1114–1123.
[5] T.K. Rosengart, L.Y. Lee, S.R. Patel, T.A. Sanborn, M. Parikh, G.W. Bergman, R. Hachamovitch, M. Szulc, P.D. Kligfield, P.M. Okin, R.T. Hahn, R.B. Devereux, M.R. Post, N.R. Hackett, T. Foster, T.M. Grasso, M.L. Lesser, O.W. Isom, R.G. Crystal, Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease, Circulation 100 (1999) 468–474.
[6] R.J. Lee, M.L. Springer, W.E. Blanco-Bose, R. Shaw, P.C. Ursell, H.M. Blau, VEGF gene delivery to myocardium: deleterious effects of unregulated expression, Circulation 102 (2000) 898–901.
[7] P. Carmeliet, VEGF gene therapy: stimulating angiogenesis or angioma-genesis?, Nat Med. 6 (2000) 1102–1103.
[8] G. Thurston, C Suri, K. Smith, J. McClain, T.N. Sato, G.D. Yancopoulos, D.M. McDonald, Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1, Science 286 (1999) 2511–2514.
[9] T. Couffinhal, M. Silver, L.P. Zheng, M. Kearney, B. Witzenbichler, J.M. Isner, Mouse model of angiogenesis, Am. J. Pathol. 152 (1998) 1667–1679.
[10] A. Rivard, M. Silver, D. Chen, M. Kearney, M. Magner, B. Annex, K. Peters, J.M. Isner, Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF, Am. J. Pathol. 154 (1999) 355–363.
[11] A. Rivard, J.E. Fabre, M. Silver, D. Chen, T. Murohara, M. Kearney, M. Magner, T. Asahara, J.M. Isner, Age-dependent impairment of angiogenesis, Circulation 99 (1999) 111–120.
[12] F.M. Faraci, C.D. Sigmund, Vascular biology in genetically altered mice. Smaller vessels, bigger insight, Circ. Res. 85 (1999) 1214–1225.
[13] E. Marshall, The rise of the mouse, biomedicine's model animal, Science 288 (2000) 248–257.
[14] S.G. Gregory, M. Sekhon, J. Schein, S. Zhao, K. Osoegawa, C.E. Scott, R.S. Evans, P.W. Burridge, T.V. Cox, C.A. Fox, R.D. Hutton, I.R. Mullenger, K.J. Phillips, J. Smith, J. Stalker, G.J. Threadgold, E. Birney, K. Wylie, A. Chinwalla, J. Wallis, L. Hillier, J. Carter, T. Gaige, S. Jaeger, C. Kremitzki, D. Layman, J. Maas, R. McGrane, K. Mead, R. Walker, S. Jones, M. Smith, J. Asano, I. Bosdet, S. Chan, S. Chittaranjan, R. Chiu,

C. Fjell, D. Fuhrmann, N. Girn, C. Gray, R. Guin, L. Hsiao, M. Krzywinski, R. Kutsche, S.S. Lee, C. Mathewson, C. McLeavy, S. Messervier, S. Ness, P. Pandoh, A.L. Prabhu, P. Saeedi, D. Smailus, L. Spence, J. Stott, S. Taylor, W. Terpstra, M. Tsai, J. Vardy, N. Wye, G. Yang, S. Shatsman, B. Ayodeji, K. Geer, G. Tsegaye, A. Shvartsbeyn, E. Gebregeorgis, M. Krol, D. Russell, L. Overton, J.A. Malek, M. Holmes, M. Heaney, J. Shetty, T. Feldblyum, W.C. Nierman, J.J. Catanese, T. Hubbard, R.H. Waterston, J. Rogers, P.J. de Jong, C.M. Fraser, M. Marra, J.D. McPherson, D.R. Bentley, A physical map of the mouse genome, Nature 418 (2002) 743–750.

[15] M. Sata, H. Nishimatsu, E. Suzuki, S. Sugiura, M. Yoshizumi, Y. Ouchi, Y. Hirata, R. Nagai, Endothelial nitric oxide synthase is essential for the HMG-CoA reductase inhibitor cerivastatin to promote collateral growth in response to ischemia, FASEB J. 15 (2001) 2530–2532.

[16] H.H. Marti, W. Risau, Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors, Proc. Natl. Acad. Sci. USA 95 (1998) 15809–15814.

[17] M. Sata, K. Walsh, Endothelial cell apoptosis induced by oxidized LDL is associated with the downregulation of the cellular caspase inhibitor FLIP, J. Biol. Chem. 273 (1998) 33103–33106.

[18] H. Aihara, J. Miyazaki, Gene transfer into muscle by electroporation in vivo, Nat. Biotechnol. 16 (1998) 867–870.

[19] T. Couffinhal, M. Silver, M. Kearney, A. Sullivan, B. Witzenbichler, M. Magner, B. Annex, K. Peters, J.M. Isner, Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE-/- mice, Circulation 99 (1999) 3188–3198.

[20] J.M. Isner, A. Pieczek, R. Schainfeld, R. Blair, L. Haley, T. Asahara, K. Rosenfield, S. Razvi, K. Walsh, J.F. Symes, Clinical evidence of angiogenesis after arterial gene transfer of phVEGF$_{165}$ in patient with ischaemic limb, The Lancet 348 (1996) 370–374.

[21] M.R. Freeman, F.X. Schneck, M.L. Gagnon, C. Corless, S. Soker, K. Niknejad, G.E. Peoples, M. Klagsbrun, Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis, Cancer Res. 55 (1995) 4140–4145.

[22] M. Inoue, H. Itoh, M. Ueda, T. Naruko, A. Kojima, R. Komatsu, K. Doi, Y. Ogawa, N. Tamura, K. Takaya, T. Igaki, J. Yamashita, T.H. Chun, K. Masatsugu, A.E. Becker, K. Nakao, Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis, Circulation 98 (1998) 2108–2116.

[23] P. Guo, Q. Fang, H.Q. Tao, C.A. Schafer, B.M. Fenton, I. Ding, B. Hu, S.Y. Cheng, Overexpression of vascular endothelial growth factor by MCF-7 breast cancer cells promotes estrogen-independent tumor growth in vivo, Cancer Res. 63 (2003) 4684–4691.

[24] I. Spyridopoulos, E. Brogi, M. Kearney, A.B. Sullivan, C. Cetrulo, J.M. Isner, D.W. Losordo, Vascular endothelial growth factor inhibits endothelial cell apoptosis induced by tumor necrosis factor-A: balance between growth and death signals, J. Mol. Cell. Cardiol. 29 (1997) 1321–1330.