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Involvement of cysteinyl leukotriene receptors in angiogenesis in rat thoracic aortic rings

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Cysteinyl leukotrienes (CysLTs) are potent inflammatory mediators that induce inflammation through the activation of $CysLT_1$ and $CysLT_2$ receptors. It has been reported that inflammatory mediators, such as prostaglandins, play an important role in angiogenesis. However, whether CysLTs and the receptor subtypes are involved in angiogenesis is not clarified. Here, we determined the effects of CysLT receptor agonist leukotriene D_4 (LTD_4) and antagonists on angiogenesis by rat thoracic aortic ring assay. We found that the microvessel growth in 25% serum-containing cultures was significantly inhibited by the $CysLT_1$ receptor antagonist montelukast (0.1–1 μM), but not by the $CysLT_2$ receptor antagonist BAY cyslt2 (0.1–1 μM). The microvessel growth in serum-free culture was affected neither by montelukast (0.01–1 μM) nor by BAY cyslt2 (0.1–1 μM). Furthermore, LTD_4 at 100 nM significantly enhanced the microvessel growth in serum-free culture and LTD_4 at 10–100 nM significantly enhanced the microvessel growth in 25% serum-containing cultures. The enhancement was abrogated by both montelukast and BAY cyslt2. Thus, $CysLT_1$ receptors may mediate endogenously regulated microvessel growth in normal culture; whereas the exogenous agonist LTD_4 induces angiogenesis through the activation of both $CysLT_1$ and $CysLT_2$ receptors. The CysLT receptor antagonists can be developed as angiogenesis inhibitors.

1. Introduction

Cysteinyl leukotrienes (CysLTs, include LTC_4, LTD_4, and LTE_4) are potent pro-inflammatory mediators, which are metabolized from arachidonic acid by 5-lipoxygenase (Brink et al. 2003). CysLTs induce inflammation and immune response as well as vascular permeability and contraction via the activation of $CysLT_1$ and $CysLT_2$ receptors (Rovati et al. 2007). Inflammation is a process that appears in many chronic disorders including tumor, rheumatoid arthritis and diabetes (Majno 1998; Costa et al. 2007), in which angiogenesis is largely involved (Majno 1998; Costa et al. 2007). The angiogenesis can be triggered by inflammatory mediators such as prostaglandins which were found to promote tumor angiogenesis (Shitivelband et al. 2003). Therefore, it is important to elucidate whether CysLTs are inflammatory mediators triggering angiogenesis.

It has been shown that $CysLT_1$ and $CysLT_2$ receptors may play an important role in angiogenesis. $CysLT_1$ and $CysLT_2$ receptors are constitutively or inducibly expressed in vascular endothelia cells and in vascular smooth muscle cells (Gronert et al. 2001; Sjostrom et al. 2003; Zhang et al. 2004; Hu et al. 2005; Uzonyi et al. 2006; Kaetsu et al. 2007; Rovati and Capra 2007). $CysLT_1$ receptor mediates the proliferation and migration of murine vascular smooth muscle cells, and the migration of vascular endothelial cells (Kaetsu et al. 2007; Yuan et al. 2009). The activation of $CysLT_2$ receptor in vascular endothelial cells increase intracellular Ca^{2+} levels and induce the expression of early genes, inflammatory genes and cytokines (Uzonyi et al. 2006; Thompson et al. 2008). Thus, the functional regulations of vascular cells by CysLT receptors imply that CysLTs may promote angiogenesis. Indeed, it has been reported that LTC_4 and LTD_4 induce angiogenesis in the chick chorioallantoic membrane through a receptor-mediated action in an earlier study (Tsopanoglou et al. 1994). However, the role of CysLTs and the relevant receptor subtypes in angiogenesis have not been clarified and identified.

To address these questions, we characterized the effects of CysLT receptor antagonists and the exogenous agonist LTD_4 on angiogenesis. We used a rat thoracic aortic ring assay, which has been proven useful for evaluating the angiogenic process (Auerbach et al. 2003; Reed et al. 2007; Aplin et al. 2008). To mimic the high- or low-perfusion situation during inflammatory disorders, we cultured the aortic ring in the presence or absence of serum.

2. Investigations and results

2.1. Microvessel growth from rat thoracic aortic rings

Microvessels sprouted from the edge of the aortic ring and gradually generated networks of neovessels (Fig. 1A). In the medium containing 0%, 10% or 25% serum, the microvessels showed different growth patterns. In serum-free or 10% serum-containing cultures, microvessels grew slower with a maximum of 11 or 14 sprouts per mm during the 8–10th day of culture (Fig. 1B). In 25% serum-containing culture, the sprouts appeared on the second day of culture, rapidly increased to 34.8 microvessels per mm on the sixth day of culture, and became too dense to be counted afterwards (Fig. 1A and 1B).

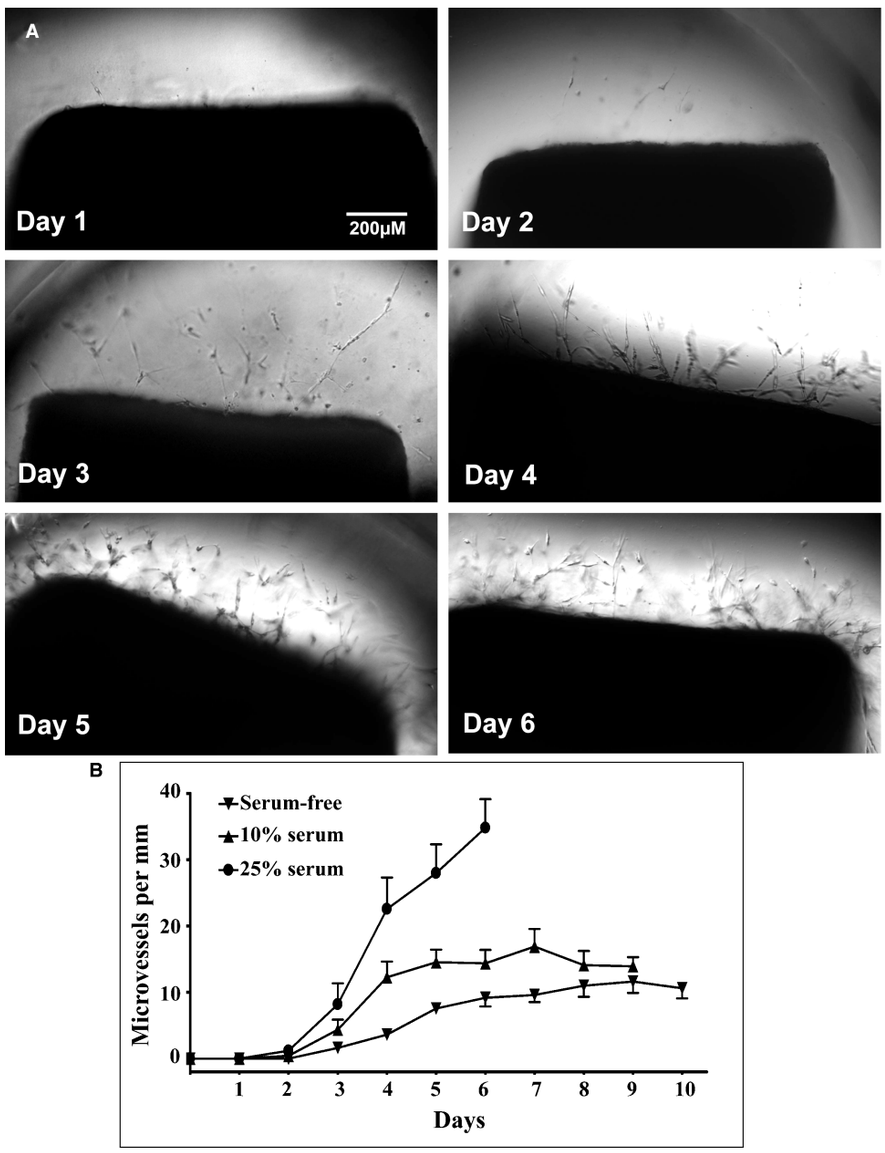

Fig. 1: Microvessel growth from rat thoracic aortic rings. The rat thoracic aortic rings were embedded in matrigel and cultured in medium containing 0%, 10% or 25% fetal bovine serum. The sprout of microvessels from the edge of aortic ring was imaged and counted every day. A. Representative images of microvessel growth in 25% serum-containing culture. B. The microvessel growth in 0%, 10% or 25% serum-containing culture. N = 6-14 rings from six rats

2.2. Role of $CysLT_1$ and $CysLT_2$ receptor in microvessel growth

To determine the role of $CysLT_1$ and $CysLT_2$ receptor in endogenously regulated microvessel growth, we used montelukast, a selective $CysLT_1$ receptor antagonist, and BAY cyslt2, a selective $CysLT_2$ receptor antagonist. In serum-free culture, neither BAY cyslt2 (0.1–1 μM) nor montelukast (0.01–1 μM) affected microvessel growth (Fig. 2A and B). In contrast, in 25% serum-containing culture, montelukast (0.1 and 1 μM), but not BAY cyslt2 (0.1–1 μM), significantly inhibited microvessel growth on the forth day of culture (Fig. 2C and D).

2.3. $CysLT_1$ and $CysLT_2$ receptor mediate LTD_4- induced microvessel growth

We determined the effect of the exogenous agonist LTD_4 (1–100 nM) on microvessel growth and the involved receptor subtypes. To avoid the inhibition from antagonists, we used the concentrations of montelukast (0.01 μM) and BAY cyslt2 (1 μM) that did not affect microvessel growth. In serum-free culture, LTD_4 (100 nM) significantly enhanced the microvessel growth only on the 6th day of culture (Fig. 3A), which was abrogated by both montelukast and BAY cyslt2 (Fig. 3B). In 25% serum-containing culture, LTD_4 (10–100 nM) significantly increased the microvessels growth on the third and forth day of culture (Fig. 3C). Montelukast and BAY cyslt2 abrogated this enhancement on the forth day of culture (Fig. 3D).

3. Discussion

The present study indicated that the $CysLT_1$ receptor mediates the endogenous microvessel growth in the presence of serum. Moreover, exogenous LTD_4 induces angiogenesis that is mediated by both $CysLT_1$ receptor and $CysLT_2$ receptors. These results show a different regulation of microvessel growth by $CysLT_1$ and $CysLT_2$ receptors endogenously and exogenously. For endogenously regulated microvessel growth, we found that the $CysLT_1$ receptor (not the $CysLT_2$ receptor) plays a

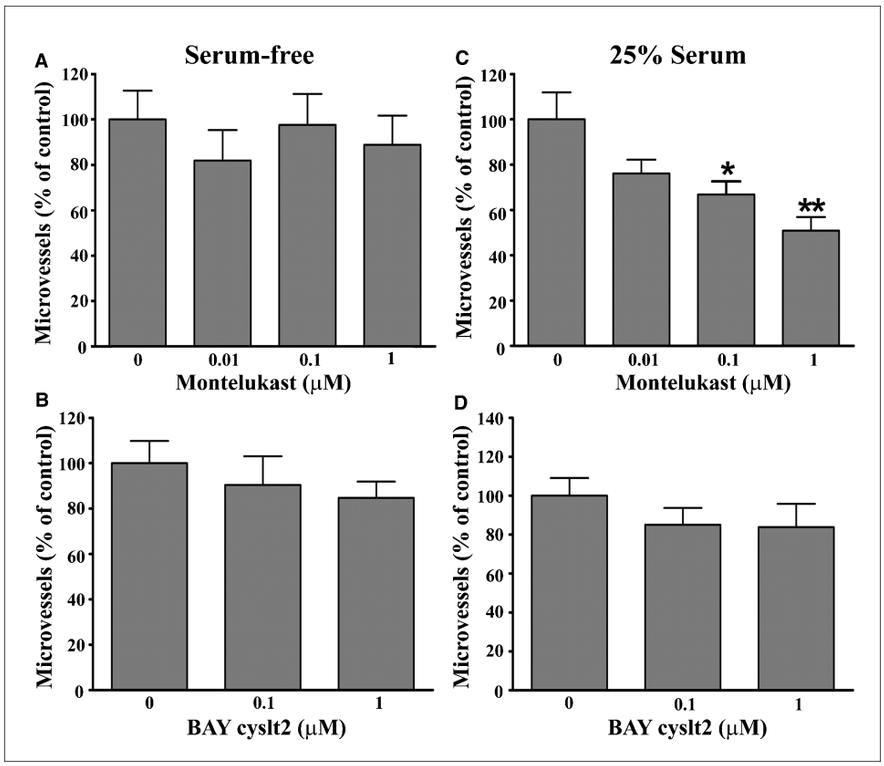

Fig. 2: Role of CysLT receptors in microvessel growth. The aortic rings were cultured in serum-free medium (A and B) or 25% serum-containing medium (C and D). Montelukast (a selective $CysLT_1$ receptor antagonist) at 0.1 and 1 μm inhibited microvessel growth in 25% serum-containing culture, but not in serum-free culture (A and C). BAY cyslt2 (a selective $CysLT_2$ receptor antagonist) at 0.1–1 μm had no effect on microvessel growth (B and D). N = 8–10 rings from 3–4 rats. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* control, one-way ANOVA

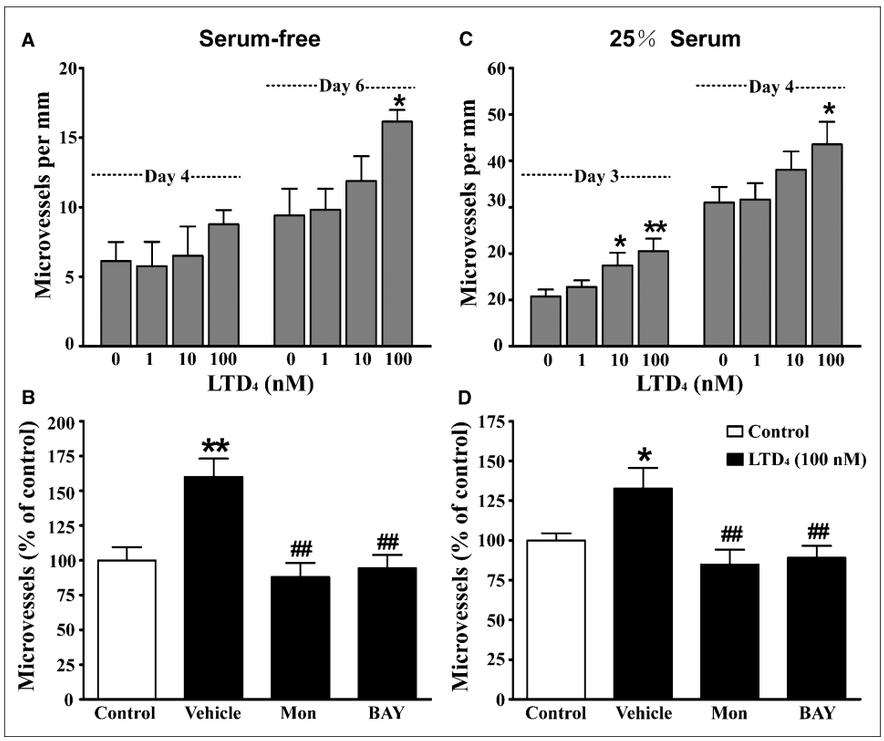

Fig. 3: LTD_4 increases microvessel growth via $CysLT_1$ and $CysLT_2$ receptor. LTD_4 (100 nM) significantly enhanced microvessel growth on the 6th day of serum-free culture (A). This enhancement was abrogated by both montelukast (Mon, 0.01 μM) and BAY cyslt2 (BAY, 1 μM) (B). LTD_4 (10 and 100 nM) significantly enhanced microvessel growth on the 3rd day and LTD_4 (100 nM) significantly enhanced microvessel growth on the 4th day of 25% serum-containing culture. The enhancement was also abrogated by both montelukast (Mon, 0.01 μM) and BAY cyslt2 (BAY, 1 μM) (D). N = 9–16 rings from 3–4 rats. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control, one-way ANOVA; $^{\#\#}P < 0.01$ *vs* vehicle, one-way ANOVA

role in 25% serum-containing cultures but not in serum-free culture. It has been shown that 5-LOX mRNA is not expressed in the endothelial and smooth muscle cells in cultured vessels, but expressed in the vascular wall biopsies (Ost et al. 1998). Thus, endogenous CysLTs are probably generated in the vascular wall during culture. Furthermore, $CysLT_1$ (not $CysLT_2$) receptor interacts with growth factors including VEGF (Auerbach et al. 2003; Kanazawa et al. 2004; Bosse et al. 2008; El-Swefy and Hassanen 2009) that can be generated in the fresh cutting of the aortic ring (Nicosia et al. 1997). LTD_4 activates $CysLT_1$ receptor and results in prime effects on other mediators, such as LPS, IL-4 (Giron-Calle et al. 2002). Therefore, the endogenous CysLTs may activate $CysLT_1$ receptor and mediate microvessel growth by interacting with growth factors or other prime inflammatory mediators. The reason for that the $CysLT_1$ receptor regulated the angiogenesis only in 25% serum-containing culture but not in serum-free culture could be due to the lack of endogenous CysLTs in serum-free culture. We have reported that CysLTs released from primarily cultured neurons with oxygen-glucose-deprivation (OGD) and reperfusion, but there is no CysLTs release during OGD alone (Ge et al. 2006). Five-LOX can be activated by OGD and reperfusion in PC12 cell (Li et al. 2009), but cannot be activated by serum deprivation (unpublished data).

For exogenously regulated microvessel growth, we found that LTD_4 induces angiogenesis both in high-serum and serum-free cultures, which mimic high- and low-perfusion situation during inflammatory disorders, respectively. Both $CysLT_1$ and $CysLT_2$ receptors might mediate LTD_4-induced angiogenesis. The involvement of $CysLT_1$ receptor in angiogenesis is supported by the findings that $CysLT_1$ receptor expresses in vascular endothelial cells (Gronert et al. 2001; Zhang et al. 2004) mediates migration and proliferation of vascular smooth muscle cells as well as endothelial cells (Kaetsu et al. 2007; Yuan et al. 2009), and regulates growth factors production, such as VEGF (Nicosia and Ottinetti 1990; Kanazawa et al. 2004; El-Swefy and Hassanen 2009). On the other hand, $CysLT_2$ receptor is expressed in vascular endothelial cells (Sjostrom et al. 2003; Hu et al. 2005; Rovati and Capra 2007), and its activation results in the intracellular Ca^{2+} accumulation and multiple gene expression (Sjostrom et al. 2003; Uzonyi et al. 2006; Thompson et al. 2008). $CysLT_2$ receptor activation by LTD_4 induces COX-2-dependent prostaglandin formation in HUVEC cells, which is positively correlated with vascular endothelial growth factor (VEGF) production (Lotzer et al. 2007). The reason that $CysLT_2$ receptor is regulated exogenously upon LTD_4-induced angiogenesis but not endogenously angiogenesis is unknown. A possible explanation is that the concentration of endogenously released CysLTs is too low within the area where angiogenesis occurs. However, this need to be further studied.

In summary, our findings indicate that both $CysLT_1$ and $CysLT_2$ receptors play critical roles in angiogenesis. Since CysLTs and CysLT receptors have been found to mediate inflammatory responses of many diseases including ischemic diseases and tumors (Rovati and Capra 2007), our present results imply that they may also be responsible for the angiogenic responses of those diseases. Thus, CysLTs and their receptors can be targeted for the intervention of both inflammation and angiogenesis – CysLT receptor antagonists can be developed not only as anti-inflammatory agents but also as angiogenesis inhibitors.

4. Experimental

4.1. Animals

Male Sprague-Dawley rats (250–300 g) were purchased from the Experimental Animal Center, Zhejiang Academy of Medicine Sciences. Rats were housed under a controlled environment: temperature $22 \pm 1\,^{\circ}C$, 12 hour light/dark cycle, and free access to food and water. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

4.2. Materials

LTD_4 was purchased from Sigma (St. Louis, USA); montelukast was a kind gift from Dr. John Obenchain (Merck Research Laboratories, Whitehouse Station, N.J, USA); pranlukast was a kind gift from Dr. Masami Tsuboshima (Ono Pharmaceutical Co. Ltd., Osaka, Japan); BAY cyslt2 was a kind gift from Dr. T. Jon Seiders (Amira Pharmaceuticals Inc., San Diego, USA); VEGF was purchased from PeproTech Asia (Yavne, Israel).

4.3. Rat aortic ring preparation

The rat thoracic aortic ring was prepared according to what previously reported (Aplin et al. 2008) with slight modification. Briefly, 10 μl matrigel was put into a plastic circle and let the liquid matrigel solidify in 37 °C. Rat thoracic aortas were isolated and rinsed in serum-free MCDB131 medium (GIBCO). Subsequently, the thoracic aortas were cut into 1–2 mm length rings. Each aortic ring was put onto one prepared matrigel and covered with 5 μl additional matrigel. The aortic ring-contained matrigel was then put in 37 °C for 30 min. The aortic rings were finally cultured in 200 μl MCDB131 supplied with 0%, 10% or 25% fetal bovine serum and incubated at 37 °C in a humidified atmosphere (5%CO_2 and air). The culture media was changed every other day.

4.4. Microvessel growth evaluation

The aortic rings were imaged under a microscope (Olympus, IX81). Angiogenesis was evaluated by counting the number of microvessel sprouts from the edge of aortic ring by using ImageTool software (University of Texas Health Science Center, San Antonio, USA) according to the published criteria (Aplin et al. 2008). The number of branches from one microvessel was counted as additional two or more microvessels. Each microvessel loop was counted as two sprouts.

4.5. Drug treatment

In serum-free culture, LTD_4 (1–100 nM), montelukast (0.01–1 μM) and BAY cyslt2 (0.1–1 μM) were applied at the beginning of culture and was replenished every other day. In 25% serum-containing culture, LTD_4, montelukast and BAY cyslt2 were applied one time at the beginning of culture. Montelukast and BAY cyslt2 were applied 30 min before LTD_4.

4.6. Statistical analysis

Values are presented as mean ± S.E.M. Statistical analyses were performed by using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

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References

Aplin AC, Fogel E, Zorzi P, Nicosia RF (2008) The aortic ring model of angiogenesis. Methods Enzymol 443: 119–136.

Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N (2003) Angiogenesis assays: a critical overview. Clin Chem 49: 32–40.

Bosse Y, Thompson C, McMahon S, Dubois CM, Stankova J, Rola-Pleszczynski M (2008) Leukotriene D4-induced, epithelial cell-derived transforming growth factor beta1 in human bronchial smooth muscle cell proliferation. Clin Exp Allergy 38: 113–121.

Brink C, Dahlen SE, Drazen J, Evans JF, Hay DW, Nicosia S, Serhan CN, Shimizu T, Yokomizo T (2003) International Union of Pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors. Pharmacol Rev 55: 195–227.

Costa C, Incio J, Soares R (2007) Angiogenesis and chronic inflammation: cause or consequence? Angiogenesis 10: 149–166.

El-Swefy S, Hassanen SI (2009) Improvement of hepatic fibrosis by leukotriene inhibition in cholestatic rats. Ann Hepatol 8: 41–49.

Ge QF, Wei EQ, Zhang WP, Hu X, Huang XJ, Zhang L, Song Y, Ma ZQ, Chen Z, Luo JH (2006) Activation of 5-lipoxygenase after oxygen-glucose

deprivation is partly mediated via NMDA receptor in rat cortical neurons. J Neurochem 97: 992–1004.

Giron-Calle J, Srivatsa K, Forman HJ (2002) Priming of alveolar macrophage respiratory burst by H(2)O(2) is prevented by phosphatidylcholine-specific phospholipase C inhibitor Tricyclodecan-9-yl-xanthate (D609). J Pharmacol Exp Ther 301: 87–94.

Gronert K, Martinsson-Niskanen T, Ravasi S, Chiang N, Serhan CN (2001) Selectivity of recombinant human leukotriene D(4), leukotriene B(4), and lipoxin A(4) receptors with aspirin-triggered 15-epi-LXA(4) and regulation of vascular and inflammatory responses. Am J Pathol 158: 3–9.

Hu H, Chen G, Zhang JM, Zhang WP, Zhang L, Ge QF, Yao HT, Ding W, Chen Z, Wei EQ (2005) Distribution of cysteinyl leukotriene receptor 2 in human traumatic brain injury and brain tumors. Acta Pharmacol Sin 26: 685–690.

Kaetsu Y, Yamamoto Y, Sugihara S, Matsuura T, Igawa G, Matsubara K, Igawa O, Shigemasa C, Hisatome I (2007) Role of cysteinyl leukotrienes in the proliferation and the migration of murine vascular smooth muscle cells *in vivo* and *in vitro*. Cardiovasc Res 76: 160–166.

Kanazawa H, Yoshikawa T, Hirata K, Yoshikawa J (2004) Effects of pranlukast administration on vascular endothelial growth factor levels in asthmatic patients. Chest 125: 1700–1705.

Li CT, Zhang WP, Lu YB, Fang SH, Yuan YM, Qi LL, Zhang LH, Huang XJ, Zhang L, Chen Z, Wei EQ (2009) Oxygen-glucose deprivation activates 5-lipoxygenase mediated by oxidative stress through the p38 mitogen-activated protein kinase pathway in PC12 cells. J Neurosci Res 87: 991–1001.

Lotzer K, Jahn S, Kramer C, Hildner M, Nusing R, Funk CD, Habenicht AJ (2007) 5-Lipoxygenase/cyclooxygenase-2 cross-talk through cysteinyl leukotriene receptor 2 in endothelial cells. Prostaglandins Other Lipid Mediat 84: 108–115.

Majno G (1998) Chronic inflammation: links with angiogenesis and wound healing. Am J Pathol 153: 1035–1039.

Nicosia RF, Lin YJ, Hazelton D, Qian X (1997) Endogenous regulation of angiogenesis in the rat aorta model. Role of vascular endothelial growth factor. Am J Pathol 151: 1379–1386.

Nicosia RF, Ottinetti A (1990) Growth of microvessels in serum-free matrix culture of rat aorta. A quantitative assay of angiogenesis *in vitro*. Lab Invest 63: 115–122.

Ost M, Uhl E, Carlsson M, Gidlof A, Soderkvist P, Sirsjo A (1998) Expression of mRNA for phospholipase A2, cyclooxygenases, and lipoxygenases in cultured human umbilical vascular endothelial and smooth muscle cells and in biopsies from umbilical arteries and veins. J Vasc Res 35: 150–155.

Reed MJ, Karres N, Eyman D, Vernon RB (2007) Culture of murine aortic explants in 3-dimensional extracellular matrix: a novel, miniaturized assay of angiogenesis *in vitro*. Microvasc Res 73: 248–252.

Rovati GE, Capra V (2007) Cysteinyl-leukotriene receptors and cellular signals. ScientificWorldJournal 7: 1375–1392.

Shtivelband MI, Juneja HS, Lee S, Wu KK (2003) Aspirin and salicylate inhibit colon cancer medium- and VEGF-induced endothelial tube formation: correlation with suppression of cyclooxygenase-2 expression. J Thromb Haemost 1: 2225–2233.

Sjostrom M, Johansson AS, Schroder O, Qiu H, Palmblad J, Haeggstrom JZ (2003) Dominant expression of the CysLT2 receptor accounts for calcium signaling by cysteinyl leukotrienes in human umbilical vein endothelial cells. Arterioscler Thromb Vasc Biol 23: e37–41.

Thompson C, Cloutier A, Bosse Y, Poisson C, Larivee P, McDonald PP, Stankova J, Rola-Pleszczynski M (2008) Signaling by the cysteinyl-leukotriene receptor 2. Involvement in chemokine gene transcription. J Biol Chem 283: 1974–1984.

Tsopanoglou NE, Pipili-Synetos E, Maragoudakis ME (1994) Leukotrienes C4 and D4 promote angiogenesis via a receptor-mediated interaction. Eur J Pharmacol 258: 151–154.

Uzonyi B, Lotzer K, Jahn S, Kramer C, Hildner M, Bretschneider E, Radke D, Beer M, Vollandt R, Evans JF, Funk CD, Habenicht AJ (2006) Cysteinyl leukotriene 2 receptor and protease-activated receptor 1 activate strongly correlated early genes in human endothelial cells. Proc Natl Acad Sci U S A 103: 6326–6331.

Yuan YM, Fang SH, Qian XD, Liu LY, Xu LH, Shi WZ, Zhang LH, Lu YB, Zhang WP, Wei EQ (2009) Leukotriene D4 stimulates the migration but not proliferation of endothelial cells mediated by the cysteinyl leukotriene cyslt(1) receptor via the extracellular signal-regulated kinase pathway. J Pharmacol Sci 109: 285–292.

Zhang WP, Hu H, Zhang L, Ding W, Yao HT, Chen KD, Sheng WW, Chen Z, Wei EQ (2004) Expression of cysteinyl leukotriene receptor 1 in human traumatic brain injury and brain tumors. Neurosci Lett 363: 247–251.