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Shear Stress and Oxidized LDL Regulates Endothelial Cell Tube Formation through VEGF Signaling

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Abstract: Shear stress and oxidized low-density lipoprotein (oxLDL) caused by abnormal blood is critical to angiogenesis for atherosclerosis. However, the mechanism in shear stress or ox-LDL regulated angiogenesis is still not well understood. There is the hypothesis that shear stress or oxLDL regulates angiogenesis through the vascular endothelial growth factor (VEGF) signaling pathway. It is discovered that both high shear stress and low concentration of oxLDL contribute to angiogenesis, which is inhibited once the VEGF or VEGFR expression is knocked down. The expression of p-FAK and p-paxillin is regulated by the VEGF/VEGFR signal axis. VEGFR2, p-FAK, p-paxillin and VEGFR1 are VEGF-responsive proteins, and they are also upregulated by high shear stress and low concentration of oxLDL. If the VEGF or VEGFR2 is knocked down, phosphorylation of FAK and paxillin induced by high shear stress and low concentration of oxLDL are also significantly inhibited. In summary, present studies have demonstrated that high shear stress and low concentration of oxLDL induces angiogenesis through the VEGFR2/FAK/paxillin signaling pathway.

Keywords: Shear stress, Oxidized low-density lipoprotein (Ox-LDL), VEGF, VEGFR2, angiogenesis.

1 Introduction

Shear stress and lipoprotein is the critical regulating factor of angiogenesis and atherosclerosis [Wang, Qiu and Luo (2016); Fong (2015)]. High shear stress is an important protector for endothelial cells (ECs) through inducing the ECs proliferation and migration [Brown, Teng, Evans et al. (2016); Fey, Schubert, Schneide et al. (2016)]. Oxidized low-density lipoprotein (oxLDL) leads to endothelial dysfunction, induction of ECs apoptosis and adhesion, as well as inhibition of migration and angiogenesis [Børsum,

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Henriksen, Reisvaag et al. (1985); Valente, Irimpen, Siebenlist et al. (2014)]. Recent researches have proved that low concentration of oxLDL lead to low level of ROS and initiates angiogenesis [Dandapat, Hu, Sun et al. (2007)]. Previous findings showed that low concentration of oxLDL induces ECs migration and angiogenesis through the inhibitor of differentiation 1 (Id1) [Qiu, Wang, Zheng et al. (2011); Qiu, Peng, Zheng et al. (2012)]. It is well known that angiogenesis is an important element of atherosclerosis [Guo, Harari, Virmani et al. (2017)]. Therefore, further investigations of the precise mechanism of shear stress and oxLDL in angiogenesis regulation play an important part for treating cancer or atherosclerosis.

Vascular endothelial growth factor (VEGF) is the main signal for ECs proliferation and migration. VEGF also can produce angiogenesis and vascular permeability. In tumor research, VEGF signaling is transduced by VEGF receptors VEGFR1, VEGFR2 and VEGF3 [Heinolainen, Karaman, D'Amico et al. (2017)]. In fact, oxLDL impairs angiogenesis through decreasing VEGFR2 expression in HUVECs [Zhang and Jiang (2016)]. Shear stress also contributes to the expression of VEGFR2 and is associated with angiogenesis.

FAK/paxillin is an important signaling pathway for ECs migration [Shen, Ma, Gao et al. (2013); Chen, Tang, Huang et al. (2011)]. VEGF binding with VEGFR might directly activate focal adhesion kinase FAK or active FAK through VEGFR2/FAK/ axis, which is significant for the angiogenesis [Saraswati and Agrawal (2013); Zhao, Wang, Liu et al. (2017); He, Zhang, Miao et al. (2015)]. Activated p-FAK further initiates the downstream protein paxillin to regulate the ECs adhesion and migration [Yang, Li, Zhong et al. (2017)]. In the end, the VEGFR2/FAK/paxillin axis results in the ECs angiogenesis. This study is carried out with focus on the regulatory effects of shear stress and oxLDL on ECs angiogenesis and the underlying mechanism. High shear stress and low concentration of oxLDL increases the ECs angiogenesis through VEGF-VEGFR signaling axis. The underlying mechanism may induce cell migration and angiogenesis by FAK and paxillin phosphorylation.

2 Materials and Methods

2.1 Cell lines and oxLDL

HUVECs-human umbilical vein endothelial cells are obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China). All of them grow in RPMI 1640 media containing 10% fetal bovine serum. OxLDL is gained from the Technology Co., Ltd. (Guangzhou, China).

2.2 Construction of shear stress loading system

The shear stress loading system-Parallel-Plate Flow Chamber is composed of silicone tube, roller pump, parallel-plate flow chamber, collection reservoir, and pouring reservoir. Collection reservoir and pouring reservoir are received from Glass Instrument Factory (Beibei, Chongqing, China). Roller pump (BT100-300, 2515 pump head) and silicone tube are from Lange Pump Co (Baoding, China). The parallel-plate flow chamber is

achieved from Shengde Plastic Co., Ltd. (Shanghai, China).

2.3 Cell migration assay

The migration ability of cells is measured by wound healing assay. The HUVECs are seeded at 2×10^5 cells/well in 12-well plates. After 24 h, the cells have completely covered the plate, forming a monolayer of ECs. After the cells have been fully integrated, they are scratched vertically with 10-microliter pipette tip. Cells are washed 3 times with PBS (0.01 M, pH=7.4) and blew gently with a dropper to remove the scraped cells completely, then added with the serum-free medium. The HUVECs are incubated at 37°C, with 5% CO_2 and 95% air humidity, then sampled and photographed at 1st, 6th, 12th, and 24th h, respectively.

2.4 Tube formation assay

The ability of cells tube formation was once tested with Matrigel (BD Biosciences, Franklin Lakes, NJ), with Matrigel being thawed in at 4°C overnight. To follow that, the dropwise Matrigel at 200 μL well in 24-well plate was solidified at 37°C for 30 mins in a humid environment. The HUVECs were seeded at 10^4 cells/well in 24-well plates. The plates containing Matrigel and HUVECs were incubated at 37°C with 5% CO_2 and 95% air humidity, and then sampled and photographed at the 6th, 12th, and 24th h, respectively. The tube formation ability was verified by analysis of the number of lumens.

2.5 Western blotting

Cells are lysed in 0.4 ml of lysis buffer. Lysates are separated by electrophoresis, blotted to a membrane and reacted with specific antibodies. The primary antibodies and appropriate secondary antibodies are from Abcam and SANTA CRUZ: Anti-VEGF 164 antibody (ab53465), Anti-VEGF Receptor 1 antibody (ab2350), Anti-VEGF Receptor 2 antibody (ab2349), Anti-FAK antibody [EP695Y] (ab40794), Anti-FAK (phospho Y397) antibody [EP2160Y] (ab81298), Anti-Paxillin antibody [Y113] (ab32084), Anti-Paxillin (phospho Y118) antibody (ab4833), Goat Anti-Rabbit IgG H&L (HRP) (ab205718) and GAPDH antibody (FL-335) (sc-25778).

2.6 Transfection

The siRNA was synthesized by Shanghai GenePharma Ltd. VEGF siRNA applied the following primers: 5’-GAUCAAACCUCACCAAGGCUU-3’ (forward primer), 5’-GCCUUGGUGAGGUUUGAUCUU -3’ (reverse primer). VEGFR2 siRNA applied the following primers: 5’-CGGAGAAGAAUGUGGUUAAdTdT-3’ (forward primer), 3’-dTdTGCCUCUUCUUACACCAAUU-5’ (reverse primer). VEGFR1 siRNA applied the following primers: 5’-CAGGAUGGUAAAGACUACA-3’ (forward primer), 5’-GTCCTACCATTTCTGATGT-3’ (reverse primer). The synthesis of VEGF recombinant protein was generated by Sigma.

2.7 Statistical analysis

There has been Data indicated as the mean ±SD. Statistical comparisons have been done

with an unpaired or paired student's *t*-test or ANOVA. $P<0.05$ is deemed of significance.

3 Results

3.1 Low concentration of oxLDL stimulates HUVEC tube formation

To explore the potential effects of oxLDL on HUVEC angiogenesis, different concentrations (0 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml) of oxLDL have been selected to stimulate HUVECs for the purpose of observing the tube formation. As shown in Figure 1, after being treated with 20 μg/ml oxLDL for 24 h, HUVECs could form more tubes than the groups treated with other concentrations (0 μg/ml, 5 μg/ml, 10 μg/ml, 40 μg/ml, 80 μg/ml). The 20 μg/ml oxLDL boosts the reach to the maximum number of HUVECs to form vessel tubes, while other concentrations (0 μg/ml, 5 μg/ml, 10 μg/ml, 40 μg/ml, 80 μg/ml) of oxLDL are with significantly smaller number. As the concentration of oxLDL (0-20 μg/ml) increases, the number of lumens greatly raises. The results indicate that low concentrations (20 μg/ml) can potentially improve the tube formation of HUVECs, whereas high concentrations (more than 20 μg/ml) of oxLDL suppress the tube formation.

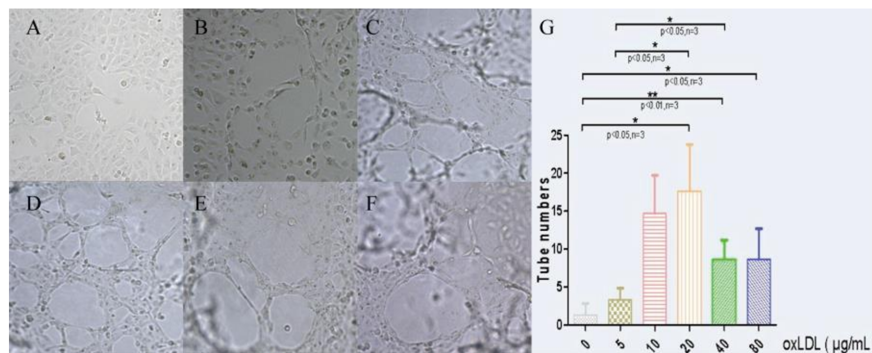

Figure 1: Low concentration of oxLDL induces tube formation of endothelial cells A. 0 μg/ml oxLDL, B. 5 μg/ml oxLDL C. 10 μg/ml oxLDL D. 20 μg/ml oxLDL E. 40 μg/ml oxLDL F. 80 μg/ml oxLDL. G. the statistic data of the tube number in A-F

3.2 Higher shear stress stimulates HUVECs tube formation

To explore the potential impacts of shear stress on HUVEC angiogenesis, a tube formation assay was performed. In this research, different shear stress (0 dyn/cm^2, 5 dyn/cm^2, 15 dyn/cm^2, 25 dyn/cm^2) were selected to stimulate HUVECs for observation on the tube formation. As shown in Figure 2, HUVECs treated with 25 dyn/cm^2 shear stress for 72 h could induce more tube formation than those under other shear stress stimulation (0 dyn/cm^2, 5 dyn/cm^2, 15 dyn/cm^2), implying that higher shear stress potentially improved the tube formation of HUVECs.

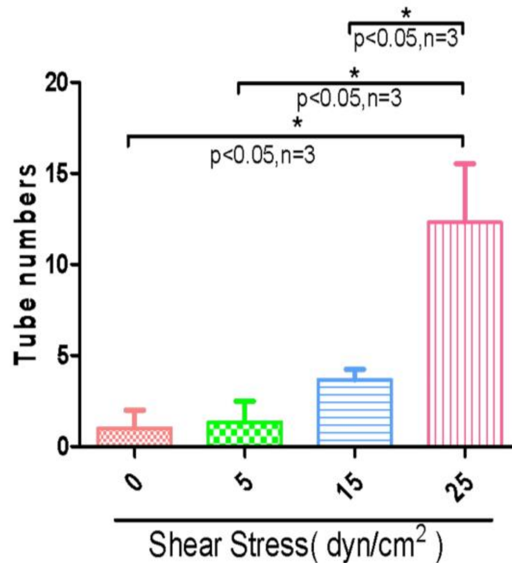

Figure 2: High shear stress induces tube formation of endothelial cells. The statistic data of the tube number in each group

3.3 OxLDL enhances VEGF, VEGFR1 and VEGFR2 expression in HUVEC

VEGF, VEGFR1 and VEGFR2 are the key regulatory proteins in angiogenesis. Therefore, there has been the investigation on the relationship among VEGF, VEGFR1, VEGFR2 and oxLDL in HUVEC tube formation and migration. Western blot results showed that lower concentrations (0-20 µg/ml) of oxLDL stimulates the VEGF, VEGFR1 and VEGFR2 expression in HUVECs, and the expression is significantly enhanced along with the concentration increase ($p<0.05$, Figure 3). In contrast, a higher concentration (20-80 µg/ml) of oxLDL decreases VEGF, VEGFR1 and VEGFR2 expression. Additionally, it is found that VEGF, VEGFR1, and VEGFR2 expression of HUVECs is treated with 20 µg/ml oxLDL at different time points (0 h, 12 h, 24 h, 48 h, and 72 h). As shown in Figure 4, 20 µg/ml oxLDL could increase VEGF, VEGFR1 and VEGFR2 expression at 48th hour. Relatively low concentration (20 µg/ml) of oxLDL could enhance VEGF, VEGFR1 and VEGFR2 expression in HUVECs at the 48th h.

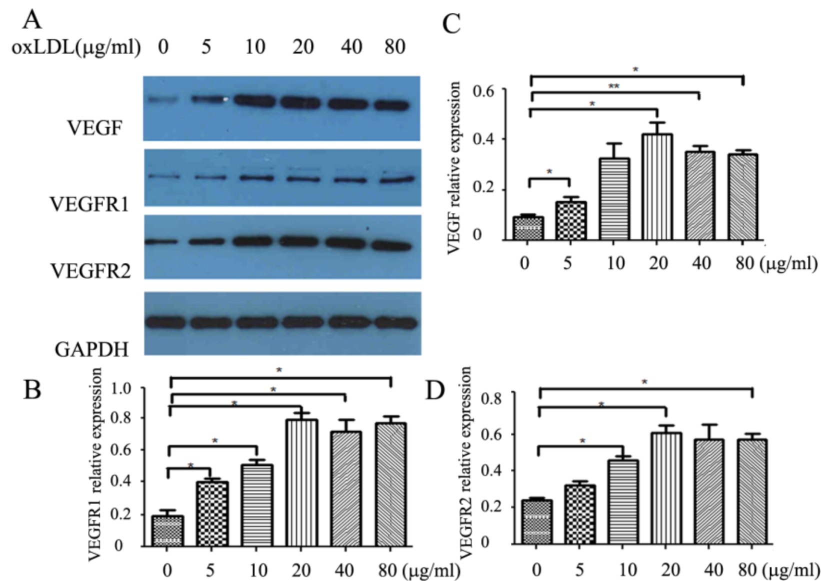

Figure 3: Different concentration of oxLDL regulates VEGF, VEGFR1, and VEGFR2 protein expression in EC. A. Western blotting detects expression of the VEGF, VEGFR1, and VEGFR2 protein in EC treated with different concentration of oxLDL (0 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml). B-D. The statistic of the expression of VEGF, VEGFR1, and VEGFR2 protein in EC

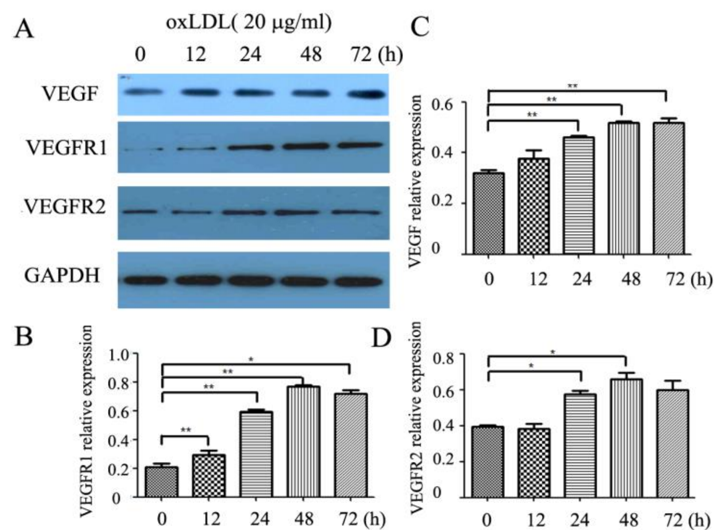

Figure 4: Expression of VEGF, VEGFR1, and VEGFR2 protein at different time point induced by 20 μg/ml oxLDL. A. Western blotting detects expression of the VEGF, VEGFR1, and VEGFR2 protein in EC treated at different times（0 h、12 h、24 h、48 h、

72 h）induced by 20 μg/ml oxLDL. B-D. The statistic of the expression of VEGF, VEGFR1, VEGFR2 protein expression in EC

3.4 Shear stress enhances VEGF, VEGFR1 and VEGFR2 expression in HUVECs

To further analyze the relationship of VEGF, VEGFR1, VEGFR2 and shear stress in HUVEC tube formation, VEGF, VEGFR1 and VEGFR2 production in shear stress-treated HUVECs were measured by Western blot. The results (Figure 5) showed that lower shear stress (5 dyn/cm^2) suppresses the VEGF, VEGFR1 and VEGFR2 expression in HUVECs. In contrast, higher shear stress (25 dyn/cm^2) comparatively increases the expression in HUVEC than those in other level (0 dyn/cm^2, 5 dyn/cm^2, 15 dyn/cm^2). Additionally, VEGF, VEGFR1, and VEGFR2 expression at the different time points (0 h, 12 h, 24 h, 48 h and 72 h) in HUVECs are detected after being treated with 25 dyn/cm^2. As shown in Figure 6, 25 dyn/cm^2 shear stress at the 72nd h is an optimal setting to improve VEGF, VEGFR2 and VEGFR1 expression (p<0.05).

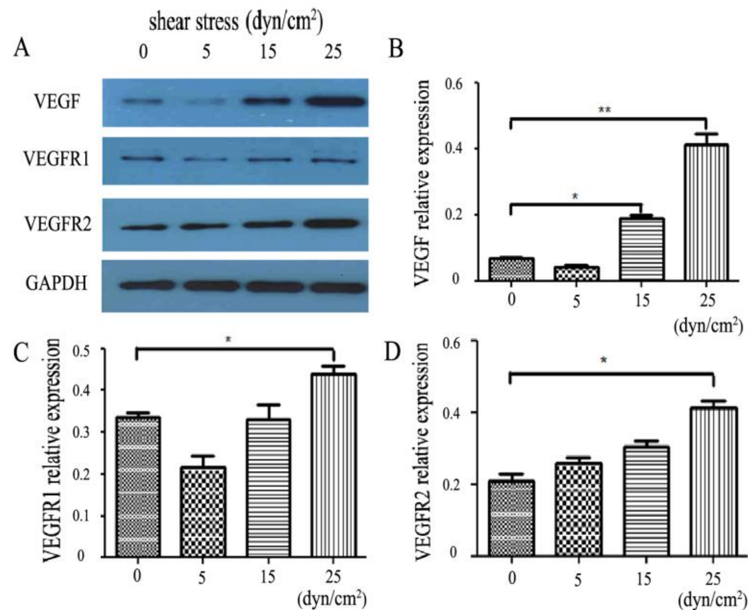

Figure 5: Different shear stress regulates VEGF, VEGFR1, VEGFR2 protein expression in EC. A. Western blotting detects expression of the VEGF, VEGFR1, VEGFR2 protein in EC. B-D. The statistic of the expression of VEGF, VEGFR1, VEGFR2 protein expression in EC

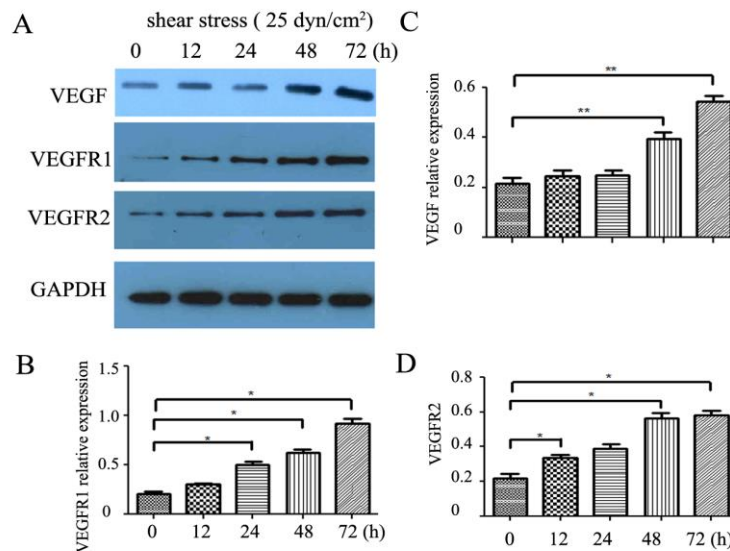

Figure 6: Expression of VEGF, VEGFR1, VEGFR2 protein at different time point induced by shear stress (25 dyn/cm^2). A. Western blotting detects expression of the VEGF, VEGFR1, VEGFR2 protein in EC treated at different times (0 h, 12 h, 24 h, 48 h, 72 h) induced by shear stress (25 dyn/cm^2). B-D. The statistic of the expression of VEGF, VEGFR1, VEGFR2 protein expression in EC

3.5 Low concentration of oxLDL and high shear stress regulates HUVEC tube formation through VEGF signaling

To investigate whether the potential effects of oxLDL and shear stress on HUVEC tube formation are mediated by VEGF, HUVECs were treated with VEGF recombinant protein (10 ug/L) to observe the tube formation (Figure 7). Firstly, the results indicate that HUVECs treated with 10ug/L VEGF could form more tubes than those without VEGF. Secondly, VEGF was knocked down by siRNA in HUVECs, then stimulated with 20 μg/ml oxLDL or 25 dyn/cm^2 shear stress. As shown in Figure 7, the transfection of VEGF siRNA into HUVECs could sharply decrease the tube formation by 20 μg/ml oxLDL and 25 dyn/cm^2 shear stress. Therefore, low concentration of oxLDL or high shear stress could promote the capacity of tube formation in HUVECs by modulating VEGF expression.

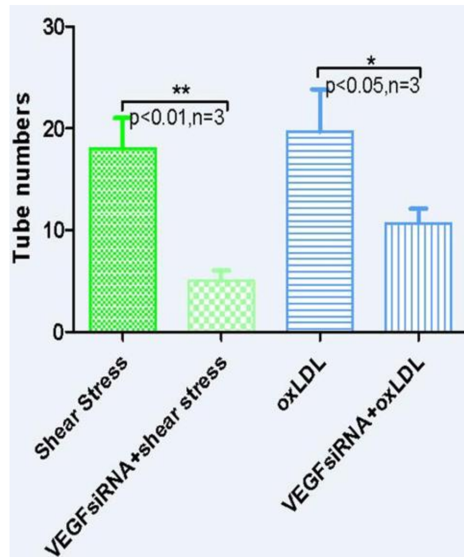

Figure 7: Shear stress or oxLDL induces tube formation in endothelial cells through VEGF. The statistic data of the tube number in each group

3.6 VEGF-VEGFR signaling participates in oxLDL or shear stress-mediated HUVEC tube formation

Previous studies have shown that VEGF could be involved in the tube formation of oxLDL and shear stress-treated HUVECs. In addition, VEGFR1 and VEGFR2 are the critical receptors of VEGF to promote angiogenesis, leading to the attempt to investigate whether VEGF-VEGFR and its related pathways regulate tube formation in oxLDL and shear stress-treated HUVECs. Our previous studies have demonstrated that low concentration of oxLDL and high shear stress can enhance VEGFR1 and VEGFR2 expression in HUVECs. VEGFR1 siRNA and VEGFR2 siRNA are transfected into HUVECs, and then the cells are stimulated with VEGF recombinant protein (10 ug/L) for observing the tube formation. As shown in Figure 8, the knockdown of VEGFR2 or VEGFR1 in HUVECs significantly decreases the VEGF-induced tube formation.

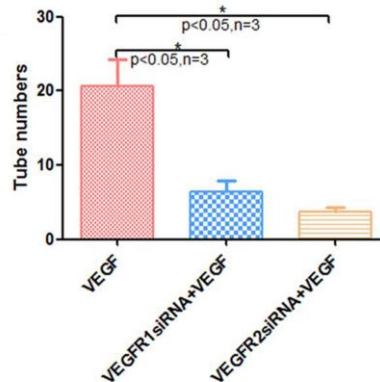

Figure 8: VEGF induces tube formation of endothelial cells through VEGFR1, VEGFR2. Quantitative analysis of the number of lumens in each group

3.7 oxLDL or shear stress regulate FAK/paxillin through the VEGF-VEGFR2 pathway

VEGFR2-FAK/paxillin was originally identified in angiogenesis. This study aims at determining how oxLDL and shear stress could assist FAK and paxillin activation (phosphorylation) by the VEGF-VEGFR2 pathway. After the cultured HUVECs being incubated for 2 h with VEGF recombinant protein (10 ng/ml), VEGFR2, VEGFR1 FAK and paxillin phosphorylation are significantly increased (Figure 9). Furthermore, 48 h after VEGFR2 siRNA transfection, FAK and paxillin phosphorylation sharply decrease (Figure 9). However, VEGFR1 siRNA transfection could not affect the FAK and paxillin phosphorylation of HUVECs. From the above-mentioned results, it is summarized that VEGF could enhance FAK and paxillin phosphorylation through VEGFR2 mediation. Finally, VEGF siRNA is transfected into HUVECs, then stimulated with 20 μg/ml oxLDL and 25 dyn/cm^2 shear stress to monitor the expression of VEGFR2, VEGFR1, FAK and paxillin phosphorylation. The results (Figure 10) showed that transfection of VEGF siRNA into HUVECs could block the VEGFR2, VEGFR1, FAK and paxillin phosphorylation stimulated by oxLDL or shear stress. Thus, oxLDL or shear stress promoted VEGFR2, VEGFR1, FAK and paxillin phosphorylation by VEGF. These results suggest that oxLDL or shear stress enhances FAK and paxillin phosphorylation of HUVECs by modulating VEGF-VEGFR2, and may promote HUVEC migration and tube formation by the VEGF-VEGFR2-FAK/paxillin pathway.

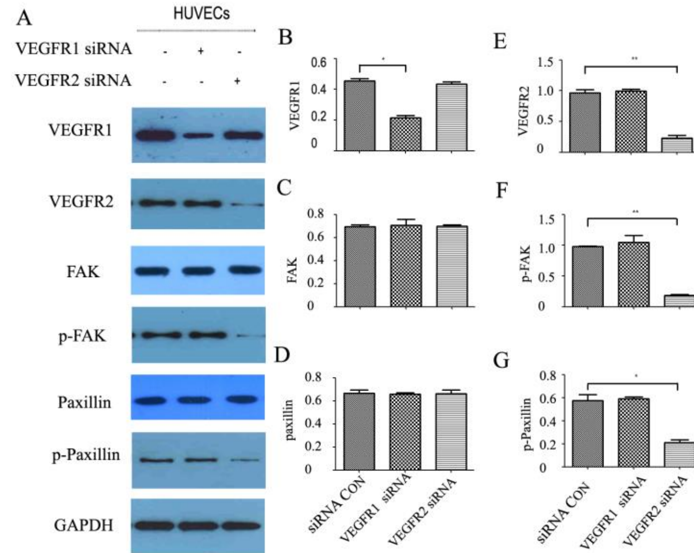

Figure 9: Adhesion protein is regulated by VEGF. A. Expression of VEGFR1, VEGFR2, FAK, p-FAK, Paxillin, p-paxillin protein detected by western blot. B-G. Quantitative analysis of VEGFR1, VEGFR2, FAK, p-FAK, Paxillin, p-paxillin protein expression in each treatment group

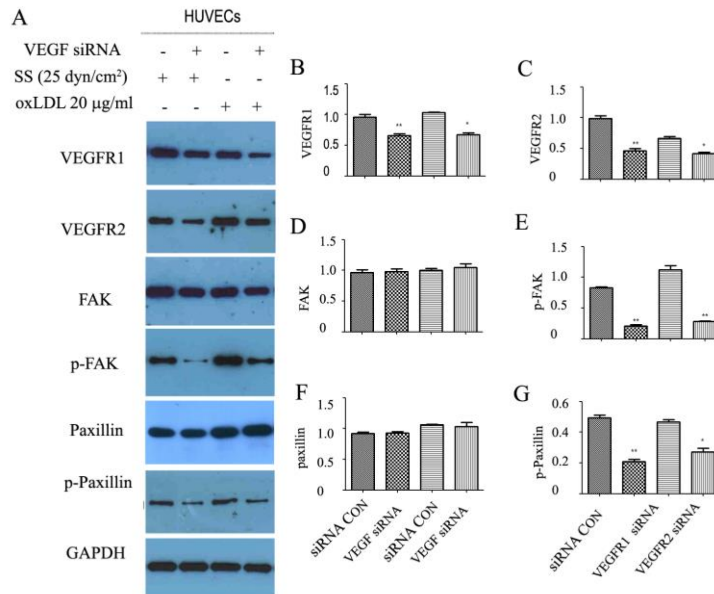

Figure 10: Shear stress or oxLDL regulated FAK and Paxillin expression are VEGFR dependent. A. Expression of VEGFR2, VEGFR1, FAK, p-FAK, Paxillin, p-paxillin protein in VEGF knockdown cells was detected under shear stress or oxLDL by western blot. B-G. Quantitative analysis of VEGFR1 expression in in each treatment group

4 Discussion

The aim of this study is to define the roles of oxLDL and shear stress in endothelial cell angiogenesis and the related mechanism. Additionally, this study has generated the following findings: high shear stress or low concentration of oxLDL induces angiogenesis; the VEGFR2/FAK/paxillin signaling pathway that participates in high shear stress or low concentration of oxLDL induces angiogenesis.

Angiogenesis takes a part in atherosclerosis and cancer development to provide oxygen and inflammation. Under the physiological condition, ECs stay in the shear stress-regulated environment. Recent studies prove that shear stress does not only control tumor cells and ECs [Chang, Chang, Lee et al. (2008)], but also the ECs in the tumor microenvironment. Meanwhile, the relationship between shear stress and angiogenesis has been explored by detecting the different roles of shear stress on the formation of lumen. It is also indicated that high shear stress induces ECs migration and angiogenesis. In the study of atherosclerosis, oxLDL has been commonly used as high-risk factor of plaque formation; it regulates endothelial cell injury, permeability, survival, apoptosis, proliferation and migration [Inoue, Itoh, Tanaka et al. (2001); Shiraki, Aoyama, Yokoyama et al. (2014)]. Previous studies have indicated that low concentrations of oxLDL (≤20 μg/mL) can accelerate vascular endothelial cell migration and proliferation, whereas high concentrations of oxLDL (>20 μg/mL) inhibit endothelial cell proliferation and migration [Dandapat, Hu, Sun et al. (2007); Qiu, Wang, Zheng et al. (2011); Qiu, Peng, Zheng et al. (2012); Khaidakov, Mitra, Wang et al. (2012)]. In this paper, different concentrations of oxLDL contributed to the finding that 20 μg/mL oxLDL can induce angiogenesis, which echoes with previous studies.

Vascular endothelial growth factor (VEGF) and its downstream signaling proteins play an essential role in endothelial cell function, especially in the angiogenesis. Many studies have found that VEGF binds to VEGFR2 receptor on the surface of cell membrane to induce phosphorylation of downstream FAK and paxillin, which affects the process of angiogenesis [Luedde (2010); Luan, Gao, Guan et al. (2014)]. In this study, the effect of oxLDL and high-shear stress on the expression of VEGF and its downstream pathway proteins are inspected, which reveals the role of VEGFR2/FAK/paxillin signaling pathway in oxLDL and angiogenesis induced by high shear stress.

The study in this paper implies the potential role of shear stress and oxLDL in atherosclerosis and atherosclerotic rupture-prone plaque development. Rupture-prone plaque development is localized in the high shear stress region, and angiogenesis increase is also observed in the high shear stress region [Gijsen, van der Giessen, van der Steen et al. (2013); Dolan, Kolega and Meng (2013); Wentzel, Chatzizisis, Gijsen et al. (2012); Pedrigi, Silva, Bovens et al. (2014)]. Therefore, this study results can explain why rupture-prone plaques are developed in the higher shear stress, and indicate that shear stress and oxLDL induces angiogenesis. The new bone angiogenesis paves the road for lipid and inflammation cells into atherosclerotic plaque [Sluimer, Kolodgie, Bijnens et al. (2009)].

5 Conclusion

In summary, the present studies have demonstrated that high-shear stress and low concentration of oxLDL induces angiogenesis through the VEGFR2/FAK/paxillin signaling pathway. It is well accepted that abnormal shear stress and oxLDL in the vascular vessel fundamentally determines vessel remodeling and atherosclerosis [Mehta and Li (2002)]. In addition, the current studies provide a cellular mechanism to understand how high shear stress and low concentration of oxLDL induces angiogenesis. The data on ECs angiogenesis in this paper suggest that mechanical microenvironment and oxLDL microenvironment of ECs may significantly contribute to atherosclerosis development.

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Conflict of interest: The authors hereby declare no competing financial interests.

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