The Degradation of G3BP2 by Trim-away Method Inhibits Oscillatory Shear Stress induced Endothelial Cell Inflammation

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Abstract. Progress in medical science has shown that cardiovascular pathogenesis is a complex disease with multiple underlying factors. Stress factors such as oxidative stress and oscillatory shear stress (OSS) play a crucial role in the occurrence and development of atherosclerosis as the major cause of cardiovascular diseases (CVD). However, study related the effect of stress on the development of human coronary atherosclerosis is not yet fully understood. Ras-GTPase-activating protein SH3 domain-binding proteins 2 (G3BP2) are multifunctional RNA binding proteins in which biomechanically sensitive gene that known important in cancer progression. Here in this study, we further explore the roles of G3BP2 protein especially their relation with atherosclerosis diseases, by investigating the G3BP2 regulation under biomechanical stress stimuli follows with the strategy of exploring the effect of G3BP2 disruption in endothelial cells by the application of new protein depletion technology called Trim-away. The result showed that oxidative stress and oscillatory shear stress (OSS) stimuli activated the expression of G3BP2 protein in primary endothelial cells. Moreover, the western blot analysis revealed that degradation of G3BP2 protein using Trim-away in endothelial cells induced OSS inhibits the release of the pro-inflammatory cytokines (VCAM-1 and ICAM-1). The compelling evidence demonstrated that suppression of G3BP2 protein expression in HUVECs potentially inhibits the development of atherosclerosis by decreased endothelial inflammation response.

Keywords: G3BP2, Atherosclerosis, oxidative stress, oscillatory shear stress (OSS), Trim-away technology

1. Introduction
Cardiovascular diseases (CVD) are recognized as one of the most global health problems. The main cause of CVD is atherosclerosis, which characterized by excessive recruitment of white blood cells and accumulation of lipids in the walls of blood vessels, which hinder the process of blood flow to its target tissues [1]. Certain factors of health conditions such as hypertension, high cholesterol, and diabetes are some risk factors of atherosclerosis development [2].
In addition, oxidative stress also crucial during the formation of atherosclerosis, that highly related to vascular inflammation [3]. Nevertheless, some local hemodynamic shear stress factors also play an important role in maintaining the homeostasis of endothelial cells by regulating the local fragility of endothelial cells in response to specific types of shear stress. Generally, atherosclerotic-prone sites developed in oscillatory shear stress (OSS) regions, while more atheroprotective in high and laminar shear stress regions. Oscillatory shear stress drives atherosclerosis by inducing vascular inflammation and excessive endothelial cell proliferation [4, 5]. Meanwhile, laminar shear stress provides the protective effect through induced the releasing of nitric oxide (NO) [6].

Interestingly, when mammalian cells are subjected to various types of stress from the environment, the cells have to adapt. One of their strategies is forming cytoplasm protein and RNA aggregates called stress granules (SGs) [7]. SGs contain various RNA binding proteins, several types of translation initiation factors, and many non-RNA binding proteins [8]. Among them, G3BP2 protein plays a key role in the formation of SGs, which indicates that inhibition of G3BP2 reduces the assembly of SGs that affect breast cancer progression [9]. Given that, since the chronic nature of many vascular degenerative diseases has some similarities with cancer, atherosclerosis in particular, we hypothesize that identifying the link between atherosclerosis with SGs formation and regulation of SGs components such as G3BP2 is an interesting area to study.

2. Materials and Method

2.1. Cell culture and oscillatory shear stress application on endothelial

Primary HUVECs (ScienCell, Cat#8000) cultured in ECM (ScienCell, Cat#1001) with 10% FBS (ScienCell, Cat#0025), 1% penicillin/streptomycin (ScienCell, Cat#0503) at incubator 37°C with 5% CO₂. Before subculture the culture flask was coated with fibronectin (ScienCell, Cat#8248) (40 μg/ml) for 12 h.

2.2. Cell culture and oscillatory shear stress application on endothelial

Cells were seed on the fibronectin coated six well plates, incubated for 12h then the OSS was applied using an orbital shaker. The rotational frequency of the orbital shaker machine was set at 150 rpm in which represents ~4 dyne/cm². Samples were collected at 0, 1, 3, and 6 h after exposed to shear stress.

2.3. Western Blotting

The protein was extracted from HUVECs using cold lysis buffer (Beyotime Biotechnology, P0013) and the protein concentration was identified using BCA Protein assay kit (Beyotime Biotechnology, P0010). Approximately 40 μg of total protein was loaded by SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to a PVDF membrane and blocked with 5% BSA for 1 hour then incubates with a specific primary antibody. The primary antibodies used include anti-GAPDH (Cell signalling technology, #5174), anti-ICAM (10020-1-AP), VCAM (66294-1-lg), and anti-G3bp2 (ab86135). After being washed in TBS for 5 times, membranes were incubated with horseradish-peroxidase conjugated secondary antibodies and detected by using the BeyoECL Plus Kit (Beyotime Biotechnology, P0018S).

2.4. Immunofluorescence

After treatment, the cells were fixed with 4% PFA for 10 minutes, and then blocked with 5% BSA (blocking) diluted with PBS + 0.1% Triton-X100. After blocking, incubate the cells with the first G3BP2 (Ab86135) antibody at a ratio of 1:50, and place in a refrigerator at 4°C overnight. Then incubated with second antibody Donkey anti-rabbit IgG H&L (Alexa Fluor® 555) with ratio1: 500. The immunofluorescence visualization was processed using confocal microscope (Leica-German).

2.5. Statistical Analysis
All results are reported as mean SEM of at least 3 independent experiments. Data were analyzed using unpaired, two-tailed Student’s t-test considered significant if p<0.05 (*p<0.05; **p<0.01; ***p<0.005).

3. Results and Discussion

3.1. Oscillatory Shear Stress (OSS) Stimuli Regulated G3BP2 Protein Expression on Endothelial Cells

G3BP2 protein has familiar with various diseases related to stress, especially cancer diseases. Several studies have been reported that G3BP2 protein is highly overexpressed in cancer cells, like breast cancer, and prostate cancer [10]. Excessive cell proliferation during cancer progression promotes cells hypoxia condition in which leads to mitochondrial dysfunction and created high levels of oxidative stress in cancer. On the other hand, there is also evidence shows that exert oxidative stress to the arterial wall accelerate atherosclerosis. In addition, several studies have shown that hemodynamic stress is an important factor of endothelial phenotype that is involved in endothelial cell proliferation and inflammation activation [11,12].

In this experiment G3BP2 protein level and stress granule (SG) formation was investigated at three conditions. HUVECs without any treatment as a control, compared to HUVECs treated with ATO 10 μM for induced cells oxidative stress and 6h oscillatory shear stress (OSS) treatment. Western blot analysis is shown in (Figure 1A) that OSS and ATO stimuli have been shown regulated G3BP2 protein expression on endothelial cells. In addition, immunofluorescence experiment was undertaken to confirm G3BP2 as the major component of SGs. Upon cell stress exposure (Figure 2B) the formation of stress granules was observed. Thus, support the idea that G3BP2 expression increased under stresses both oxidative and OSS, which is one of the atherosclerosis pathological inducing factors.
Figure 1. G3BP2 protein level in HUVEC after treated with oscillatory and oxidative stress stimuli. (A) After HUVEC cells were treated with ATO 10 μM and OSS 6h, G3BP2 protein expression level was detected with western blots analysis. Whereas, (B) the stress granules (SGs) formation was visualized with immunofluorescent staining represent by G3BP2 (red) and DAPI (blue). Data presents the average of three independent studies.

3.2. Trim-away Technology for G3BP2 Depletion in HUVECs

In order to study G3BP2 function, the Trim-away application was applied to deplete G3BP2 in primary human umbilical vein endothelial cells (HUVECs). Trim-away was applied by incubated the HUVECs overexpressed Trim-21 with recombinant antibody G3BP2-TAT (100µg/mL) for 6 hours at incubator 37 °C, with 5% CO2. TAT is cell penetrating peptide that has been effectively used to offer a wide variety of cargoes into cells from small particles to proteins, peptides, nucleic acids and even drug Nano-carriers [9, 13, 14]. Here, after the Trim-away application, the G3BP2 and TRIM21 protein levels were investigated and immunofluorescence analysis was carried out in HUVECs. Untreated HUVECs as a control, HUVECs induced oscillatory shear stress and HUVECs fused with anti-G3BP2 TAT plus treated with oscillatory shear stress (OSS).
Figure 2. The effect of Trim-away application in mediated G3BP2 depletion in HUVECs induced OSS. (A) Western blot result with (G3BP2/GAPDH) represent protein relative expression and (B) immunofluorescence confocal analysis of G3BP2 protein expression in static untreated HUVECs and HUVECs induced oscillatory shear stress (OSS) with and without the addition of anti-G3BP2 TAT. Data presents the average of three independent studies.

The western blot analysis data (Figure 2A) supported with immunofluorescence image (Figure 2B) have shown that the increasing G3BP2 protein expression by oscillatory shear stress treatment was suppressed after the application of Trim-away technology in HUVECs.

3.3. G3BP2 Protein Degradation inhibits the endothelial Inflammation Response

Cytokines are involved in all stages of atherosclerosis, and have a significant effect on atherosclerosis disease pathogenesis. Atherosclerotic plaques tend to form at the internal curvature and branch points of the arteries, which is usually associated with turbulent blood flow. Activated endothelial cell (EC) due to shear stress exposure, triggered releases a series of chemokines and cytokines, and also causes the recruitment of circulating immune cells (especially monocytes and T lymphocytes) [13]. During inflammation states endothelial cells express adhesion proteins, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These molecules are involved in the immune cells recruitment and can promote pro-inflammatory cytokines releases [15].
Figure 3 The G3BP2 proteins in the modulation of the flow-dependent inflammatory response. This figure showed relative protein expression of HUVECs overexpressed TRIM21 under static conditions or subsequently exposed to oscillatory shear stress (OSS) for 6h with or without prior incubation of anti-G3BP2 TAT (6h). The level of G3BP2, VCAM-1 and ICAM-1 protein expression level were determined by using western blot analysis, normalized to the levels of GAPDH. Data presents the average of three independent studies.

Several studies have been reported that the atherosclerosis regions are characterized by disrupted oscillatory flow, which induces pro-inflammatory adhesion molecules such as ICAM-1 and VCAM-1 upregulation. A critical feature of atherosclerosis involves the increased release of adhesion molecules via the activated endothelium. Furthermore, numerous studies have shown that reducing cytokines favourable to slow down the inflammation and the formation of plaque in atherosclerosis, and many therapies targeting anti-inflammatory cytokines targeting atherosclerosis. [16], [17].

As shown in Figure 3, the upregulation of G3BP2 protein due to OSS stimulation leads to increased protein levels of inflammatory biomarkers VCAM-1 and ICAM-1. In addition to confirming the role of G3BP2 mediates endothelial cell inflammation, we performed Trim-away technology to deplete G3BP2 expression in the endothelial cell by introduced the anti-G3BP2 TAT. Western blot analysis confirmed that the expression of G3BP2 in HUVEC incubated with anti-G3BP2 TAT was significantly reduced. Thus, from this result, we found that the depletion of G3BP2 protein along with reduction the expression levels of VCAM-1 and ICAM-1 expression level.

4. Conclusion
In conclusion, our current research indicates that G3BP2 protein might involve in inhibits the development of atherosclerosis induced by oscillatory shear stress (OSS). This study found that in activated endothelial cells, G3BP2 expression increased significantly compared to control. Furthermore, the depletion of G3BP2 protein by Trim-away method causes a decrease in the pro-inflammatory ICAM-1, VCAM-1 response in cultured endothelial cells.

Conflict of Interest
The authors declare no conflict of interest

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