Original article

Hepatocyte steatosis increases the expression of adhesion molecules in endothelial cells

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Background: Non-alcoholic fatty liver disease is considered a hepatic manifestation of the metabolic syndrome. It is associated with endothelial dysfunction as an early event of generalized atherosclerosis. However, it is unclear whether steatotic hepatocytes influence endothelial function directly.

Objective: Explore the influence of hepatocyte steatosis on the function of endothelial cells.

Methods: Oleic and palmitic acid (2:1 mixture, final concentration: 1 mM for 24 hours) was used to induce a normal adult hepatocyte strain (L-02) for transformation into steatosis cells. This was followed by oil red O staining and transmission electron microscopy (TEM) for verification. The culture solution of steatotic L-02 cells was filtered and collected, and added into the culture substrate of human umbilical vein endothelial cells (HUVECs). The expression of vascular cellular adhesion molecule -1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin in HUVECs was detected by real-time polymerase chain reaction and Western blot assays. The apoptosis and proliferation of HUVECs was determined using flow cytometry. The experimental results were compared with the controls.

Results: Oil red O staining and microscopic observation showed that the cytoplasm of induced L-02 cells contained a large amount of red lipid droplets. TEM results showed that the cytoplasm had lipid accumulation, swelling mitochondria, fewer cristae, and reduced number of rough endoplasmic reticula accompanied with degranulation. However, these changes were not observed in normal L-02 cells. As to the group of HUVECs treated by the filtrate of steatosis L-02 cells, the mRNA and protein expression of VCAM-1, ICAM-1, and E-selectin was higher than that in the control group. The difference was statistically significant ($p < 0.01$). No significant difference was found when HUVECs apoptosis and proliferation were assessed by flow cytometry.

Conclusion: Secretion from steatotic hepatocytes could boost the expression of VCAM-1, ICAM-1, and E-selectin in endothelial cells, indicating that hepatocyte steatosis could induce endothelial cell dysfunction. The proliferation and apoptosis of endothelial cells did not change, suggesting that hepatocyte steatosis had no influence on the viability of endothelial cells under this condition.

Keywords: Adhesion molecules, dysfunction, endothelial cell, hepatocyte, steatosis

The prevalence of non-alcoholic fatty liver disease (NAFLD) has been increasing worldwide in recent years. It affects 15-30% of the general population in various countries [1-3]. NAFLD is not a benign condition and has become the most common cause of abnormal liver function tests in clinical practice [4].

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Aside from progressing to cirrhosis and hepatocellular carcinoma, NAFLD is also closely related to cardiovascular disease (CVD) risk factors, such as obesity, insulin resistance, hypertension, dyslipidemia, and metabolic syndrome [2]. Therefore, the relationship between NAFLD and CVD is of major concern.

A series of studies have shown that NAFLD patients have higher presentations of sub-clinical and clinical atherosclerosis (As) than age and sex-matched patients without NAFLD, independently of the

classical risk factors of the metabolic syndrome [5-7]. To most NAFLD patients, the prevalence of CVD events is 10-20 times higher than that of liver disease. CVD has become the second major cause of death in NAFLD patients [8]. These novel findings suggest a more complex picture and raise the possibility that NAFLD might not merely be a marker but also an early mediator of CVD [9]. Therefore, a study of the relationship between NAFLD and CVD is of clinical importance. However, the relationship between NAFLD and CVD is still controversial. It remains unknown whether NAFLD is the result or cause of CVD, or participates in the cascade reaction of metabolic disorders that lead to As [8].

In this study, we explored the influence of hepatocyte steatosis on the function of endothelial cells. Since endothelial dysfunction is a factor to trigger As [10], we applied the secretions of steatotic hepatocytes on endothelial cells to determine the existence of any influence on function of endothelial cells. Endothelial dysfunction manifested itself as lower levels of anticoagulation, anti-cell adhesion, and anti-oxidation [11]. It is well-known that the damaging factors of endothelial cells can cause increased expression of adhesion molecules, and protective factors can reduce expression levels, indicating that increased secretion of adhesion molecules may be responsible for endothelial dysfunction [11]. Three types of adhesion molecules, vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, are major adhesion molecules that support the adhesion and migration of leukocytes [12, 13]. Hence, in this study, we used these three adhesion molecules as indicators of endothelial dysfunction.

Materials and methods
Materials

Human hepatocyte cells (L-02) were purchased from China Cell Culture Center (Shanghai, China). Human umbilical venous endothelial cells (HUVECs) were obtained from ATCC (Rockville, USA). Cell culture reagents were purchased from Invitrogen (Carlsbad, USA). Antibodies against VCAM-1, ICAM-1 and E-selectin were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Unless stated otherwise, all other reagents were purchased from Sigma Chemical Company (St Louis, USA).

Culture, fat-overloading induction of L-02 cells

L-02 cells were routinely cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Before each experiment, cell viability was determined by trypan blue exclusion, and was routinely greater than 90%. Fat overloading induction of cells was done mainly according to previously established methods [14, 15], where L02 cells at 80% confluency were exposed to HFFA, a mixture of oleate (OA) and palmitate (PA), at the final ratio of 2:1 and final concentration of 1 mM for 24 hours. Then, cells were incubated with non-HFFA DMEM containing 10% FBS for 16 hours. The conditioned medium was collected, sterile-filtered through a 0.22 μm filter, and kept frozen at -20 °C until use.

Oil Red O staining and transmission electron microscopy observation

Oil red O staining was performed as previously described [16]. Briefly, cells were washed gently twice with phosphate buffer solution (PBS), fixed with 3.7% fresh formaldehyde in PBS for one hour at room temperature, and stained with filtered Oil red O solution (60% isopropanol and 40% water) for at least one hour. After staining, the Oil Red O staining solution was removed, and the plates were rinsed with water and dried. Images of lipid droplets in L-02 cells were collected using a microscope (Olympus, Tokyo, Japan).

The changes of cellular ultramicrostructure were assessed by transmission electron microscopy (TEM), which was performed by the Electron Microscopy Center of the Shanghai Jiao Tong University School of Medicine.

Culture and treatment of HUVECs

HUVECs were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Before treatment experiments, cells were grown to 80-90% confluence and serum-starved overnight. Treatments were performed in the medium of steatotic L-02 cells or normal L-02 cells as detailed above.

Real-time polymerase chain reaction and Western blot analysis

After treatment of HUVECs for four hours, expression of VCAM-1, ICAM-1, and E-selectin was assessed by real-time polymerase chain reaction (qPCR) and Western blot. HUVECs cultured in steatotic, normal, and control media were harvested.

For qPCR, total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instruction. Concentration of the RNA samples was estimated spectrophotometrically at OD 260/280, and cDNA was synthesized using MMLV reverse transcriptase (Epicenter Biotechnologies, Madison, USA) in the presence of oligo-dT primer (Promega BioSciences, Sunnyvale, USA). The sequences of PCR primers were as follows: VCAM-1, sense 5'-GGATGCAAAATAGAGCACGAGA-3' and
antisense 5'-CAGTTGAAGGATGCGGGAGT-3'; ICAM-1,
sense 5'- GTGCCAGTTCCACCCGTTC -3' and antisense 5'-TGTGCAAGAAGATAGCCAACCA-3'; E-selectin,
sense 5'- TAACGGAAACTGCCAGAAGC -3' and antisense
5'-CCCAGGTTGAATGCACCAC -3'. The expression of β-actin
(sense 5'- TGACGTGGACATCCGCAAAG -3' and antisense 5'- CTGGAAGGTGGACAGCGAGG -3') was used as an internal control to normalize specific gene expression in each sample. Q-PCR was performed using cDNA samples with SYBR Green PCR master mix (Applied Biosystems, Foster City, USA) and carried out in the ABI 7300 Real-Time PCR system (Applied Biosystems).

Western blotting was performed according to a standard procedure [17]. Briefly, cells were lyzed in RIPA lysis buffer at 4 °C for 30 minutes. Cell lysates (25μg) were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK), blocked with 5% no-fat milk and hybridized with primary antibodies (diluted 1: 800). After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature, immunoreactive proteins were detected using a chemiluminescence ECL assay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. Western blot bands were visualized using a LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

Proliferation and apoptosis assays

After treatment for 24 hours, HUVECs grown in 60 mm culture dish were harvested for proliferation and apoptosis assays [18], respectively. Cell proliferation was determined by staining DNA with propidium iodide (PI), using CycleTestTM Plus (Becton Dickinson, Franklin Lakes, USA), as described in the manufacturer's protocol. The DNA content was determined by using FACStar flow cytometer (Becton Dickinson, Franklin Lakes, USA). Apoptosis was evaluated by staining cells with annexin V-FITC and PI (Becton Dickinson). To quantify apoptosis, prepared cells were washed with cold PBS and re-suspended in binding buffer [10mMHEPES/ NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2] at a concentration of 1×10^{6} cells/mL. Then, 5 μL of annexin V-FITC and 10 μL of PI were added to these cells, and were analyzed with FACStar flow cytometer (Becton Dickinson, Franklin Lakes, USA).

Statistical analysis

Each experiment was performed in at least triplicate and data were expressed as means ± SD (standard deviation). Differences between various treatments were analyzed by unpaired Student's t-tests or oneway ANOVA as applicable with p-values <0.01 considered highly significant and less than 0.05 considered significant.

Results
Verifying hepatocyte steatosis

After 24 hours induction of L-02 cells by oleic and palmitic acid, the cells were stained with oil red O and observed under a light microscope. Orange red or red granules appeared in the cytoplasm, suggesting the retention of lipid droplets in the cells. **Figure 1** shows oil red O staining of L-02 cells from control and HFFA incubated groups. The model group cells had a large accumulation of lipid droplets (big or small) in **B**, while the normal group cells had very few or almost no lipid droplets in **A**.

When they were further observed under TEM, changes of ultramicrostructure of the cells were found. As shown in **Fig. 2**, lipid micro-vesicae were accumulated in the cytoplasm. The number of RERs was reduced accompanied with degranulation, mitochondria increased, and cristae were reduced. These changes support the formation of fatty liver, suggesting that a hepatocyte steatosis model was successfully established in the experiment.
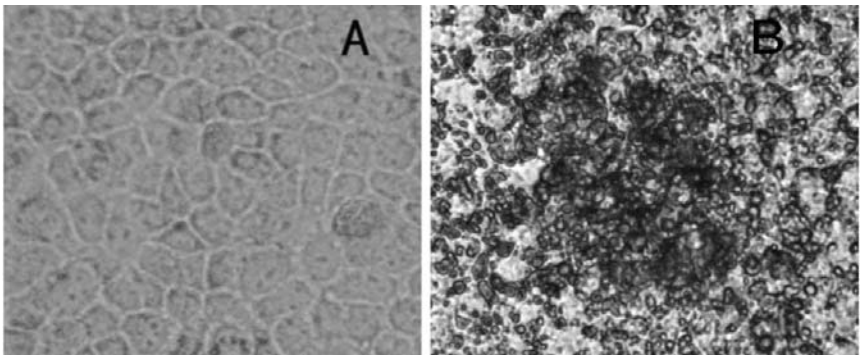
Fig. 1 Oil red O staining of L-02 cells from control (**A**) and incubated with HFFA for 24 hours (**B**). Original magnifications: x 200.
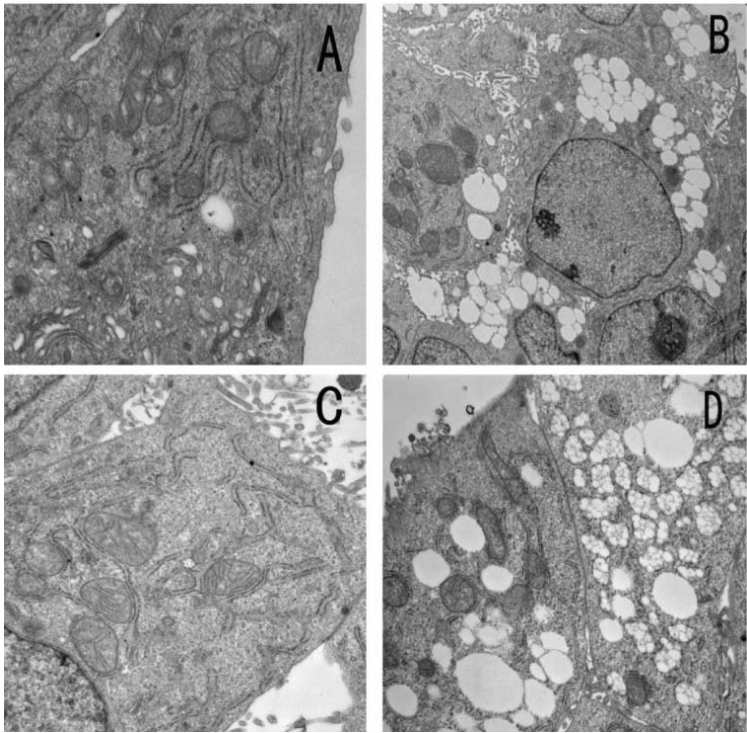

Fig. 2 Changes of ultramicrostructure in L-02 cells assessed using transmission electron microscopy. **A**: control, **B**, **C**, **D**: incubated with HFFA for 24 hours. Original magnifications: x 17,500.

Detecting the expression of VCAM-1, ICAM-1, and E-selectin

After HUVEC was treated by the secretion from hepatocyte steatosis for four hours, the expression of mRNA in adhesion molecules were detected by qRT-PCR method. **Figure 3** shows expression of VCAM-1, ICAM-1, and E-selectin mRNA in HUVECs cultured in DMEM, secretion from normal hepatocyte, and secretion from steatotic hepatocyte for four hours. The group treated by the steatosis filtrate had a higher expression level of VCAM-1, ICAM-1, and E-selectin mRNA than the group treated by normal filtrate and the control group. The difference was statistically significant ($p < 0.01$). No significant difference between the normal and control groups was observed ($p > 0.05$).
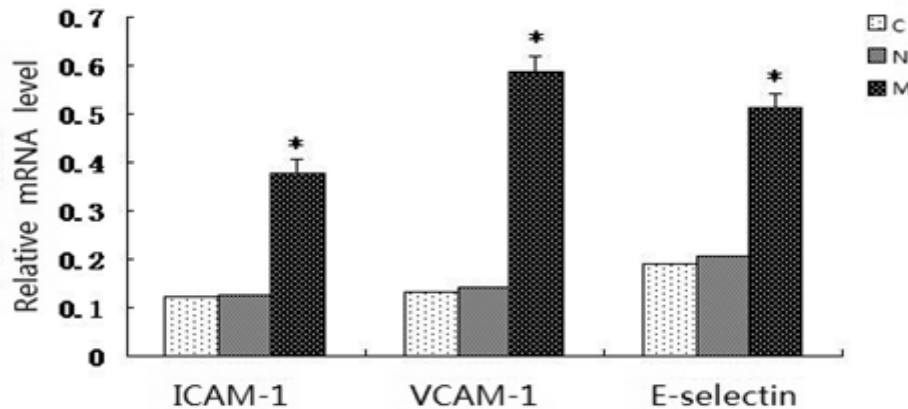
Fig. 3 Expression of VCAM-1, ICAM-1, and E-selectin mRNA in HUVECs cultured in DMEM (**C**), secretion from normal hepatocyte (**N**) and secretion from steatotic hepatocyte (**M**) for four hours. Data represent means ± SD and each experiment was performed at least six times. *denotes p <0.01 comparing **M** with **C** and **N**.

After the treatment, Western blot examination was conducted by taking GADPH as the internal control protein, and the relative expression level of protein in the adhesion molecules was analyzed. **Figure 4** shows expression of VCAM-1, ICAM-1, and E-selectin by Western blotting. The group treated by steatosis filtrate had a significantly higher average level of protein (VCAM-1, ICAM-1, and E-selectin) than the group with normal filtrate treatment and the control group. The difference was statistically significant, but between the normal and control groups, no significant difference was found (p >0.05).

HUVEC apoptosis and proliferation

The result using flow cytometry demonstrated that the percentage of apoptotic cells in the control, normal,

and steatosis groups were 5.36 ± 1.06, 5.05 ± 1.06, and 5.03 ± 1.02, respectively. Analysis showed no significant increase of HUVEC apoptosis in the three groups (p >0.05). Cell proliferation in all groups as detected by flow cytometry had no statistical significance (p >0.05).

Discussion

In the present experiment, after HUVECs were treated with the secretion from steatotic hepatocyte induced by fatty acids for four hours, the expression level of ICAM-1, VCAM-1, and E-selectin were higher than that in the control group (**Fig. 3** and **4**). As well established, cell adhesion molecules mediate the adhesion and migration of monocytes and leukocytes to and across the endothelium. This is a crucial early
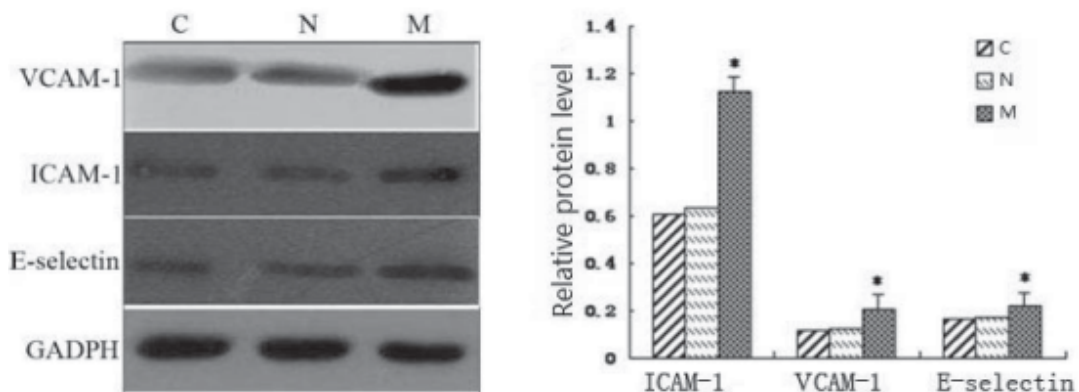

Fig. 4 Expression of VCAM-1, ICAM-1, and E-selectin by Western blotting (left). HUVECs were treated with DMEM (**C**), secretion from normal hepatocyte (**N**) and secretion from steatotic hepatocyte for four hours (**M**) (left). Relative protein level for VCAM-1, ICAM-1, and E-selectin in **C**, **N** and **M** (right). Data represent means ± SD and each experiment was performed at least six times. *denotes p < 0.01 comparing **M** with **C** and **N**.

step in the atherogenic process [19]. Davies et al. [20] demonstrated increased expression of ICAM-1, VACM-1, and E-selectin in As in the clinical study, and suggested that adhesion molecules participate in the As inflammation process. O'Brien et al. [21] also reported that the expression of ICAM-1, VCAM-1, and E-selectin in neovessels of atheromatous plaque was about twice as high as that in the endothelial cells of a lumen artery. The increased density of macrophages in the inner membrane of the plaque was apparently related to the expression of VCAM-1 in endothelial cells of neovessels. According to a prospective study on 1246 patients with coronary heart disease by Blankenberg et al. [22], the level of VCAM-1, ICAM-1, and E-selectin in the plasma increased. Soluble VCAM-1 was the strongest independent predictor of fatal cardiovascular events in patients suffering from coronary heart disease. Therefore, the not pre-regulated expression of the three adhesion molecules, as found in this study, could provide a theoretical basis to explain that hepatocyte steatosis could cause endothelial dysfunction. Ivan et al. [23] applied human hyperlipidemia serum to stimulate vascular endothelial cells, and found that expression of VCAM-1 increased and lipid droplets increased in cells could be transformed into foam cells. This is consistent with the present results.

Since endothelial dysfunction is an early event in the development of the atherosclerotic process [24], the influence of hepatocyte steatosis on HUVEC, as shown in this study, suggests the conclusion that NAFLD promotes the formation of As, and supports the conclusion that NAFLD is an early risk factor of As [9]. The mechanism may involve the induction and aggravation of insulin resistance and increasing metabolic disorder, systematic inflammation, oxidative stress, hypohepatia, and influence of adipocytokines, such as high tumor necrosis factor alpha (TNF-α) and hypoadiponectinemia. All these facilitate the formation of As [8, 9, 25].

The changing micro-environment during hepatocyte steatosis may influence the growth of endothelial cells [25, 26]. Thus, this study adopted flow cytometry to assess the apoptosis and proliferation of HUVECs. Interestingly, we did not find any influence of treatment on the apoptosis and proliferation of HUVECs. This suggests that hepatocyte steatosis had no influence on the viability of HUVEC, being consistent with the study result of Kralisch et al. [15]. However, we must note that the present experiment was done during a short treatment period (only 24 hours) where HUVEC proliferates slowly outside the body [27], and the sophisticated environment of hepatocyte steatosis inside the body cannot be simulated perfectly .

Conclusion

Secretion from hepatocyte steatosis on HUVECs could enhance the expression of VCAM-1, ICAM-1, and E-selectin. This suggests that hepatocyte steatosis could induce endothelial cells dysfunction. It supports the viewpoint that NAFLD facilitates the formation of As and can be regarded as an early risk factor of As. It provides a basis to monitor and prevent CVD-related complications in NAFLD patients. However, the reason why hepatocyte steatosis has no effect on the proliferation and apoptosis of endothelial cells and the mechanisms underlying NAFLD's causing endothelial cells dysfunction require further investigation.

The authors have no conflict of interest to declare.

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