Fibronectin-containing High-Density Lipoprotein is Associated with Cancer Cell Adhesion and Proliferation

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A large amount of evidence suggests that high-density lipoprotein (HDL) has anti-atherosclerotic properties. HDL-cholesterol (HDL-C) has also been widely used as a marker of cardiovascular disease. Recently, it was reported that plasma HDL-C levels are inversely correlated with cancer risk. However, the relationship between HDL and cancer pathophysiology remains unknown.

Here, we sought to investigate the effect of HDL on cancer progression. First, we focused on fibronectin—an essential extracellular matrix glycoprotein—as an HDL-associated protein and found that only 7.4% of subjects in this study had fibronectin in HDL isolated from their plasma. The fibronectin-containing HDL (FN-HDL) increased the phosphorylation of focal adhesion kinase (FAK) in HeLa cells compared to HDL without fibronectin, further inducing the phosphorylation in a dose-dependent manner. Second, we found that fibronectin-treated HDL activated the phosphorylation of FAK, and its upstream effector blocked the phosphorylation induced by FN-HDL. Finally, we demonstrated that FN-HDL promoted cancer cell proliferation and adhesion compared to HDL without fibronectin. Our study showed the possible mechanism by which FN-HDL enhanced cancer cell proliferation and adhesion via the FAK signaling pathway. Further investigation of the roles of HDL components in tumorigenesis might provide novel insight into cancer pathophysiology.

A large amount of evidence has demonstrated that high-density lipoprotein (HDL) has anti-atherosclerotic properties and high-density lipoprotein cholesterol concentration (HDL-C) inversely correlates with the risk for cardiovascular disease (1-3). The athero-protective effect of HDL is due to its key role in reverse cholesterol transport (RCT) (4), whereas it is well known that in response to cardiovascular disease, HDL has anti-inflammatory, anti-oxidative, anti-apoptotic, and anti-thrombotic effects and vasodilatory properties independent of RCT (5, 6). In fact, HDL has a cardioprotective effect beyond its athero-protective properties, leading to reduction in the incidence of heart failure in humans (7, 8). These beneficial effects of HDL largely depend on the multiple components of HDL including paraoxonase-1 (PON1), platelet-activating factor acetylhydrolase (PAF-AH), and sphingosine-1-phosphate (S1P) (9). In contrast, several studies have indicated a negative impact of HDL components on atherosclerosis. For instance, serum amyloid A (SAA) and myeloperoxidase (MPO), carried on HDL, generate dysfunctional HDL, leading to multiple components of HDL including paraoxonase activating factor acetylhydrolase (PAF-AH), and sphingosine-1-phosphate (S1P) (9). In contrast, several studies have indicated a negative impact of HDL components on atherosclerosis. For instance, serum amyloid A (SAA) and myeloperoxidase (MPO), carried on HDL, generate dysfunctional HDL, leading to...
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from the subjects with acute coronary syndrome (22). Fibronectin participates in multiple stages of cancer progression, namely cancer growth, migration, and invasion (23-26). Mitra, et al. found that fibronectin upregulated the c-Met/FAK/Src signaling pathway by binding to cellular α5β1-integrin in ovarian cancer cells (26) and suggested that fibronectin promotes ovarian cancer invasion and metastasis. However, how HDL with fibronectin affects cancer progression is still unknown. Here, we sought to elucidate the biological mechanism of action of fibronectin-containing HDL (FN-HDL) in the process of tumorigenesis.

MATERIALS AND METHODS

Clinical participants and HDL preparation

The Kobe Cardiovascular Marker Investigation (CMI) registry is a single-center registry of patients referred to Kobe University Hospital with cardiovascular disease, which is conducted to identify blood-based biomarkers that have utility in predicting cardiovascular disease. The study protocol was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. The study was approved by the Ethics Review Committee at Kobe University (Japan) and was registered at the UMIN Clinical Trials Registry (identification number 000030297). Written informed consent was obtained from all patients prior to enrollment in the study. We randomly enrolled 27 patients with stable angina who did not have a medical history of cancer in the past 5 years, from CMI registry for the present study and isolated HDL from their plasma as previously reported (27). In brief, HDL fraction was isolated by ultracentrifugation, and its purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Coomassie staining.

Cell culture

HeLa cells were purchased from the European Collection of Authenticated Cell Cultures (Porton Down, UK) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (WAKO, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin (P/S) (each from Sigma-Aldrich, St. Louis, MO, USA) at 37 °C and 5% CO2.

Treatment of HDL with fibronectin

To test whether HDL isolated from the subjects contains fibronectin, we performed western blot analysis as described below. We incubated Non-FN-HDL (100 μg/ml) with fibronectin (10 μg/ml) overnight and then used that for stimulation of HeLa cells.

Western blotting

Western blotting was performed as previously reported (28). In brief, cells were washed twice with cold phosphate-buffered saline (PBS) (-) and harvested with ice-cold lysis buffer (20-mM HEPES (pH 7.4), 1% NP40, 1% SDS, and 150-mM NaCl). Proteins from the lysates were separated using SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Thermo Fisher, Tokyo, Japan). After blocking with 5% milk for 1 h, the membranes were incubated with primary antibodies overnight at 4 °C and then with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The signals were detected using the Amersham Imager 600 (GE Healthcare, Tokyo, Japan). The following antibodies were used in this study: anti-phosphor-FAK antibody (Thermo Fisher, #44-624G), anti-FAK antibody (BD, #610087), anti-Fibronectin antibody (Sigma Aldrich, #F0916), and anti-ApoA1 antibody (Santa Cruz, #sc-376818).

Cell proliferation assay

Three hundred cells were placed into a 60 mm dish and incubated in DMEM with 10% FBS and 1% P/S at 37 °C and 5% CO2 overnight. Subsequently, cells were cultured with 10 μg/ml HDL or vehicle for 7 days, followed by crystal violet staining. Cells were washed twice with PBS (-) and fixed for 5 minutes with 4% paraformaldehyde. After fixation, cells were stained with 0.1% crystal violet.

Cell adhesion assay

Twenty thousand cells were placed into a Matrigel (CORNING, Tokyo, Japan) coated 96 well plate and incubated in serum-free medium. Afterwards, the cells were treated with 10 μg/ml HDL and incubated for 20 min. After washing the cells 4 times with PBS (-), cell adhesion was assessed using a cell counting kit (CCK)-8 assay kit (Cell Counting Kit-8, DOJINDO, Kumamoto, Japan) according to manufacturer’s protocol.
RESULTS

In order to replicate a study by Alwaili, et al. (22), we examined whether HDL contained fibronectin by western blotting. Interestingly, HDL from only 2 subjects, out of 27, contained fibronectin (Figure 1A). Although the sample size was too small to draw any definite conclusions, the comparisons of patient profiles and conventional lipid profiles between patients with and without fibronectin in their HDL particles are shown in Table I. The FN-HDL showed greater phosphorylation of focal adhesion kinase (FAK) compared to HDL without fibronectin (Non-FN-HDL) in HeLa cells (Figure 1B). FAK is known to be a non-receptor protein tyrosine kinase and contributes to cancer progression and metastasis by activating cell proliferation, migration, and adhesion (29).

Figure 1. Fibronectin-containing HDL (FN-HDL) highly enhances the phosphorylation of focal adhesion kinase (FAK). A) Fibronectin contained in HDL was detected by western blot analysis with anti-Fibronectin antibody. B) HeLa cells were incubated with 10 μg/ml HDL, which either contained fibronectin (FN-HDL) or not (Non-FN-HDL). Afterwards, phosphor-FAK was evaluated by western blotting.

To investigate whether the FAK phosphorylation depends on the presence of fibronectin on HDL, we stimulated HeLa cells with fibronectin-treated HDL and showed that it increased the phosphor-FAK (Figure 2A). On the other hand, we confirmed that there was no effect on the FAK phosphorylation when fibronectin alone was used for the stimulation to the cells (Figure 2B). Additionally, FN-HDL upregulated the phosphorylation of FAK; however, it was inhibited by a calcium chelator ethylene glycol tetra-acetic acid (EGTA), which is known to inactivate integrin, an upstream effector in FAK signaling (Figure 2C).

To test the dose dependency of FN-HDL on the phosphorylation of FAK, we stimulated HeLa cells with FN-HDL and found that FN-HDL treatment upregulated the phosphorylation of FAK in a dose dependent manner (Figure 3A). On the other hand, treatment with ApoA1, a main protein moiety in HDL, did not enhance the phosphorylation of FAK (Figure 3A).

Next, we examined the effects of FN-HDL on cancer cell properties. FN-HDL activated the proliferation in HeLa cells better than Non-FN-HDL (4A). Moreover, FN-HDL also upregulated the adhesion of HeLa cells as compared to Non-FN-HDL (4B). These findings suggested that FN-HDL promoted cancer cell proliferation and adhesion via the FAK signaling pathway.
Figure 2. Phosphorylation of FAK due to FN-HDL is blocked by its upstream inhibitor.
A) Non-FN-HDL (100 μg/ml) was incubated with 10 μg/ml fibronectin. Subsequently, HeLa cells were incubated with the fibronectin-treated HDL (+) or non-treated HDL (-), followed by western blotting with antibodies as indicated. B) HeLa cells were incubated with the 10 μg/ml fibronectin alone, followed by western blotting using same antibodies as Figure 2A. C) HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with FN-HDL or phosphate-buffered saline (PBS) (-) containing 2 mM ethylene glycol tetra-acetic acid (EGTA), a calcium chelator. Subsequently, western blot analysis was performed using antibodies as indicated.

Figure 3. HDL increases phosphor-FAK in a dose dependent manner.
A) HeLa cells were stimulated with recombinant ApoA1 (200 μg/ml) or FN-HDL (0, 50, 100, and 200 μg/ml). Phosphorylation of FAK was evaluated by western blotting.
Figure 4. FN-HDL promoted cancer cell proliferation and adhesion.

A) Three hundred HeLa cells were placed into a 60mm dish and cultured with FN-HDL or Non-FN-HDL for 7 days, followed by crystal violet staining. B) HeLa cells were seeded into 96 well plate coated by Matrigel. Twenty minutes after incubation, cells were washed 4 times with PBS (-). Afterwards, cells adhered on the plate was quantified by Cell Counting Kit-8 (CCK-8) assay. C) A scheme showing the possible mechanism by which FN-HDL activates FAK signaling pathway.

Table I. Patient Characteristics

<table>
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<th>FN-HDL (n = 2)</th>
<th>Non-FN-HDL (n = 25)</th>
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<tr>
<td>Age (years)</td>
<td>53±7</td>
<td>68.3±11.8</td>
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<tr>
<td>Gender (Male %)</td>
<td>100.0</td>
<td>72.0</td>
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<tr>
<td>BMI (kg/m2)</td>
<td>26.7±0.75</td>
<td>25.2±3.1</td>
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<td>Current smoking (%)</td>
<td>50.0</td>
<td>24.0</td>
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<tr>
<td>Hypertension (%)</td>
<td>100.0</td>
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<tr>
<td>Diabetes mellitus (%)</td>
<td>0.0</td>
<td>36.0</td>
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<tr>
<td>Dyslipidemia (%)</td>
<td>100.0</td>
<td>88.0</td>
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<tr>
<td>HbA1c (%)</td>
<td>5.95±0.15</td>
<td>6.3±1.2</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>106.5±3.5</td>
<td>106.3±30.2</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>169.5±6.5</td>
<td>159.7±41.0</td>
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<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>104.0±12.0</td>
<td>89.6±33.3</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>45.0±6.0</td>
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</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>133.5±1.5</td>
<td>140.5±87.8</td>
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<td>Apolipoprotein AI (mg/dL)</td>
<td>127.5±11.5</td>
<td>128.8±28.4</td>
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<tr>
<td>hs-CRP (mg/dL)</td>
<td>0.045±0.025</td>
<td>0.260±0.473</td>
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FN-HDL; Fibronectin-containing HDL, Non-FN-HDL; HDL without fibronectin, BMI; body mass index, LDL; low density lipoprotein, HDL; high density lipoprotein, hs-CRP; high sensitivity C-reactive protein.

DISCUSSION

Here, we evaluated the effect of FN-HDL on the pathological mechanisms of cancer progression. We found that HDL isolated from only 7.4% of subjects contained fibronectin, and FN-HDL increased the phospho-FAK levels, promoting cancer cell adhesion and proliferation (Figure 4C).
Low HDL-C level is related to the incidence of several cancers such as breast, prostate, and lung cancers (30-32). One of the possible reasons why low HDL-C correlates with the increasing risk of cancers is thought to be the contribution of Apolipoprotein A-I (ApoA-I), the major protein constituent of HDL. Many studies reported that ApoA-I suppressed cancer development and progression both in vitro and in vivo (33). Su, et al. revealed that ApoA-I and ApoA-I mimetic peptides inhibit tumor development in a mouse model of ovarian cancer by removing lysophosphatidic acid, a bioactive lysophospholipid which induces cell migration and invasion in ovarian cancer (34). Zamanian-Daryoush, et al. demonstrated that ApoA-I suppressed tumor growth and metastasis in multiple animal tumor models (35). In their study, a transgenic mouse model overexpressing the human apoA1 presented increased infiltration of M1 macrophages, known as an anti-tumor phenotype, and decreased tumor burden and metastasis. In contrast, apoA1-deficient mice showed highly enhanced tumor growth and reduced survival (35). On the other hand, several studies revealed the negative impact of HDL on cancer progression. Hayat, et al. demonstrated that presence of SAA on HDL altered HDL binding to the cell surface, possibly leading to cancer cell proliferation owing to the change in HDL-mediated cholesterol delivery to cells (36). Similar to the results for SAA, our data indicated that fibronectin on HDL mediated cancer cell proliferation and adhesion. Taken together, the results suggest that the pro- or anti-tumorigenic effects of HDL are highly likely to be attributed to the proteins carried by HDL.

Our study revealed that FN-HDL upregulated the phosphorylation of FAK in HeLa cells, a cervical cancer cell line. Fibronectin is overexpressed in many tumors and associated with survival and metastasis in patients with cancer (23-26). Zhou, et al. reported that fibronectin was activated in patients with cervical cancer, and its higher expression was associated with a poor prognosis for patients with cervical cancer (37). Moreover, numerous studies revealed the association between FAK and various cancers and the biological mechanisms by which FAK promotes cancer development and progression (38). FAK plays an important role in cell signaling systems and is highly expressed and phosphorylated in several cancers, including lung, pancreatic, gastric and ovarian cancers (39-42). Considering that fibronectin is one of the inducers of the FAK signaling pathway (26), it seems to be convincing that FN-HDL activates this pathway. Although there is not enough evidence that demonstrates involvement of HDL or HDL components in cancer pathophysiology, investigating the association between characteristics of HDL constituents and tumorigenesis might have significant implications in cancer treatment.

There are some limitations to our study. First, although we used HDL isolated from CAD patients who did not have a medical history of cancer in the past 5 years, we could not follow the incidence of cancer in these patients prospectively. Second, only 7.4% of the patients had fibronectin in their HDL; further elucidation of the pathological role of FN-HDL, using more patient samples is needed. Finally, we could not assess the concentration of fibronectin contained in FN-HDL and clarify why fibronectin on HDL activated the FAK signaling pathway, not the circulating fibronectin which is not carried by HDL (Figure 2A, B). There might be several mechanisms by which HDL affects the affinity of fibronectin to its receptors or the tissue transitivity of that.

In summary, we identified fibronectin in HDL particles, further demonstrating that FN-HDL promoted cancer cell proliferation and adhesion through the FAK signaling pathway. In the field of cardiovascular disease, several studies show the adverse role of dysfunctional HDL on atherogenesis and heart disease. Correspondingly, HDL has positive and negative effects on tumorigenesis and may work as a mediator of systemic diseases. Elucidation of the detailed mechanism by which HDL and its components regulate cancer development and progression might help develop a novel strategy in cancer therapeutics.

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