HDL of Patients with Type 2 Diabetes Mellitus Elevates the Capability of Promoting Breast Cancer Metastasis

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Abbreviations:

HDL: high-density lipoprotein; N-HDL: normal high-density lipoprotein; D-HDL: diabetic high-density lipoprotein; G-HDL: glycated high-density lipoprotein; Ox-HDL: oxidized high-density lipoprotein; LDL: low-density lipoprotein; apoA-I: apolipoprotein A-I; DM: diabetes mellitus; T2DM: type 2 diabetes mellitus; TC: tumor cells; HUVEC: human umbilical vein endothelial cells; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ECM: extracellular matrix; PKC: protein kinase C.

Translational Relevance

Our study has shown that modifications (such as glycation and oxidation) are associated with the abnormal functions of diabetic HDL in type 2 diabetes on promoting breast cancer cell metastasis potency in vivo and the abilities of adhesion to HUVEC and ECM in vitro, involving integrin upregulation and PKC activation in the process. This provides substantial supporting evidence that type 2 diabetes is closely related to breast cancer development, especially the metastasis progression, which may be partly due to the modifications of HDL in the diabetic patients. It is suggested that diabetic HDL can be considered as potential risk markers and targets in the treatment of breast cancer with type 2 diabetes and the prospective HDL-based strategies in the therapy are in urgent need.
Abstract

Purpose:

Epidemiological studies suggested complicated associations between type 2 diabetes mellitus and breast cancer. HDL is inversely associated with the risk and mortality of breast cancer. Our study is to determine the different effects of normal and diabetic HDL on breast cancer cell metastasis.

Experimental Design:

MDA-MB-231 and MCF7 cells were treated with N-HDL, D-HDL, G-HDL and Ox-HDL. Cell metastasis potency was examined using a tail-vein injection model and cell adhesion abilities to HUVEC and ECM were determined in vitro. Integrins expression and PKC activity were evaluated, and PKC inhibitor was applied.

Results:

D-HDL dramatically promoted cell pulmonary metastasis (103.6% increase at $P<0.001$ for MDA-MB-231 with $1\times10^5$ cell injection; 157.1% increase at $P<0.05$ for MCF7 with $4\times10^5$ cell injection) and hepatic metastasis (18.1-fold increase at $P<0.001$ for MCF7 with $4\times10^5$ cell injection), and stimulated higher TC-HUVEC adhesion (21.9% increase at $P<0.001$ for MDA-MB-231; 23.6% increase at $P<0.05$ for MCF7) and TC-ECM attachment (59.9% and 47.9% increase respectively for MDA-MB-231 and MCF7, both at $P<0.01$) compared with N-HDL. D-HDL stimulated higher integrins ($\beta_1$, $\beta_2$, $\beta_3$, $\alpha v$) expression on cell surface and induced higher PKC activity. Increased TC-HUVEC and TC-ECM adhesion induced by D-HDL, G-HDL and Ox-HDL could be inhibited by staurosporine.

Conclusions:
Our study demonstrated that glycation and oxidation of HDL in diabetic patients could lead to abnormal actions on breast cancer cell adhesion to HUVEC and ECM, thereby promoting metastasis progression of breast cancer. This will largely draw the attention of HDL-based treatments in the diabetes patients with breast cancer.
Introduction

Both cancer and diabetes are prevalent diseases whose incidence is increasing globally and have a tremendous impact on health worldwide (1-3). Epidemiologic evidence suggests that people with diabetes are at a higher risk of many forms of cancer (including breast, colon, pancreas and etc.) (1). Moreover, some studies show that patients diagnosed with cancer who have preexisting diabetes are at increased risk for long-term, all-cause mortality compared with those without diabetes (4). In terms of breast cancer, diabetes was associated with a close to 40% increase in mortality within the first 5 year following breast cancer (5).

Metastasis of breast cancer is the major cause of tumor-related morbidity and mortality (6, 7), which involves multistep processes and various cytophysiological changes including altered intercellular interaction between circulating tumor cells and the endothelial cells of blood vessels, and changed adhesion ability between tumor cells and subendothelial extracellular matrix (ECM) (8). These interactions are considered as important events during tumor metastasis (9). Multiple and diverse adhesion molecules play a pivotal role in the intercellular and cell–ECM interactions of cancer (10), such as integrins, the immunoglobulin supergene family, selectins, cadherines and etc (11). Integrins, which consist of an α subunit noncovalently linked to a β subunit, are members of a glycoprotein family that forms heterodimeric receptors via which cells attach to ECM, to each other’s surfaces or to different cell types (12). They have been implicated in a wide variety of cellular functions, including cell adhesion, migration, invasion, proliferation and survival (12, 13). Protein kinase C (PKC) activity has been shown to be important in the regulation of integrins expression, localization (14, 15) and activity (16).
High-density lipoprotein (HDL) contributes importantly to cardiovascular disease risk, with a significant inverse relationship between HDL levels and the risk of cardiovascular disease (17-20). Similarly, it has been reported that HDL levels are inversely related to the rates of incident cancer, including breast cancer (21-24). However, emerging evidence indicates that HDL can be modified under certain circumstances, such as in diabetes (25) and cardiovascular diseases (26), and lose its protective effects or even become proatherogenic (25, 27). HDL isolated from patients with T2DM can be modified into glycated HDL and oxidized HDL (25, 28) and exhibits deficient activities in comparing with normal HDL of healthy subjects (25). Due to the difference between HDL from healthy subjects and T2DM patients, we speculate that normal and diabetic HDL may have different effects in the metastasis of breast cancer cells and finally lead to the increased mortality of breast cancer in patients with diabetes.
Materials and Methods

Patient Characteristics

Written informed consent was obtained from all participants, and the study protocol was approved by the local ethics committee. Each patient volunteer underwent a medical history, physical examination, screening laboratory tests and a 75 g oral glucose tolerance test. Patients were stable on antihypertensive, hypoglycemic, and lipid-lowering medications. There was no treatment change during the study. The healthy subjects had no family history of diabetes and had normal glucose tolerance. Characteristics of the study participants are shown in Table 1.

Animals

4 week old female BALB/c nude mice were obtained from the animal house, Academy of Military Medical Sciences, China. Throughout the experiments, mice were maintained with free access to pellet food and water. Animal welfare and experimental procedures were performed strictly in accordance with the related ethical regulations.

Cell Lines and Cell Culture

The hormone-independent MDA-MB-231 and hormone-dependent MCF7 human breast cancer cell lines were from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, UK) containing 10% fetal bovine serum (FBS; GIBCO, UK) in a humidified incubator at 37 °C with an atmosphere of 5% CO₂. Human umbilical vein
endothelial cells (HUVEC) were isolated by collagenase digestion of umbilical veins from fresh cords (29). The cells were plated on gelatin-coated culture dishes in Endothelial Cell Medium (ECM; Sciencell, USA) consisting of 500 ml of basal medium, 25 ml of fetal bovine serum, 5 ml of endothelial cell growth supplement and 5 ml of penicillin/streptomycin solution and then cultured in a humidified incubator at 37 °C with an atmosphere of 5% CO₂. HUVEC were used at passages 2–5.

**Isolation, Glycation and oxidation of HDL**

Fresh, fasting plasma was separated by centrifugation from peripheral blood obtained from healthy subjects (n=102) and type 2 diabetic patients (n=107). LDL (1.019–1.063) and HDL (1.063–1.210) were isolated from fresh plasma by ultracentrifugation as previously described (30). HDL from each individual were dialyzed against 3×1 L of endotoxin-free PBS (pH=7.4) and 100 μM diethylenetriamine pentaacetic acid (DTPA) (Sigma, USA), sterilized with 0.22 μm filter, stored in sealed tubes at 4 °C in dark and used within 2 months. To create glycated HDL, fresh human HDL (5 mg protein) was incubated with 25 mmol/L glucose in PBS under sterile conditions for 1 week at 37 °C (referred to as G-HDL) (31). Glycation of the apoA-I components of HDL was measured using mass spectrometry. Oxidative modification of HDL was performed by dialysis at lipoprotein concentration of 0.8 mg protein/mL against PBS containing 5 μM CuSO4 for 24 hours at 37 °C (referred to as Ox-HDL) (32). Lipid peroxidation of normal, diabetic, and oxidized HDL was determined by TBARS (Nanjing Jiancheng Bioengineering Institute, China) kit which quantifies the malondialdehyde (MDA) content. Thiobarbituric acid reactive substances (TBARS) were expressed as nanomoles of MDA.
per milligram apoA-I.

**Tail Vein Metastasis Assay**

MDA-MB-231 and MCF7 cells were pretreated with normal, diabetic, glycated and oxidized HDL for 24 hours. To produce experimental metastasis, BALB/c nude mice were injected intravenously with MDA-MB-231 cells (1x10⁵) or MCF7 cells (1x10⁵ or 4x10⁵) in 0.2 ml DMEM via tail veins. After 20 days, the mice were sacrificed, their lungs and livers were resected and photographs were taken after fixation in buffered formalin. The numbers of metastatic nodules on the surface of the lungs and livers were counted.

**Histology**

Tissues were fixed in buffered formalin for 24–48h. After washing in fresh PBS, fixed tissues were processed and embedded in paraffin. Sections (5 milli micron) were collected on microscope slides, deparaffinized and stained with H&E. The images were captured at 10x magnification.

**TC-HUVEC Adhesion Assay**

TC-HUVEC adhesion was measured using rose Bengal stain as previously described method (33). Briefly, HUVEC were seeded onto 96-well plates at a density of 5x10⁴ cells/well. Then tumor cells (pretreated with or without HDL for 6 hours) were plated (5x10⁴ cells/well) and incubated for 30 minutes at 37 °C. Then unattached cells were gently washed twice with 10% FBS-containing DMEM, and 100 μl of 0.25% rose Bengal (Sigma, USA) was added. After 5
minutes, cells were gently washed twice, and then 200 μl ethanol/PBS (1:1) was added to each well. After 30 minutes, the absorbance at 570 nm was recorded. Five parallel wells were set up for each group.

**TC-ECM Adhesion Assay**

Cell adhesion was measured using MTT assay as previously described method (34). Briefly, 96-well flat bottom plates were coated with 2 μg/well basement membrane matrix (Matrigel, BD, Germany) for a hour at 37 °C then blocked with 2% BSA for 2 hours at 37 °C followed by washing twice. Tumor cells seeded on 12-well plate were treated with serum-free DMEM alone or HDL (normal, diabetic, glycated or oxidized) for 6 hours. After detachment with trypsin, the cells (1×10^4/well) were plated on ECM-precoated 96-well plates and then washed twice with PBS to remove non-adherent cells after 45 minutes. MTT colorimetric assays at 490 nm were employed to measure the absorbance of adhesive cells. Four parallel wells were set up for each group.

**Antibody Blocking Experiments**

To determine the contribution of individual integrins to the interaction of MDA-MB-231 and MCF7 cells with HUVEC and ECM, monoclonal antibodies against integrin β1, integrin β2, integrin β3 and integrin αv (Abcam, Hong Kong) were applied. Human polyclonal antibody of IgG (Boster, China) was served as negative control. The detached cells were treated with 5 μg/ml monoclonal antibodies for 30 minutes at 37 °C and then allowed to adhere to HUVEC and ECM for 30 minutes. Five parallel wells were set up for each group.
**Determination of Cell Surface Expression of Adhesion Molecules by Enzyme Immunoassay**

Cells in 96-well plates were grown till 70% confluency, then treated with HDL (normal, diabetic, glycated or oxidized) in serum-free medium for 6 hours at 37 °C. Cells were washed with PBS and fixed with methanol for 5 minutes and then washed with PBS 3 times and blocked with 2% BSA for 2 hours at 37 °C. Cell surface expression of adhesion molecules were determined by primary binding with specific monoclonal antibodies for integrin β1, integrin β2, integrin β3, integrin αv (1:200; Abcam, Hong Kong) followed by secondary binding with a horseradish peroxidase-conjugated antibody (1:3000; Boster, China) as described previously (35). Quantification was performed by determination of colorimetric conversion at an optical density at 450 nm of 3,3,5,5-tetramethylbenzidine using TMB peroxidase EIA substrate kit (Bio-Med innovation, China).

**PepTag Assay for Nonradioactive Detection of PKC Activity**

The PKC activity in the MDA-MB-231 and MCF7 cell lysates was determined by non-radioactive detection kit of protein kinase (PepTag Corporation, USA), following the manufacturer's instructions. Briefly, PKC in the HDL-treated cell lysates was separated by column of DEAE cellulose and was incubated with the brightly colored, fluorescent peptide substrates. Then phosphorylation by PKC of its specific substrate alters the peptide’s net charge from +1 to −1, which allows the phosphorylated and non-phosphorylated versions of the substrate to be rapidly separated on an agarose gel.
Statistical Analysis

The results of multiple observations are presented as the means ± SD and as a representative result of two or three different separate experiments, unless otherwise stated. Data were analyzed using Student’s t test and ANOVA test and values were considered significant at $P < 0.05$. 
Results

Participants

Baseline demographic and clinical characteristics of healthy subjects and patients with type 2 diabetes mellitus are shown in Table 1. Participants were included in the study between May 2009 and Jul 2011.

Pretreatment with diabetic, glycated and oxidized HDL promotes metastasis of MDA-MB-231 and MCF7 cells.

We previously found that diabetic HDL has elevated ability in promoting breast cancer cells proliferation, migration and invasion compared with normal HDL in vitro (36), so we speculated that diabetic HDL may promote breast cancer cell metastasis progression. In order to test our hypothesis, we first checked the metastasis ability of HDL-treated MDA-MB-231 and MCF7 cells in nude mice by tail vein injection, and 6 mice were set for each group (Figure 1A). Due to the higher levels of glycation and oxidization as we previously determined in the diabetic HDL (36), we also tested glycated and oxidized HDL to partly mimic HDL in diabetes individuals. Twenty days after the injection of $1 \times 10^5$ MDA-MB-231 cells, it is observed that D-HDL, G-HDL and Ox-HDL significantly enhanced the lung homing of MDA-MB-231 cells as counted by the number of colonies on the surface of whole lung, with 84.3%, 64.2% and 71.6% increase as compared with control (all at $P<0.001$) and with 103.6%, 81.4% and 89.6% increase as compared with N-HDL treated cells (all at $P<0.001$). By contrast, N-HDL reduced metastasis by 9.5% as compared with control, though no statistic significance was found (Figure 1B). However, no visible nodules on the livers
were found in the mice treated with 1x10^5 MDA-MB-231 cells (data not shown). In addition, no pulmonary and hepatic metastasis was shown in the groups injected with 1x10^5 MCF7 cells (data not shown). Then 4x10^5 MCF7 cells were injected for further study. It is suggested that D-HDL, G-HDL and Ox-HDL significantly promoted both pulmonary and hepatic metastasis of MCF7 cells. The nodules on the lungs of D-HDL, G-HDL and Ox-HDL groups increased by 111.8%, 168.6% and 149.1% as compared with control (P<0.05 for D-HDL; P<0.01 for G-HDL and Ox-HDL) and by 157.1%, 226.1% and 202.4% as compared with N-HDL group (P<0.05 for D-HDL, P<0.001 for G-HDL, P<0.01 for Ox-HDL; Figure 1C); the nodules on the livers of D-HDL, G-HDL and Ox-HDL groups increased by 275.3%, 229.2% and 320.2% as compared with control (all at P<0.01) and by 18.1, 15.7 and 20.4 fold as compared with N-HDL group (P<0.01 for G-HDL; P<0.001 for D-HDL and Ox-HDL; Figure 1D). On the contrary, normal HDL reduced metastasis of MCF7 cells by 7.1% in the lung and 95.9% in the liver as compared with control though without statistic significance. Consistent results were obtained in the hematoxylin and eosin stained sections (Figure 1E). Weight of mice during the study was shown (Supplement Figure 1A, B).

The diabetic, glycated and oxidized HDL-treated MDA-MB-231 and MCF7 cells have increased capacities of adhesion to HUVEC and attachment to ECM.

Tumor cells adhesion to vascular endothelial cells and attachment to ECM are considered as important steps in the metastatic processes of malignant tumor cells. Therefore, corresponding experiments were carried out. Adhesion of MDA-MB-231 (hormone-independent) and MCF7 (hormone-dependent) cells pretreated with diabetic, glycated and oxidized HDL, but not
pretreated with normal HDL, to both HUVEC (Figure 2A) and ECM (Figure 2B) markedly increased. In the TC-HUVEC adhesion assay, the adhesion of MDA-MB-231 cells pretreated with D-HDL, G-HDL and Ox-HDL increased by 24.4%, 32.8% and 32.1% as compared with control respectively (all at $P<0.001$), and increased by 21.9%, 30.2% and 29.5% as compared with N-HDL respectively (all at $P<0.001$); the adhesion of MCF7 cells pretreated with D-HDL, G-HDL, and Ox-HDL increased by 22.7%, 31.0% and 32.6% as compared to control respectively (all at $P<0.01$), and increased by 23.6%, 31.9% and 33.6% as compared with N-HDL respectively ($P<0.05$ for D-HDL; $P<0.01$ for G-HDL and Ox-HDL). In the TC-ECM adhesion assay, the attachment of MDA-MB-231 cells treated with D-HDL, G-HDL and Ox-HDL has increased by 33.7%, 40.3% and 39.0% as compared with control respectively without statistical significance, and have increased by 59.9%, 67.8% and 66.2% as compared with N-HDL respectively (all at $P<0.01$); the attachment of MCF7 cells pretreated with D-HDL, G-HDL, and Ox-HDL increased by 29.4%, 22.1% and 29.0% as compared to control respectively without statistical significance, and increased by 47.9%, 39.6% and 47.4% as compared with N-HDL respectively ($P<0.05$ for G-HDL; $P<0.01$ for D-HDL and Ox-HDL).

By contrast, normal HDL reduced MDA-MB-231 and MCF7 cells adhesion to ECM by 16.4% and 12.5% respectively as compared with control, though no statistic significance was found. Furthermore, HDL from 6 normal controls and 6 diabetic patients at 100 μg/ml apoA-I were used for confirmation. It was suggested that normal HDL hardly stimulate MDA-MB-231 and MCF7 cells adhesion to both HUVEC and ECM, whereas diabetic HDL substantially enhanced the two adhesion capabilities. The adhesion of MDA-MB-231 and MCF7 cells which were induced by diabetic HDL was 40.6% ($P<0.001$) and 20.5% ($P<0.01$)
higher respectively in the TC-HUVEC adhesion assay (Figure 2C), and the attachment of MDA-MB-231 and MCF7 cells induced by diabetic HDL was 27.6% \( (P<0.01) \) and 23.5% \( (P<0.05) \) higher respectively in the TC-ECM adhesion assay (Figure 2D).

**Anti-integrin antibodies inhibit MDA-MB-231 and MCF7 cell adhesion to HUVEC and to ECM.**

Blocking antibodies to integrins were used to determine the roles of integrin \( \beta_1 \), integrin \( \beta_2 \), integrin \( \beta_3 \) and integrin \( \alpha_\nu \) in the adhesion of MDA-MB-231 and MCF7 cells to HUVEC and ECM. Adhesion of MDA-MB-231 and MCF7 cells to HUVEC (Figure 3A) and ECM (Figure 3B) was significantly inhibited in the presence of anti-integrin antibodies. The adhesion of untreated cells was considered as 100%. The adhesion of MDA-MB-231 cells treated with monoclonal antibodies of integrin \( \beta_1 \), integrin \( \beta_2 \), integrin \( \beta_3 \) and integrin \( \alpha_\nu \) to HUVEC was \( (44.51 \pm 19.25\%) \), \( (62.09 \pm 4.75\%) \), \( (45.43 \pm 18.48\%) \) and \( (43.24 \pm 29.15\%) \) at \( P=0.004 \), \( P=0.0031 \), \( P=0.0038 \) and \( P=0.0135 \) respectively; the adhesion of MCF7 cells treated with monoclonal antibodies of integrin \( \beta_1 \), integrin \( \beta_2 \), integrin \( \beta_3 \) and integrin \( \alpha_\nu \) to HUVEC was \( (68.91 \pm 6.59\%) \), \( (59.97 \pm 16.08\%) \), \( 68.49 \pm 14.29\% \) and \( (71.25 \pm 5.75\%) \) at \( P=0.0472 \), \( P=0.019 \), \( P=0.0395 \) and \( P=0.0292 \) respectively. The adhesion of MDA-MB-231 cells treated with monoclonal antibodies of integrin \( \beta_1 \), integrin \( \beta_2 \), integrin \( \beta_3 \) and integrin \( \alpha_\nu \) to ECM was \( (86.56 \pm 5.92\%) \), \( (83.42 \pm 10.62\%) \), \( 76.37 \pm 8.29\% \) and \( (87.33 \pm 7.71\%) \) at \( P=0.0092 \), \( P=0.0264 \), \( P=0.0021 \) and \( P=0.026 \) respectively; the adhesion of MCF7 cells treated with monoclonal antibodies of integrin \( \beta_1 \), integrin \( \beta_2 \), integrin \( \beta_3 \) and integrin \( \alpha_\nu \) to ECM was \( (71.53 \pm 5.71\%) \), \( (69.77 \pm 10.10\%) \), \( 67.56 \pm 8.25\% \) and \( (79.25 \pm 14.52\%) \) at \( P=0.0024 \), \( P=0.0038 \), \( P=0.0017 \) and...
Diabetic, glycated and oxidized HDL induce higher integrins expression on the MDA-MB-231 and MCF7 cell surface.

Integrins have been reported to play a key role in TC-HUVEC and TC-ECM adhesion. Diabetic, glycated and oxidized HDL induced elevated expressions of integrin β1 (Figure 4A), integrin β2 (Figure 4B), integrin β3 (Figure 4C) and integrin αv (Figure 4D) compared with normal HDL on the MDA-MB-231 and MCF7 cell surface. Specifically, for MDA-MB-231 cells, integrin β1 was increased by 21.1%, 22.2% and 24.5% respectively (all at $P<0.001$); integrin β2 was increased by 21.2%, 19.7% and 27.9% respectively (all at $P<0.001$); integrin β3 was increased by 20.0%, 21.8% and 19.3% respectively (all at $P<0.01$); integrin αv was increased by 18.7%, 17.3% and 18.1% respectively (all at $P<0.01$); and for MCF7 cells, integrin β1 was increased by 30.1%, 27.8% and 41.1% respectively ($P<0.05$ for G-HDL, $P<0.01$ for D-HDL and Ox-HDL); integrin β2 was increased by 24.3%, 18.4% and 17.1% respectively ($P<0.001$ for D-HDL, $P<0.01$ for G-HDL and Ox-HDL); integrin β3 was increased by 25.5%, 23.1% and 27.1% respectively (all at $P<0.001$); integrin αv was increased by 35.6%, 40.6% and 52.1% respectively (all at $P<0.001$). Furthermore, 6 normal and 6 diabetic HDL samples at 100 μg/ml apoA-I were used for confirmation. It was suggested that normal HDL hardly stimulated integrins expression, whereas diabetic HDL induced higher integrins expression both on the hormone-independent and hormone-dependent human breast cancer cells. For MDA-MB-231 cells, diabetic HDL induced 17.4% higher integrin β1 ($P<0.001$), 33.5% higher integrin β2 ($P<0.001$), 23.4%
higher integrin β3 (P<0.001) and 25.5% higher integrin αv (P<0.01) compared with normal HDL respectively; for MCF7 cells, diabetic HDL stimulated 30.3% higher integrin β1 (P<0.01), 17.5% higher integrin β2 (P<0.001), 25.5% higher integrin β3 (P<0.001) and 17.6% higher integrin αv (P<0.001) compared with normal HDL respectively (Figure 4E).

**Diabetic, glycated and oxidized HDL-induced MDA-MB-231 and MCF7 cell metastasis involves PKC pathway.**

It is reported that PKC activity is associated with the regulation of integrin-related adhesion. Therefore, PKC activity induced by normal, diabetic, glycated and oxidized HDL was examined. It was shown that diabetic, glycated and oxidized HDL stimulated higher PKC activity compared with normal HDL both in MDA-MB-231 and MCF7 cells (Figure 5A). So far, the results indicated that diabetic, glycated and oxidized HDL may promote MDA-MB-231 and MCF7 cell metastasis via PKC pathway. Therefore, MDA-MB-231 and MCF7 cells were treated with staurosporine, a PKC inhibitor, in order to determine whether the suppression of the PKC pathway would result in the inhibition of breast cancer cell adhesion promoted by diabetic, glycated, and oxidized HDL. Staurosporine presented to be powerful in inhibiting HDL-induced (D-HDL, G-HDL and Ox-HDL) breast cancer cell adhesion to HUVEC (Figure 5B) and to ECM (Figure 5C). After pretreatment with staurosporine, MDA-MB-231 and MCF7 cell adhesion to both HUVEC and ECM was substantially reduced, and no statistical significance was found among the groups of staurosporine-treated cells in both of the adhesion assays. It was suggested that the increased abilities of diabetic, glycated, and oxidized HDL in promoting MDA-MB-231 and MCF7 cell adhesion to HUVEC and ECM were mainly owing to the increased PKC activity. Then next,
the inhibitory effects of staurosporine on integrins expression on the MDA-MB-231 and MCF7 cell surface were examined. Integrin β1 (28.41% decrease, \( P<0.01 \) for MDA-MB-231 cells; 26.49% decrease, \( P<0.01 \) for MCF7 cells), integrin β2 (33.27% decrease, \( P<0.01 \) for MDA-MB-231 cells; 57.57% decrease, \( P<0.001 \) for MCF7 cells), integrin β3 (15.15% decrease, \( P<0.01 \) for MDA-MB-231 cells; 57.11% decrease, \( P<0.001 \) for MCF7 cells) and integrin αv (18.36% decrease, \( P<0.01 \) for MDA-MB-231 cells; 22.47% decrease, \( P<0.05 \) for MCF7 cells) were all significantly suppressed by staurosporine (Figure 5D).
Discussion

Previous study suggests complex associations between T2DM and breast cancer (37). Diabetes may have direct biologic effects on breast cancer risk, clinical and pathological characteristics and outcome. Moreover, certain antidiabetic therapies may have direct activity against breast cancer. Diabetes may also affect breast cancer outcome indirectly, and has been shown to influence medical decision-making regarding screening and management of breast cancer (37). In addition, some studies pointed that it is possible that cancer is well-known to be a pro-inflammatory state while the decreased level of HDL is inefficient to exert its anti-inflammatory effects, which allows tumor cell proliferation, survival and migration (38, 39). Our study provides a novel view and demonstrates the significant difference between diabetic and normal HDL on breast cancer cell metastasis progression.

We previously found that HDL in type 2 diabetes mellitus can be glycated and oxidized, and diabetic HDL was found to promote proliferation, migration and invasion of breast cancer cells \textit{in vitro} as compared with normal HDL (36). Glycated and oxidized HDL produced \textit{in vitro} were used to partly mimic diabetic HDL. Diabetic, glycated and oxidized HDL could induce higher synthesis and secretion of VEGF-C, MMP-2 and MMP-9 from MDA-MB-231 cells (36). It was indicated that diabetic, glycated and oxidized HDL promote MDA-MB-231 cell migration and invasion through ERK and p38 MAPK pathways, and Akt pathway plays an important role as well in MDA-MB-231 cell invasion (36). It drove us to speculate that diabetic HDL may have elevated ability of promoting breast cancer cell metastasis.

In our present study, we have demonstrated that diabetic, glycated and oxidized HDL, which were compared with normal HDL, could promote breast cancer cells metastasis \textit{in vivo}. 
and cell adhesion to HUVEC and ECM in vitro in similar ways, and could induce much higher expression of integrins on the cell surface, and increase the activity of PKC as well. Moreover, we observed that staurosporine, a PKC inhibitor, inhibited breast cancer cell adhesion to HUVEC and ECM which were induced by diabetic, glycated and oxidized HDL, as well as the expression of integrins. The present data demonstrated that the increased capabilities of diabetic, glycated, and oxidized HDL in promoting breast cancer cell adhesion to HUVEC and ECM is mainly due to the elevated PKC activity. The activated PKC could in turn stimulate secretions of integrin β1, integrin β2, integrin β3 and integrin αv, which are of vital importance in promoting breast cancer cell metastasis. Our study used a tail-vein injection model to investigate the effects of normal, diabetic, glycated and oxidized HDL on breast cancer cell metastasis in vivo. A spontaneous metastatic model can be further employed to study the effects of different forms of HDL on breast cancer development in nude mice, including tumor growth and metastasis (40). In addition, fluorescence imaging techniques can be applied to visualize the interaction of breast cancer cells with vasculature and ECM in nude mice, including tumor cell mobility, invasion, metastasis and angiogenesis (41, 42). All the studies were performed on both hormone-independent (MDA-MB-231) and hormone-dependent (MCF7) cell lines. We found that MDA-MB-231 cells presented to have stronger abilities of metastasis both in vivo and in vitro for the reason that MCF7 cells seldom have lung or liver homing after injected with $1 \times 10^5$ cells and that the absolute OD value of the adhesive MDA-MB-231 cells in both TC-HUVEC and TC-ECM assay was higher than that of MCF7 cells (data not shown). It has been reported that the effects of HDL on breast cancer cell proliferation have a higher response in the hormone-independent cells such as MDA-MB-231.
cell line (43). However, our study observed similar effects of diabetic, glycated and oxidized HDL on the cell adhesion to HUVEC and to ECM between hormone-independent (MDA-MB-231) cells and hormone-dependent (MCF7) cells compared with normal HDL.

HDL plays an extremely important role of protecting the cardiovascular system from atherosclerosis, including reversing cholesterol transport (RCT), anti-inflammatory, anti-oxidant properties, anti-thrombotic and etc. For instance, HDL can mediate reverse cholesterol transport through its receptors of SR-BI and ABCAI (44). Also, HDL is potential carriers of enzymatic proteins, such as paraoxonase and platelet-activating factor (PAF)-acetyl hydrolase, which can hydrolyze phospholipid peroxides and cholesteryl ester peroxides (44).

In addition, HDL may inhibit adhesion molecule expression through SR-BI and S1P receptors (especially S1P-1) when an inflammatory cytokine such as TNF-α is a pro-atherogenic stimulant (45). Furthermore, a recent study found that HDL can mediate signal transduction of extracellular signal-regulated kinase (ERK) 1/2 and Akt by an ABCA1-dependent mechanism, leading to increased proliferation and migration of prostate cancer cells (46). However, HDL is found to be modified in many ways under some diseases and such modification could impair the cardiovascular-protective abilities of HDL. For example, glycated HDL may lead to the deterioration of vascular function through altered production of reactive oxygen species and reactive nitrogen species in endothelial cells (47); oxidized HDL induces a dose-dependent increase in reactive oxygen species (ROS) production and elicits a marked increase in the activation of the NF-κB pathway in the endothelial cells, a key player in the inflammatory response (48). Furthermore, oxidized HDL can promote the activation of a network of intracellular kinases including ERK1/2 and p38 MAPK in the endothelial cells.
(48). In our present study, we found glycated and oxidized HDL, as well as diabetic HDL have increased capability of promoting breast cancer metastasis through upregulating the PKC pathway related integrins expression. All the experimental evidence allows us to speculate that the promoting effects of diabetic HDL on breast cancer cell metastasis, at least partially, may be attributed to the glycation and oxidation of HDL. Owing to the complicated composition of HDL, it needs further intensive research to determine the altered parts of HDL (both in quality and in quantity) in diabetic patients and how they act in different ways to advance breast cancer development.

Evidence from epidemiologic studies also demonstrates that some pathologic alterations including changes of lipids profile levels (22, 49) in T2DM could raise higher risk of breast cancer (43). Notably, low level of HDL, frequently presented in the patients with T2DM and metabolic syndrome, is strongly linked with breast cancer (49, 50). Our study here provided supporting biological evidence (Supplement Figure 2A, B) in vitro that low concentration of normal HDL could promote MDA-MB-231 breast cancer cell capacities of adhesion to HUVEC and attachment to ECM, while high concentration of normal HDL is slightly inhibitory.

All the evidence substantially supported the concept that T2DM is closely related to breast cancer, which could at least be partially attributed to the alterations of HDL in T2DM, not only to the declined quantity but also to the altered structures and compositions. Our study demonstrates that the modifications of HDL in the diabetic patients could lead to accelerated breast cancer metastasis progression. This will bring more attention on the therapeutic strategies based on HDL function, especially in the patients of diabetes with breast cancer.
Acknowledgements

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Figure Legends

Figure 1. Effects of the different types of HDL on the pulmonary and hepatic metastasis of human breast cancer cells.

(a) MDA-MB-231 and MCF7 cells were left untreated (C), or treated with normal (N), diabetic (D), glycated (G) and oxidized (Ox) HDL. Control and HDL-treated cells (1×10^5 for MDA-MB-231 cells and 4×10^5 for MCF7 cells) were intravenously injected into BALB/c nude mice via tail vein. After 20 days, lungs and livers were resected and analyzed for metastasis. Representative pictures of lungs and livers were shown.

(b, c, d) Quantitative evaluation of macroscopically detectable metastasis nodules on the surface of the whole lungs and livers. Data is expressed as mean±SD. (***, P<0.001 by one-way ANOVA).

(e) Representative histological photomicrographs of lung and liver tissue sections stained with H&E (10×). Arrows indicate tumor islands.

Figure 2. Diabetic, glycated and oxidized HDL promote more MDA-MB-231 and MCF7 breast cancer cell adhesion to HUVEC and attachment to ECM.

(a) MDA-MB-231 and MCF7 cells were pretreated with normal, diabetic, glycated and oxidized HDL at 100 μg/ml apoA-I for 6 hours and then seeded onto HUVEC-coated 96-well plate for 30 minutes. Relative cell adhesion was determined by Rose Bengal assay and expressed as percentage of HDL treated cells in comparison to control. Data is expressed as mean±SD with five parallel wells. (*, P<0.05; **, P<0.01; ***, P<0.001 by one-way ANOVA).
(b) MDA-MB-231 and MCF7 cells were pretreated with normal, diabetic, glycated and oxidized HDL at 100 μg/ml apoA-I for 6 hours and then plated onto matrigel-coated 96-well plate for 30 minutes. Relative cell adhesion was determined by MTT assay and expressed as percentage of HDL treated cells in comparison to control. Data is expressed as mean±SD with four parallel wells. (*, P<0.05; **, P<0.01 by one-way ANOVA).

(c) MDA-MB-231 and MCF7 cells were pretreated with HDL from 6 normal subjects and 6 patients with type 2 diabetes for 6 hours at 100 μg/ml apoA-I. Cell adhesion to HUVEC stimulated by normal HDL vs. diabetic HDL is shown. (**, P<0.01; ***, P<0.001 by student’s t-test).

(d) MDA-MB-231 and MCF7 cells were pretreated with from 6 normal subjects and 6 patients for 6 hours at 100 μg/ml apoA-I. Cell adhesion to ECM stimulated by normal HDL vs. diabetic HDL is shown. (*, P<0.05; **, P<0.01 by student’s t-test).

Figure 3. Anti-integrin antibodies inhibit MDA-MB-231 and MCF7 breast cancer cell adhesion to HUVEC and attachment to ECM.

MDA-MB-231 and MCF7 cells were incubated with no antibody, negative control antibody, or anti-integrin (β1, β2, β3, αv) antibodies for 30 minutes at 37 °C before the adhesion to HUVEC (a) and attachment to ECM (b). Antibody-blocked groups were compared with control (antibody-untreated groups). Data is expressed as mean±SD with five parallel wells. (*, P<0.05; **, P<0.01 by student’s t-test).

Figure 4. Diabetic, glycated and oxidized HDL are more efficient in stimulating
**MDA-MB-231 and MCF7 cell surface integrins expression.**

MDA-MB-231 and MCF7 cells were treated with normal, diabetic, glycated and oxidized HDL at 100 µg/ml apoA-I for 6 hours. Integrin β1 (a), integrin β2 (b), integrin β3 (c) and integrin αv (d) levels on the cell surface were measured by cell ELISA with five parallel wells. Results were expressed as percentage of HDL treated cells in comparison to control and data is expressed as mean±SD. (*, P<0.05; **, P<0.01; ***, P<0.001 by one-way ANOVA).

(e) MDA-MB-231 and MCF7 cells were treated with 6 normal and 6 diabetic HDL for 6 hours at 100 µg/ml apoA-I. Relative integrins expression on the cell surface stimulated by normal HDL vs. diabetic HDL is shown. (**, P<0.01; ***, P<0.001 by student’s t-test).

**Figure 5. Diabetic, glycated and oxidized HDL stimulate MDA-MB-231 and MCF7 cell adhesion to HUVEC and to ECM involving PKC pathway.**

(a) MDA-MB-231 and MCF7 cells were treated with normal, diabetic, glycated and oxidized HDL, and then cell lysates were subjected to the PepTag nonradioactive PKC assay.

(b, c) MDA-MB-231 and MCF7 cells were pretreated with 5 nM staurosporine, a PKC inhibitor, for 3 hours and then coincubated with normal, diabetic, glycated and oxidized HDL at 100 µg/ml apoA-I respectively for 6 hours. Cell adhesion to HUVEC (b) and attachment to ECM (c) were measured. (*, P<0.05; **, P<0.01; ***, P<0.001 by student’s t-test).

(d) MDA-MB-231 and MCF7 cells were pretreated with 5 nM staurosporine for 6 hours and then integrins expression on the cell surface was measured by cell ELISA. (*, P<0.05; **, P<0.01; ***, P<0.001 by student’s t-test).
Table 1. Patient Characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy Controls (n=102)</th>
<th>Patients With T2DM (n=107)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>46±19</td>
<td>60±13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>50:52</td>
<td>49:58</td>
<td>&gt; 0.05</td>
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<tr>
<td>Fasting glucose, mmol/L</td>
<td>5.10±0.56</td>
<td>8.95±3.95</td>
<td>&lt; 0.001</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>1.05±0.35</td>
<td>1.56±0.95</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.65±0.75</td>
<td>4.77±1.34</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.36±0.30</td>
<td>1.22±0.31</td>
<td>&lt; 0.01</td>
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<tr>
<td>LDL-C, mmol/L</td>
<td>2.55±0.60</td>
<td>2.94±1.18</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

Abbreviations: HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Values are expressed as mean ± SD.
Clinical Cancer Research

HDL of Patients with Type 2 Diabetes Mellitus Elevates the Capability of Promoting Breast Cancer Metastasis

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