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High-Density and Very-Low-Density Lipoprotein Have Opposing Roles in Regulating Tumor-Initiating Cells and Sensitivity to Radiation in Inflammatory Breast Cancer

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Abstract

Purpose—We previously demonstrated that cholesterol-lowering agents regulate radiation sensitivity of inflammatory breast cancer (IBC) cell lines in vitro and are associated with less radiation resistance among IBC patients who undergo postmastectomy radiation. We hypothesized that decreasing IBC cellular cholesterol induced by treatment with lipoproteins would increase radiation sensitivity. Here, we examined the impact of specific transporters of cholesterol (ie lipoproteins) on the responses of IBC cells to self-renewal and to radiation in vitro and on clinical outcomes in IBC patients.

Methods and Materials—Two patient-derived IBC cell lines, SUM 149 and KPL4, were incubated with low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL), or high-density lipoproteins (HDL) for 24 hours prior to irradiation (0–6 Gy) and mammosphere formation assay. Cholesterol panels were examined in a cohort of patients with primary IBC diagnosed between 1995 and 2011 at MD Anderson Cancer Center. Lipoprotein levels were then correlated to patient outcome, using the log rank statistical model, and examined in multivariate analysis using Cox regression.

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Results—VLDL increased and HDL decreased mammosphere formation compared to untreated SUM 149 and KPL4 cells. Survival curves showed enhancement of survival in both of the IBC cell lines when pretreated with VLDL and, conversely, radiation sensitization in all cell lines when pretreated with HDL. In IBC patients, higher VLDL values (>30 mg/dL) predicted a lower 5-year overall survival rate than normal values (hazard ratio [HR] = 1.9 [95% confidence interval [CI]: 1.05–3.45], $P=.035$). Lower-than-normal patient HDL values (<60 mg/dL) predicted a lower 5-year overall survival rate than values higher than 60 mg/dL (HR = 3.21 [95% CI: 1.25–8.27], $P=.015$).

Conclusions—This study discovered a relationship among the plasma levels of lipoproteins, overall patient response, and radiation resistance in IBC patients and IBC patient-derived cell lines. A more expansive study is needed to verify these observations.

Introduction

Inflammatory breast cancer (IBC) comprises approximately 5% of all breast cancer cases and is a rare, phenotypically distinct, highly aggressive form of locally advanced breast cancer (1). IBC is highly invasive and has a high propensity for invading dermal lymphatics and metastasizing to distant organs (2). These features confer an extremely high metastatic potential, which accounts for poor prognosis in patients with IBC: the 5-year overall survival (OS) rate is 40.5% (3). Even with dose escalation and modified treatment regimens, local recurrence rates after radiation remain high, and morbidity due to local failure is significant. In the search for methods to sensitize IBC tumors to conventional therapies, the plasticity of IBC tumor cells has been extensively studied (4).

IBC is enriched in gene signatures identified in tumor-initiating cells (TICs). TICs have been defined several ways but most conservatively are prospectively identified cells that self-renew to initiate tumors in transplantation assays. Enrichment for gene expression found in TICs may contribute to IBC resistance to standard therapies (4). TICs have been shown to be important in resistance to therapies such as radiation and chemotherapy (5–7), and new strategies for advanced cancer therapies increasingly include TIC-targeting drugs. Studies have shown cells that share markers with TICs have an enhancement in DNA repair, including increases in checkpoint kinase 1 (CHK1), checkpoint kinase 2 (CHK2), and RAD51 (8, 9).

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, or statins, have been shown to increase self-renewal of tumor cells via inactivation of Ras homolog family member A and increased accumulation of p27^{kip1} in the nucleus (10). Interestingly, in a retrospective analysis of 723 IBC patients from MD Anderson Cancer Center (MDACC), treatment with a hydrophilic statin was associated with significantly longer progression-free survival (PFS) than without a statin (11). Statins are known to regulate concentrations of lipoproteins in the blood, and low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very-low-density lipoprotein (VLDL) mediate cholesterol homeostasis in the blood (12). Martin and van Golen (13) recently demonstrated that IBC cells display uptake and storage characteristics of cholesterol that are different from those of non-IBC cells. IBC cells were able to maintain intracellular stores of cholesterol in

cholesterol-depleted environments, whereas non-IBC cells could not (13). Even though statins are known to decrease the “bad” cholesterol LDL and VLDL and increase the “good” cholesterol HDL (14, 15), the effects of regulating these lipoproteins has never been established at the cellular level. Furthermore, the role of specific cholesterol transport lipoproteins on TIC survival and radiation sensitivity in IBC cell lines has not been reported.

We hypothesized that altering IBC cellular cholesterol induced by treatment with lipoproteins increased radiation sensitivity. We examined the impact of specific transporters of cholesterol (ie lipoproteins) by using in vitro assays of self-renewal and radiation resistance of IBC cells and on clinical outcomes in IBC patients. Together, our analyses of patient outcomes and preclinical data specifically demonstrate that radiosensitization of IBC by statins was due to changes in HDL and VLDL levels and that dyslipidemia (ie clinically low HDL and high VLDL) was associated with worse outcomes among IBC patients.

Methods and Materials

Cell culture and reagents

The SUM 149 IBC cell line is commercially available (Asterand, Detroit, MI). Cells were incubated under normal cell culture conditions with Hams-F12 cell culture medium supplemented with 1 µg/mL hydrocortisone, 5 µg/mL insulin, and 1% antibiotic-antimycotic. The KPL4 IBC cell line was maintained in Dulbecco’s modified Eagle medium (DMEM)/F-12 medium supplemented with 10% fetal bovine serum. VLDL, LDL, and HDL were purchased from Sigma-Aldrich (St. Louis, MO). For all experiments including lipoproteins, serum was not added to the medium to remove the cholesterol and lipoprotein contained in fetal bovine serum.

Mammosphere formation assay

To generate primary mammospheres, we pretreated cells in monolayer culture with lipoproteins for 24 hours, and untreated cells were grown in standard mammosphere medium (serum-free, growth factor enriched). Low-attachment plates were used as described previously (16). For secondary mammosphere assay, cells from primary mammospheres were dispersed with 0.05% trypsin seeded in ultra-low attachment plates (20,000 cells/mL) in mammosphere medium, incubated for 7 days and counted. All 3 lipoproteins were used at a concentration of 10 µg/mL in all in vitro experiments to simulate normal cholesterol lipoprotein levels found in human blood samples.

Clonogenic survival assays

Clonogenic viability of the SUM 149 and KPL4 cells was tested in triplicate under both standard monolayer conditions (referred to here as 2-dimensional [2D] culture) and 3D culture conditions. Cells in 2D culture were incubated for 14 days after treatment with VLDL, LDL, or HDL and irradiation. Crystal violet staining (10%) was used to mark colonies with 50 or more cells (300-µm diameter). Survival curves were obtained for all groups, and curves were fitted on the basis of the linear quadratic model.

Delivery of radiation

Cell lines were grown to 75% confluence, and lipoproteins were added to the cell culture medium 24 hours prior to irradiation. Cells were irradiated using a cesium-137 source (Shepherd Irradiator; JL Sheperd and Associates, San Fernando, CA). Cells were washed twice with phosphate-buffered saline solution (PBS) and plated for 2D culture; 14 days later, colonies were stained with crystal violet. For 3D culture, cells were plated for 7 days immediately after irradiation. Spheres with a minimal size of 50 μ m were counted using a colony counter (Gelcount; Optronix, Oxford, UK).

Cholesterol staining

SUM 149 cells were incubated with HDL or VLDL for 24 hours and fixed and stained with the cholesterol-specific stain filipin III (cholesterol kit, catalog no. ab133116; Abcam), a bacterial byproduct that fluoresces after interacting with an isomer of cholesterol. Image J software (National Institutes of Health, Bethesda, MD) was used to quantify the mean fluorescence for a sample of 6 images.

Immunoblotting

Western blot analysis was performed with precast gradient gels (Novex Life Technologies) using standard methods. Briefly, cells were lysed in Radio-Immunoprecipitation Assay Buffer containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich). Membranes were probed with the specific primary antibodies, followed by peroxidase-conjugated secondary antibodies. The following antibodies against pEGFR, EGFR, Akt, pAkt, FOXO3a, and pFOXO3a (Cell Signaling) were used.

Immunofluorescence

Following irradiation at time points of 30 min, 4 hours, and 24 hours, cells were cultured in chamber slides overnight and fixed with 4% formaldehyde in PBS for 30 min, followed by permeabilization with 0.1% Triton X-100 in PBS for 1 hour. Cells were then blocked for nonspecific binding with 1% bovine serum albumin for 1 hour, and incubated with the γ H2AX antibody (1:400 dilution; Cell Signaling) overnight at 4°C. Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG; 1:400 dilution; Cell Signaling) for 1 hour at room temperature. Coverslips were mounted on slides by using antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Cell Signaling).

Patient data

Patient and tumor characteristics for women with stage III or stage IV primary IBC diagnosed between January 12, 1995, and January 27, 2011, at MDACC were reported previously under a protocol approved by the MDACC Institutional Review Board (11). Data for lipid and cholesterol levels were available for 193 of the women in this cohort and were extracted from the laboratory results database. These data were merged into the patient tumor data, which included statin use, and these 193 patients comprised the cohort examined in this analysis.

Statistical analysis

For in vitro studies, statistical significance was determined by using Student *t* test, calculated by Origin software. For patient data, associations between categorical variables were assessed via cross-tabulation and the χ^2 test or Fisher exact test, where appropriate. Five-year OS was estimated by using the Kaplan-Meier method. Both univariate and multivariate Cox proportional hazard models were applied to assess the effect of covariates of interest on OS.

Results

In vitro lipoprotein treatment of IBC cells results in changes in self-renewal and radiation sensitivity

We determined the effects of treatment with lipoproteins on the capacity of IBC cells to form secondary mammospheres under 3D culture conditions. Secondary mammosphere-forming efficiency is an in vitro assay of self-renewal, a critical attribute of stem-like cells (16). VLDL significantly increased primary and secondary mammosphere-forming efficiency in both SUM 149 and KPL4 cells compared to that in untreated cells (Fig. 1). In SUM 149 cells, the effect of VLDL on secondary mammosphere formation was slightly greater than that on primary mammosphere formation (50% vs 33% enhancement; $P<.05$). For KPL4 cells, primary and secondary mammosphere formation was enhanced 42% by VLDL treatment. Conversely, HDL treatment significantly reduced the number of mammospheres formed compared to that in untreated cells in both cell lines: mammosphere formation by SUM 149 cells was reduced by 21%, and mammosphere formation by KPL4 cells was reduced by 70% ($P<.05$) (Fig. 1). LDL treatment, on the other hand, was associated with no significant differences in mammosphere formation compared to untreated IBC cells. These findings suggest that VLDL increases and HDL decreases self-renewing cells that survive under low attachment, serum-free conditions.

In light of the radiation resistance of cells capable of growing as mammospheres and our previous work demonstrating that statin treatment sensitized mammospheres to radiation (17), we investigated the effect of lipoprotein treatments on radiation sensitivity of IBC cells. Clonogenic assays of SUM 149 and KPL4 cells treated under the same conditions as those for the mammosphere formation assay (10 $\mu\text{g/mL}$ of VLDL, HDL, or LDL) were performed. After 24 hours of lipoprotein treatment, cells were irradiated with increasing doses of radiation, between 0 and 6 Gy, and then seeded in 2D or 3D culture conditions (Fig. 2.). Consistent with the findings of mammosphere-forming efficiency, VLDL increased radiation resistance and HDL enhanced radiation sensitivity of both of the IBC cell lines. These findings led us to hypothesize that radiation sensitization of IBC could be due to the increased and decreased cellular levels of cholesterol induced by VLDL and HDL, respectively.

Decreased IBC cellular cholesterol concentrations correlate with decreased growth factor signaling and increased DNA repair following radiation

To elucidate the role of cholesterol transport in response to radiation, we sampled cells from the clonogenic assays treated with VLDL or HDL to image intracellular levels of cholesterol

before irradiation. VLDL-treated cells showed a 1.3-fold increase in cholesterol content in the intracellular environment ($P=.03$), whereas cells treated with HDL showed a 3-fold decrease in intracellular cholesterol content ($P<.01$) (Fig. 3). To explore the mechanistic connection between lipoprotein treatment and response to radiation, we examined reverse-phase protein array data from statin-treated breast cancer cells (data not shown) and observed Akt and FOXO3a to be regulated by statins. In addition, Van Laere et al (4) previously showed higher (Akt) and lower (FOXO3a) expression levels in IBC patients than in non-IBC patients. AKT and FOXO3A signaling is a key cytoprotective response in many cell types downstream of the EGFR family receptor (18, 19). SUM 149 (Fig. 4A) and KPL4 (Fig. 4B) cells treated with HDL or VLDL for 24 hours prior to EGF (15 ng/mL) stimulation displayed lower HDL and higher VLDL levels of the protein ratios pEGFR:EGFR, pAKT:Akt, and pFOXO3a:FOXO3a than control SUM 149 and KPL4 cells.

Gamma-H2AX (γ H2AX) is a marker for DNA double-strand repair after ionizing radiation treatment, and γ H2AX foci persist longer in radiation-sensitive cell lines than in radiation-resistant lines (20). We observed persistence (HDL-treated) and decreased (VLDL-treated) γ H2AX foci at 4 hours after 4-Gy ionizing radiation treatment (HDL was 4.27-fold increased, $P=.001$; and VLDL was 0.15-fold decreased, $P=.009$) and continued differences at 24 hours (HDL was 5.5-fold increased, $P=.001$; and VLDL was 0.1-fold decreased, $P=.005$) (Fig. 4C), indicating that HDL-treated IBC cells were less able to repair DNA lesions and VLDL-treated IBC cells were better able to repair DNA lesions. It is important to highlight the fact that HDL-treated SUM 149 cells had higher baseline γ H2AX foci starting at 0.5 hour following irradiation (1.8-fold increase, $P=.002$), whereas VLDL-treated SUM 149 showed similar γ H2AX foci (0.8-fold decrease, $P=.27$) after 0.5 hour.

Dyslipidemia in IBC patients predicts overall survival

To expand the relevance of this work to patients, we correlated lipoprotein levels to OS in the cohort of 193 patients with stage III or stage IV IBC. Their lipid and cholesterol panels were analyzed; patients with stage III or IV disease were analyzed together as there were no significant differences according to stage ($P=.1$). A summary of patient characteristics is presented in Table E1, available online at www.redjournal.com. Lipid panels were drawn according to physician preference and practice and presumably represent a bias toward women known to have dyslipidemia or risk factors for dyslipidemia.

The demographic and clinical characteristics of this subset of 193 women were similar to those of the larger cohort (11). The women were older (78% older than 45 years of age), demonstrated high body mass index (48% BMI >30), and were predominantly postmenopausal (60.6%). Approximately 20% of the patients had triple-negative disease, and the majority of patients received radiation therapy (75%). The patients had higher than normal LDL (63% had >100 mg/dL) and VLDL (38% had >30 mg/dL) levels and lower than normal HDL levels (73% had <60 mg/dL). Statin users comprised 24% of the total number. Statin users had significantly better locoregional control of disease (82% vs 54%, respectively, $P=.003$). No local failures occurred among statin users for a period of 36 months after treatment. Patients with HDL level higher than 30 mg/dL had a significantly higher 5-year local-regional recurrence-free survival rate than patients with HDL level less

than 30 mg/dL (98% vs 74%, respectively, $P=.003$). Kaplan-Meier curves for 5-year OS and local-regional recurrence-free survival by baseline HDL levels are shown in Figure 5.

The risk factors included in the multivariate model ($P<.25$ in univariate analysis) were estrogen receptor status ($P=.02$), progesterone receptor status ($P=.04$), *HER2* status ($P=.11$), triple-negative status ($P<.0006$), total cholesterol level ($P=.11$), HDL level ($P=.03$), VLDL level ($P=.06$), statin use ($P=.23$), and menopausal status ($P=.13$) (Table E2; available online at www.redjournal.com). In the final multicovariant Cox model, triple-negative breast cancer, HDL, and VLDL were significant predictors of 5-year OS (Table 1). Patients with higher-than-normal VLDL level had a significantly lower 5-year OS rate than patients with a normal VLDL level (hazard ratio [HR] = 1.9 [95% confidence interval [CI]: 1.05–3.45], $P=.035$). Similarly, patients with a lower-than-normal HDL level had a significantly lower 5-year OS rate than patients with a normal HDL level (HR = 3.21 [95% CI: 1.25–8.27], $P=.015$). Of the 72 patients who had died by January 27, 2011, 68 died of disease. Of the remaining 4 patients, 3 were in the no-statin group; 1 died of a fall, 1 of metastatic endometrial cancer, and 1 of unknown cause. Removing stage IV, the local-regional recurrence and OS rates remained significant for HDL versus those patients without, 97% versus 68% local-regional recurrence, $P=.004$; 84% versus 59%, respectively; $P=.015$. The trend remained for OS in stage IV patients but was not significant, suggesting use of the entire cohort makes the results more conservative rather than biased.

Discussion

In this study, we demonstrated that the lipoproteins VLDL and HDL had opposite effects on IBC cells grown as mammospheres and on these cells' radiation resistance in vitro and that the level of both lipoproteins predicted 5-year OS in IBC patients.

Previous studies have shown that cholesterol and LDL and VLDL are significantly elevated in breast cancer patients compared to those in controls (21). In addition, hypercholesterolemia was discovered to be an independent risk factor for breast cancer in postmenopausal women (22–24). The cholesterol metabolite 27-hydroxycholesterol increased estrogen-receptor-positive breast tumor growth in vivo through a liver X receptor agonist mechanism (25). Furthermore, mice fed a high cholesterol diet developed more aggressive tumors and had significantly more metastasis in a breast cancer model (26). We have previously shown statins, which inhibit de novo cholesterol synthesis, sensitize IBC cells to radiation in vitro (27). Herein we observed in vitro intracellular levels of cholesterol in IBC cells are altered by HDL and VLDL treatment, ultimately modifying response to radiation by reducing (HDL) or increasing (VLDL) self-renewal of these cells, as shown by our mammosphere-forming assay. Importantly, a clinical correlation between HDL and local control after post-mastectomy radiation is identified. Of course, independent validation is critical, and it remains to be seen whether this effect is limited to IBC as might be predicted by the in vitro work by Martin and van Golen (13) showing unique cholesterol properties in IBC cells (13).

Cholesterol plays an important role in cell membrane integrity and fluidity (28). Several growth factor signaling receptors are located in cholesterol-rich domains in the membrane,

and reverse-phase protein array analysis of cells treated or not with statin suggested signaling downstream of EGFR was regulated by statins. Interestingly, it is well known that EGFR is activated by association with cholesterol-mediated lipid rafts in the membrane (29, 30). We examined these as potentially regulated signals in direct lipoprotein treatments by HDL inhibited phosphorylation of EGFR, Akt, and FOXO3a, and VLDL was observed to increase the levels of these phosphorylated proteins. Phosphorylated Akt is downstream of EGFR and has been shown to be important in radiation resistance (31–33). FOXO3a is a tumor suppressor, inactivated by phosphorylation, which increases cell death following DNA damage from radiation (34). We showed DNA damage repair was decreased with HDL treatment and increased in VLDL treatment following radiation (Fig. 4). We speculate that cholesterol-enriched IBC membranes promote EGFR signaling through lipid raft formation and that HDL can directly reduce lipid raft-mediated signaling by removing cholesterol from the membrane.

Conclusions

In conclusion, our results demonstrate a potential role for dyslipidemia in radiation sensitivity and survival among IBC patients. We also highlighted the urgent need for further studies of the potential benefit of aggressively correcting dyslipidemia in IBC patients at the time of diagnosis and of the role of altering lipid profiles in IBC patients without clinical dyslipidemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Studies have shown that cholesterol-lowering statins taken by inflammatory breast cancer (IBC) patients increased survival. The present study investigated mediators in cholesterol transport and lipoproteins and their influence on IBC cancer cells and how dyslipidemia influences IBC patient outcomes. We show very-low-density lipoproteins (VLDL) and high-density lipoproteins (HDL) have opposite effects on IBC self-renewal and sensitivity to radiation. HDL values in patients were protective, and IBC patients with low VLDL values had worse outcomes.

Fig. 1.

The impact of lipoproteins on mammosphere formation was demonstrated in three-dimensional culture. (A) SUM 149 (left) and KPL4 (right) cells were treated with LDL, HDL, or VLDL (10 $\mu\text{g}/\text{mL}$) and incubated for 24 hours to evaluate mammosphere formation (* $P < .05$ in triplicate independent experiments). (B) Light microscopy images (magnification 40 $\times$) of the primary mammospheres in culture. VLDL was observed to increase growth of primary and secondary mammospheres and HDL to inhibit this growth. HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very-low-density lipoprotein.

Fig. 2. Effect of lipoprotein treatment on radiation response of SUM 149 (left) and KPL4 (right) cells. (A) We observed in two-dimensional (2D) cultures that VLDL caused radiation protection, HDL was sensitized to radiation, and LDL showed no effect on radiation response in either IBC cell line. (B) The same effects were observed in mammosphere 3D culture. HDL = high-density lipoprotein; IBC = inflammatory breast cancer; LDL = low-density lipoprotein; VLDL = very-low-density lipoprotein.

Fig. 3.

In vitro detection of cholesterol levels in SUM 149 cells by filipin staining. (A) Microscopy images (magnification $\times 40$) demonstrate intracellular cholesterol-rich domains after treatment with VLDL, HDL, or a combination. (B) Quantification of mean fluorescence activity is shown. HDL = high-density lipoprotein; VLDL = very-low-density lipoprotein.

Fig. 4. Immunoblotting of pEGFR, pAKT, pFOXO3a, and actin in SUM 149 (A) and KPL4 (B) cells treated with either HDL or VLDL for 24 hours. (C) γ H2AX and DAPI staining of SUM 149 cells treated with either HDL or VLDL for 0.5 hour or 4 and 24 hours after 4-Gy ionizing radiation. Original magnification $\times 100$. DAPI = 4',6-diamidino-2-phenylindole; HDL = high-density lipoprotein; VLDL = very-low-density lipoprotein.

Fig. 5.

(A) Locoregional recurrence-free survival curves compare patients with high levels of HDL (>30 mg/dL) and those with low levels of HDL (<30 mg/dL; $P=0.004$). (B) Overall survival curves compare patients with normal versus those with abnormal serum HDL levels, determined from blood samples collected at the time of IBC diagnosis.

Table 1

Multivariate Cox model for overall survival

Variable	Hazard ratio (95% confidence interval)	P value
Triple Negative Status	2.52 (1.35–4.68)	.003
HDL (<60 mg/dL)	3.21 (1.25–8.27)	.015
VLDL (>30 mg/dL)	1.90 (1.05–3.45)	.035