Cholesterol Regulates VEGFR-1 (FLT-1) Expression and Signaling in Acute Leukemia Cells

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Abstract

VEGF receptors 1 (FLT-1) and 2 (KDR) are expressed on subsets of acute myeloid leukemia (AML) and acute lymphoid leukemia cells, in which they induce cell survival, proliferation, and migration. However, little is known about possible cofactors that regulate VEGF receptor expression and activation on leukemia cells. Here we show that cholesterol accumulates in leukemia-rich sites within bone marrow of xenotransplanted severe combined immunodeficient (SCID) mice. Therefore, we hypothesized that cholesterol-rich domains might regulate FLT-1 signaling and chemotaxis of acute leukemias. We then showed that FLT-1 accumulates in discrete cholesterol-rich membrane domains where it associates with caveolin-1 and that placenta growth factor (PlGF)/VEGF stimulation promotes FLT-1 localization in such cholesterol-rich domains. Accordingly, FLT-1 localization and its phosphorylation are abrogated by methyl-β-cyclodextrin (MβCD), which removes cellular cholesterol, and by nystatin, an inhibitor of lipid-raft endocytosis. Mechanistically, cholesterol increases FLT-1 expression and promotes PlGF/VEGF-induced leukemia cells viability and also induces VEGF production by the leukemia cells in vitro. Taken together, we conclude that cholesterol regulates VEGF:VEGFR-1 signaling on subsets of acute leukemias, modulating cell migration, and viability, which may be crucial for disease progression. Finally, we provide evidence obtained from human AML samples that primary leukemia cells accumulate significantly more cholesterol than do normal cells and that cholesterol accumulation correlates with disease aggressiveness.

Introduction

Acute leukemia cells have been previously shown to express VEGF receptors (VEGFR), which can be stimulated in a paracrine or autocrine manner, resulting in increased cell survival, proliferation, and migration (1). VEGF signaling through VEGFR-1 (FLT-1) on acute leukemias was shown to involve p38 and Erk1/2 activation, resulting in caveolae formation (2). Others have shown that VEGF stimulation of subsets of leukemias results in the activation of a downstream signaling pathway which mainly involves the activation of the phosphoinositide-3 kinase pathway (3).

Concerning the function of the different VEGF receptors on leukemia cells, we have recently shown that FLT-1 mediates leukemia migration within the bone marrow microenvironment, promoting leukemia expansion and ultimate exit to colonize extramedullary sites (4). These findings led us to hypothesize that other signals within the bone marrow microenvironment might cooperate or promote VEGF signaling on leukemia cells, which, in turn, would contribute toward favoring leukemia migration and invasion, worsening disease outcome.

Plasma membrane lipid-raft domains, which contain high concentrations of cholesterol and sphingolipids, are known to function as centers of signaling complexes. The ability of lipid rafts to enhance receptor signaling has led to the concept of a signalosome, a region where proteins are localized together to facilitate receptor signaling. A vast body of literature is available about the localization of epidermal growth factor (EGF) receptors in lipid rafts (5). Much less is known about the involvement of membrane-rich lipid domains and VEGF signaling (6). Our recent data showed that inhibitors of lipid-raft assembly, including nystatin, blocked VEGF-induced leukemia migration (4), which strongly suggested that cholesterol-rich domains might in fact regulate VEGF signaling on malignant cells such as acute leukemias.
In the present report, we exploit the biochemical pathways involved in VEGF signaling in acute myeloid leukemia (AML) cells and show that FLT-1 is modulated by cellular cholesterol. We show that FLT-1 colocalizes with membrane-rich cholesterol domains, whose assembly is essential for FLT-1 expression and activation on leukemia cells. Moreover, cholesterol content of acute leukemia patient samples correlates with disease aggressiveness. As such, our data reveal novel possibilities of therapeutic intervention on subsets of acute leukemias.

Materials and Methods

Cell culture
HEL, HL60, and 697 cells were obtained from American Type Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 mmol/L L-glutamine, and 1% penicillin-streptomycin (Invitrogen Life Technologies). HUVECs were maintained in endothelial cell growth medium containing 5% FBS (EBM-2; Cambrex, Inc.) at 37°C with humidified 95% air/5% CO₂.

Human samples
Sixteen patient samples (bone marrow or peripheral blood) were used and grouped in 4 pediatric AML samples, 2 of them in clinical bone marrow remission; 4 adult samples I, between 30 and 35 years of age; 7 adult samples II, between 74 and 79 years of age; 1 in clinical bone marrow remission. Also, 1 MDS sample was used in the adult sample II group. Mononuclear cells were collected from bone marrow/peripheral blood and isolated using Ficoll/Histopaque gradient. Dry pellets were processed for cholesterol quantification according to Amplex Red kit.

RNA extraction and quantitative real-time PCR
Leukemia cells were analyzed for VEGF-1 (FLT-1) by quantitative real-time PCR (qRT-PCR; ABI PRISM 7700 Sequence Detection System and SYBR Green Master Mix Kit; Applied Biosystems). Total cellular RNA was extracted using Trizol protocol (Sigma-Aldrich), and cDNA was synthesized following conventional protocols. The 18S gene was used as a standard reference. The relative expression of FLT-1 and KDR was obtained using comparative threshold cycle (CT) method. Primer sequences used were as follows: FLT-1 sense; 5'‑GCTCGCGGAAAGTTGTAT‑3'; FLT-1 antisense; 5'-CTCCTCCCGGCAATTTCTCA‑3'; KDR sense; 5'-ATTCTCTCCCGCCCATCA‑3'; KDR antisense; 5'-GTCTCGTGGCGCAGTCCTT‑3'.

Whole-cell lysate preparation and Western blotting
Acute leukemia cells were stimulated for 30 minutes with recombinant VEGF₁₆₅ (50 ng/mL), placenta growth factor (PlGF; 100 ng/mL) and/or treated with for 2 to 4 hours with MβCD (10 or 20 mmol/L), cholesterol + MβCD (0.2 or 0.4 mmol/L), and nystatin (50 μg/mL). After stimulation/treatment, total protein extracts were obtained by suspending cell pellets in cold buffer A [50 mmol/L Tris-HCl, pH 7.4; 1% (v/v) Triton X-100; 150 mmol/L NaCl; 1 mmol/L EDTA; 0.1% (v/v) SDS], in the presence of protease and phosphatase inhibitors, for 30 minutes on ice followed by centrifugation at 12,000 × g, for 15 minutes at 4°C. Protein concentrations were determined using the Bio-Rad Laboratories DC protein assay kit and equal amounts were separated by SDS-PAGE gels, transferred onto nitrocellulose membranes, and processed for Western blotting. Primary antibodies used were as follows: anti-FLT-1 (0.5 μg/mL; Santa Cruz Biotechnology, Inc.), anti-caveolin-1 (1:100; BD-Biosciences), anti-phosphotyrosine (1:50, 0.5 μg/mL; Santa Cruz Biotechnology, Inc.), anti-β-actin (1:2,500; Sigma-Aldrich). Horseradish peroxidase–conjugated secondary antibodies were used at a dilution of 1:2,500, and the enhanced chemiluminescence detection system and Kodak films (Amersham Pharmacia Biotech) were used to visualize the presence of proteins on the nitrocellulose blots. Bands were quantified using Image J software (rsb.info.nih.gov/ij/).

Immunoprecipitation assay
HEL lysates (900 μg) were precleared (1 hour) with Protein G-Sepharose beads (Sigma Aldrich) and then incubated overnight at 4°C with anti-FLT-1 (1 mg/mL; Santa Cruz) or with rabbit IgG as a negative control of the coimmunoprecipitation procedure. Protein G-Sepharose beads were then added and mixed for 2 hours at 4°C. Beads were recovered by centrifugation, washed with cold buffer A or with buffer A supplemented with high salt concentration (500 mmol/mL NaCl), and resuspended in (20 μL) Laemmli buffer. After boiling at 95°C for 5 minutes, the immunoprecipitates were analyzed by 8% SDS-PAGE gels followed by Western blotting with anti-phosphotyrosine and anti-FLT-1 antibodies (1 μg/mL; Santa Cruz).

Sucrose density centrifugation and isolation of lipid-raft fractions
Acute leukemia cells (HEL; 1 × 10⁶) stimulated for 30 minutes with PlGF (100 ng/mL) or treated for 1 hour with IR1 inhibitor (2 μmol/L; Calbiochem), MβCD (10 mmol/mL), and cholesterol + MβCD (0.2 mmol/mL) were washed with PBS and cell pellets were suspended in 1.0 mL of 1% (v/v) Triton X-100 in TNEV buffer [100 mmol/mL Tris-HCl (pH 7.5), 150 mmol/mL NaCl, 5 mmol/L EDTA, 1 mmol/L Na₂VO₃, 1 mmol/L PMSF, 1× protease inhibitors (Roche)] on ice for 60 minutes. Cells were homogenized with 10 passages through a 22-gauge needle and nuclei were removed by centrifugation at 800 × g for 8 minutes at 4°C. The supernatants were mixed 1:1 in 85% sucrose (v/v)/TNEV buffer. The mixture (2 mL) was transferred to the bottom of the ultracentrifuge tube, and 2 solutions with different sucrose concentrations in TNEV buffer were added sequentially [6 mL of 35% (v/v) sucrose and 3.5 mL of 5% (v/v) sucrose]. The discontinuous gradients were separated by centrifugation in a swing-out rotor (SW41Ti) at 38,000 × g during 18 hours at 4°C in a
Beckman XL-80 Ultracentrifuge. One-milliliter fractions were collected sequentially from the top to the bottom of the tube, and Western blot analyses were done with antibodies against FLT-1, caveolin-1. After identification by Western blot fractions from sucrose gradients (lipid raft, 4 and 5; cytosolic fractions, 10 and 11) were concentrated by centrifugation (4,000 × g for 20 minutes at 4°C), using Amicon Ultra-4 devices with 10-kDa cutoff membranes (Millipore).

Cell viability and migration assays
Cells (1 × 10⁶/mL) were cultured in serum-free RPMI medium for 48 hours in the presence of PlGF (100 ng/mL), MβCD (10 mmol/L), and cholesterol + MβCD complexes (0.4 mmol/L). When applicable, cells were previously treated for 2 hours with 2 μmol/L IR1 (tyrosine kinase inhibitor of FLT-1; Calbiochem.) or with neutralizing antibodies directed to FLT-1 (1 μg/mL 6.12 antibody; Imclone). Cell viability was determined at 24 and 48 hours by Trypan Blue exclusion and cell counts (with the aid of a hemocytometer). Each experiment was done in triplicate. Cell migration was assayed using a modified version of a Transwell migration technique described previously (7). Serum-starved cells (1 × 10⁶ cell/mL) were treated for 4 or 18 hours with cholesterol + MβCD. Cell aliquots (100 μL) were added to 8-μm pore Transwell inserts of migration system (6.5 μm in diameter; Costar) and allowed to migrate for 4 or 6 hours toward PlGF in the absence/presence of neutralizing antibodies directed to FLT-1. Cell counts were done in 7 distinct power fields (20 × magnification) with an Olympus CK2 microscope. Experiments were done in triplicate, and results are shown as the number of migrating cells per milliliter.

Human VEGF ELISA and cholesterol measurement
VEGF production by leukemia cell lines was determined by ELISA. VEGF in serum-free medium was quantified using the Human VEGF ELISA kit (Calbiochem.). Cellular cholesterol was detected using the Amplex Red Cholesterol Assay Kit according to manufacturer’s instructions (Molecular Probes).

Immunofluorescence
Leukemia cells (5 × 10⁶) were serum starved for 16 hours and further attached to poly-l-lysine–coated coverslips for 10 minutes at 37°C. After a brief wash in PBS, cells were stimulated with PlGF (100 ng/mL) for 30 minutes and/or treated with MβCD (10 mmol/L) or cholesterol + MβCD complex (0.2 mmol/L cholesterol) for 2 to 4 hours. The cells were fixed in 2% (v/v) paraformaldehyde for 10 minutes at room temperature, washed twice with PBS, and permeabilized in 0.1% (v/v) Triton X-100 for 30 seconds. After blocking in PBS (Invitrogen Life Technologies) supplemented with 0.1% (w/v) BSA, 5% (v/v) complete goat serum, rabbit anti-human FLT-1 (1.5 μg/mL; Santa Cruz Biotechnology, Inc.), and mouse anti-caveolin-1 (1:100; BD biosciences) were used overnight at 4°C. The cells were washed and incubated with Alexa Fluor 594 or 488 secondary antibodies at 1:500 (Molecular Probes) for 90 minutes and washed with PBS. Samples were mounted in Vectashield and analyzed by confocal microscopy in a True Confocal Scanner Leica TCS SP2 (Leica Microsystems; objectives HCX PL APOCS 63 × 1.4 oil). Sets of optical sections with 0.5-μm intervals along the z-axis were obtained from the top to the bottom of cells. Z-projections of the acquired images were obtained using ImageJ software (rsb.info.nih.gov/ij/).

Bone marrow smears and Nile Red staining
Ten to 12 days after 697 and HL60 cells xenotransplantation, mice were sacrificed and bone marrow [Balb/SCID (severe combined immunodeficient) mice] was removed in toto by flushing a femoral cavity. Bone marrow smears were prepared by streaking the exposed bone marrow onto a glass slide. Pressure while executing the smears was adjusted to disperse cells in a monolayer without disrupting cells and vascular structure integrity. Bone marrow smears were air dried, fixed in cold acetone for 10 minutes, and stained with Nile Red for 15 minutes. Images were acquired on a Zeiss Axioplan microscope with a Zeiss Axioimaging MRm (amplification ×200, 630). Nile Red solution [1:6 diluted in 75% (v/v) glycerol] was prepared from a stock Nile Red solution (100 μg/mL in ethanol; N3013; Sigma). Nile Red stained neutral lipids (yellow-gold emission) with an excitation wavelength of 450 to 500 nm and polar lipids (red emission) with an excitation wavelength of 515 to 560 nm (8).

Electrophoretic mobility shift assay
Nuclear extraction and electrophoretic gel mobility shift assays were conducted following standard methodology as described elsewhere (9). Briefly, oligonucleotide probe (sequence: ACCCCTTGAGTCACCAGAAGG) was labeled with [γ32P]-ATP, using T4 polynucleotide kinase (Promega), and purified in Micro-spin G-50 columns (Bio-Rad). For the electrophoretic mobility shift assay (EMSA) analysis, 10 μg of nuclear proteins was preincubated with EMSA binding buffer (Promega) and 15 ng/μL poly(dI)-poly(dC) at room temperature 10 minutes before the addition of the radiolabeled oligonucleotide for an additional 25 minutes. For Supershift studies, before the addition of the radiolabeled probe, samples were incubated for 30 minutes with 4 μg of CREB-1 antibody (H-74; Santa Cruz).

Statistical analysis
Results are expressed as mean ± SD. Data were analyzed using the unpaired 2-tailed Student t test. The values of P < 0.05 were considered significant.

Results
Acute leukemia cells localize in cholesterol-rich niches in the bone marrow microenvironment in vivo
We have previously observed that acute leukemia cells migrate in an FLT-1 dependent manner within the bone
Avidity for cholesterol of leukemia cells

We compared cholesterol content of mononucleated cells (MNC) isolated from healthy donors with MNCs obtained from AML patients (2A). Patient samples were grouped into pediatric samples, adult sample I (AML patients between 30 and 35 years of age), and adult sample II (AML patients between 64 and 79 years of age). In peripheral blood AML patient samples (AML8 and 15), the levels of intracellular cholesterol were increased by 2-, 4- to 3-, and 4-fold in relation to healthy donor samples. AML patients display increased intracellular cholesterol, in particular older patients with 4- to 6-fold increase (adult samples II). After clinical bone marrow remission, intracellular cholesterol levels decreased to levels compared with that obtained from healthy donors (AML3, 4, and 16-BM R). Also, MDS sample (patient with myelodysplastic syndrome, MDS) has lower levels of cholesterol. Furthermore, pediatric AML samples present 3- to 4-fold increased levels of cellular cholesterol. In Figure 2B, AML cells (HEL and HL60 cell lines) showed 2- to 4-fold significant increase of cellular cholesterol (controls 1 and 2, respectively) when compared with cells isolated from healthy donors. Next, we disturbed cholesterol homeostasis of leukemia cells by exposure for 4 hours to 10 μmol/L methyl-β-cyclodextrin (MβCD), which depletes cholesterol from cellular membranes, or by increasing its cellular cholesterol levels with the use of cholesterol + MβCD complexes (0.4 μmol/L). Cholesterol + MβCD complex treatment increased by 1-, 5- to 2-, and 3-fold cellular cholesterol of leukemia cells when compared with untreated cells. This increase was abolished by both MβCD and nystatin treatments, which inhibit lipid-raft endocytosis. In addition, PlGF treatment also increased intracellular cholesterol of AML cells, an effect that was reverted to control levels (control 2; Fig. 2B) by the use of a neutralizing antibody (Ab) against FLT-1 (6.12 Ab; P < 0.02; Imclone). Acute leukemia cells possess more intracellular cholesterol than normal cells. Furthermore, FLT-1 activation by PlGF further potentiates this effect. In addition, cellular cholesterol is highly increased in AML patients, an effect that was reverted after clinical bone marrow remission.

PlGF induces FLT-1 accumulation and colocalization with caveolin-1 in lipid rafts

We have previously reported that FLT-1 associates in vitro with caveolin-1, the main component of a subtype of lipid rafts (caveolae), in AML cells (2). This suggested that FLT-1-mediated signaling in acute leukemia cells might depend on cholesterol-raft membrane domains. Therefore, in the present study, we determined a more precise membrane location of FLT-1 on leukemia cells and examined whether it was affected by PlGF stimulation and/or cholesterol disturbance. We isolated lipid rafts by sucrose density gradients, and this analysis revealed that FLT-1 and caveolin-1 colocalize in 2 distinct regions of the sucrose gradients: a caveolin-enriched membrane region (fractions 4 and 5; 6%–30% sucrose) and a cytosolic region (fractions 10–11; 35% sucrose), as assessed by caveolin-1 (a component of lipid rafts) co-sedimentation (Fig. 3A). On PlGF/VEGF stimulation, FLT-1 was localized preferentially into lipid-raft fractions (fractions 4 and 5), an effect that was reverted in the presence of the FLT-1 inhibitor IR1 (Fig. 3A). Also, inhibition of FLT-1 by IR1 treatment, removes caveolin-1 from the lipid-raft fractions. Using confocal microscopy, we...
observed that FLT-1 (in red) and caveolin-1 (in green) colocalized in lipid-raft/caveolin-1–rich structures (Fig. 3B; see FLT-1 antibody specificity in Supplementary Fig. 1). To determine whether FLT-1 distribution in lipid rafts was affected by cholesterol disturbance, sucrose gradient–generated lipid-raft fractions 4 and 5 and cytosolic fractions 10 and 11 were concentrated and analyzed by Western blot as before. As shown in Figure 3C, cholesterol enrichment accumulated FLT-1 in lipid-raft fractions, whereas MβCD extracted FLT-1 from lipid-raft fractions (see quantification in Fig. 3D). β-Actin was used as a loading control (Fig. 3C). As determined by confocal microscopy, after cholesterol enrichment, FLT-1 colocalized with caveolin-1 whereas cholesterol extraction with MβCD reduced
this colocalization (Fig. 3E). These results indicating the preferential localization of FLT-1 in lipid rafts after intracellular cholesterol enrichment suggest that FLT-1–raft interactions may regulate PlGF/VEGF-mediated signaling.

**Cholesterol enrichment of acute leukemia cells induces FLT-1 activation in lipid rafts**

In AML cells, FLT-1 is phosphorylated by PlGF/VEGF treatment for 30 minutes (Fig. 4A), as assessed by coimmunoprecipitation of FLT-1 with pan-phosphotyrosine antibody. We determined the consequences of disturbing cholesterol homeostasis for FLT-1 activation on AML cells. We observed that cholesterol extraction abolishes FLT-1 phosphorylation induced by PlGF in a dose dependent manner. In contrast, cholesterol enrichment increased FLT-1 phosphorylation (activation) in the absence or presence of PlGF (Fig. 4B). FLT-1 activation was inhibited by the use of nystatin, an inhibitor of lipid-raft formation and endocytosis, which impeded PlGF-induced FLT-1 phosphorylation (Fig. 4C). β-Actin was always used as loading control. Taken together, these data suggest that FLT-1 activation (phosphorylation) is affected by cellular cholesterol levels and, in particular, by agents that perturb the formation of lipid-rafts/cholesterol-rich membrane domains.

**Cholesterol enrichment modulates PlGF-induced AML cells properties**

To evaluate the role of cholesterol in acute leukemia cells properties, we assessed cell viability, migration, and VEGF...
production on cholesterol-enriched or -depleted cells, alone or in the presence of PIGF. Viability was assessed after 24 to 48 hours by counting cell viability with Trypan Blue exclusion dye. As previously reported, PIGF induced a modest increase in AML cells viability. However, cholesterol enrichment by leukemia cells increased cell viability after 24 hours; interestingly, cotreatment with MβCD abolishes FLT-1 phosphorylation mediated by VEGF/PIGF. In contrast, AML intracellular cholesterol increase activates FLT-1. C, inhibition of lipid-raft formation/endocytosis by nystatin impedes FLT-1 activation. Autoradiographs are representative of similar results obtained from 3 independent experiments. WB, Western blotting.

FLT-1 expression on AML cells increases on cholesterol exposure
Besides its effects in the regulation of FLT-1 activation and subcellular localization, next we examined whether cholesterol levels also affected FLT-1 expression. In fact, cholesterol enrichment upregulates FLT-1 mRNA expression in leukemia cells (Fig. 6A). PIGF further increased FLT-1 expression in cholesterol-enriched cells, an effect that was reverted by the FLT-1 inhibitors. This effect on FLT-1 mRNA expression is characteristic of leukemia cells, as FLT-1 mRNA expression remains unaltered by cholesterol disturbances in human umbilical vein endothelial cells (HUVEC; Fig. 6B). It was previously reported that CREB (cAMP responsive element binding protein)/ATF (activating transcription factor) element regulates the basal transcription of FLT-1 expression (11). Moreover, we observed that increasing intracellular cholesterol levels in AML cells further potentiate the binding of CREB complexes to the FLT-1 promoter.
region, thereby regulating FLT-1 expression (see Supplementary Fig. 3). In contrast, KDR mRNA expression was not altered by increasing cellular cholesterol in leukemia cells (Fig. 6C).

Discussion

In this report, we reveal novel molecular evidence by which cellular cholesterol regulates the function of a receptor tyrosine kinase on malignant leukemia cells. In detail, we show that cholesterol affects FLT-1 expression, localization, and activation, thereby modulating cellular phenotypes including viability, chemotaxis/migration, and VEGF production. The mechanisms by which cholesterol interferes with FLT-1 signaling and cellular localization are still not completely understood but may involve the intracellular transport machinery and the regulation/assembly of specific membrane domains, including vesicles and lipid rafts. Other receptor tyrosine kinases, most notoriously EGF receptor, have been shown to be transported intracellularly in vesicles, in and out of the cell onto the cell nucleus where EGF receptor is shown to activate transcription (5). Whether FLT-1 recycling involves a similar mechanism of intracellular transport is still undisclosed. Nevertheless, the present report reveals that FLT-1 localizes preferentially in specific lipid-enriched membrane...
domains. Detailed biochemical analysis of cellular extracts, together with the use of biochemical inhibitors, suggests that these membrane domains may be considered lipid rafts. As such, our data strongly suggest that lipid rafts are essential for VEGF receptor signaling on malignant leukemia cells.

We have previously shown that subsets of acute leukemias respond to VEGF/PlGF gradients within the bone marrow microenvironment, migrating toward the epiphysis of long bones, from where the leukemia cells leave the bone marrow onto the peripheral blood, en route to other target organs (4). From these findings, it became clear that leukemia cells migrate toward specific “niches” within the bone marrow microenvironment. Recent studies have suggested that leukemia cells “create” specific niches within the bone marrow microenvironment, where the malignant cells thrive and proliferate, eventually replacing the normal hematopoietic elements (12). The signals/molecular cues involved in this “invasion” of the bone marrow by malignant cells are still largely undisclosed but are believed to involve SDF-1:CXCR4 and possibly integrin-mediated signaling (13–15).

Our present report suggests that bone marrow cholesterol levels and the cholesterol distribution throughout the bone marrow may play an important part during leukemia engraftment, expansion, and perhaps also during leukemia spread. In detail, we provide evidence that PlGF/VEGF signaling via FLT-1 is affected by cellular cholesterol levels and that specific cholesterol accumulations are seen in leukemia-rich sites in the bone marrow, in vivo. Interestingly, we have recently discovered that systemic cholesterol levels also affect the levels of SDF-1 in the bone marrow microenvironment (16). This strongly suggests that cholesterol may affect leukemia engraftment and expansion by interfering with SDF-1:CXCR4 signaling and VEGF:FLT-1 signaling, respectively.

A link between cholesterol avidity and acute leukemias has been previously suggested. In our present study, AML patient samples show increased levels of intracellular cholesterol compared with healthy donor samples. Moreover, the bone marrow cholesterol content correlates with disease.

**Figure 6.** Acute leukemia cell exposure to cholesterol upregulates FLT-1 expression. A, as determined by real-time PCR, FLT-1 expression after AML cell treatment with cholesterol + MβCD complexes (4 hours) in the presence/absence of PIGF is significantly upregulated (without PIGF, \( P = 0.048 \); with PIGF, \( P < 0.023 \)). Inhibition of FLT-1 signaling (6.12 neutralizing antibody; IR1 chemical inhibitor) significantly abolishes the upregulated FLT-1 expression obtained after cholesterol cell exposure \( (P < 0.03) \). In contrast, FLT-1 mRNA expression was not affected by cellular cholesterol disturbance in HUVECs. B, KDR (also expressed in these cells) mRNA expression remains unaltered by enriched cellular cholesterol. C, experiments were done in triplicate and represented as the mean \( (n = 3) \).
aggressiveness (and stage); leukemia patients undergoing clinical remission show a corresponding decrease in intracellular cholesterol levels. Other studies have reported that cholesterol uptake by leukemia cells promotes their survival and resistance to chemotherapy (17, 18). These studies led to the use of statins (cholesterol-lowering agents) as therapeutic targets for subsets of acute leukemias (19, 20), with reported clinical activity and efficacy. Nevertheless, to our knowledge, there have been no reports explaining the importance of cholesterol in VEGF-mediated signaling on acute leukemia cells. All together, the findings reported in this article have implications for the understanding of the regulation of the bone marrow microenvironment during the onset/engraftment and expansion/progression of acute leukemias. The biochemical mechanisms described here, showing that lipid-enriched membrane domains regulate VEGFR signaling, may be relevant also in the context of other receptor tyrosine kinases and other tumor types.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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