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Adipose tissue attracts and protects acute lymphoblastic leukemia cells from chemotherapy

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ABSTRACT

Obesity is associated with an increased risk of acute lymphoblastic leukemia (ALL) relapse. Using mouse and cell co-culture models, we investigated whether adipose tissue attracts ALL to a protective microenvironment. Syngeneically implanted ALL cells migrated into adipose tissue within ten days. *In vitro*, murine ALL cells migrated towards adipose tissue explants and 3T3-L1 adipocytes. Human and mouse ALL cells migrated toward adipocyte conditioned media, which was mediated by SDF-1α. In addition, adipose tissue explants protected ALL cells against daunorubicin and vincristine. Our findings suggest that ALL migration into adipose tissue could contribute to drug resistance and potentially relapse.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of cancer in children. With aggressive combination chemotherapy, the overall cure rate is about 80% in children [1] but only 50% in adults [2]. Leukemia relapse continues to be a problem, and is thought to be due to drug resistance [3]. While many studies of drug resistance have focused on acquired gene mutations in leukemia cells, in some studies the leukemia microenvironment has also been shown to play a major role in *de novo* chemotherapy resistance in ALL [4]. It is thought that leukemia cells home to the bone marrow, which acts as a niche that protects ALL cells from drug-induced death [4,5]. Since bone marrow is comprised of many different cell types (*e.g.* mesenchymal stem cells, osteoblasts, endothelial cells, hematopoietic cells, adipocytes), it is not clear which marrow cells are responsible for ALL homing and induction of chemotherapy resistance.

There is growing evidence that adipocytes may interact with cancer cells to promote invasion, proliferation, and/or drug resistance [6–8]. Adipocytes secrete numerous factors which have roles in cancer cell proliferation, migration, and metastasis, such as insulin-like growth factor 1, leptin, platelet-derived growth factor, matrix metalloproteinase 11, interleukin 6 and stromal cell-derived factor 1 (SDF-1α) [9–12]. Some of these factors may contribute to the strong associations observed between obesity and cancer mortality [13] including studies which show that obesity is associated with increased risk of relapse in ALL [14,15]. However, the precise mechanisms whereby adipocytes may contribute to ALL relapse remain unknown.

Previously, we observed the presence of transplanted ALL cells in the fat depots of obese mice by fluorescence microscopy after vincristine treatment [8]. However, since these mice had developed a substantial leukemia burden, and we did not look for ALL cells in other organs, it was not known whether the leukemia cells actively and preferentially migrated into the adipose tissue. Furthermore, the mechanism(s) regulating leukemia cell migration into adipose tissue are not known. In the present study, we report that adipocyte

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secretion of SDF-1α induces ALL cells to migrate into adipose tissue, further demonstrating the importance of adipocytes in the leukemia cell microenvironment.

2. Materials and methods

2.1. Materials

AMD3100, daunorubicin, and vincristine were purchased from Sigma Chemicals (St. Louis, MO). Mouse recombinant SDF-1α, leptin, adiponectin, MCP-1, resistin and RANTES were obtained from Peprotech (Rocky Hill, NJ). All other chemicals and reagents were tissue culture grade.

2.2. Mouse tissue explants

All mouse experiments were approved by the Children's Hospital Los Angeles Institutional Animal Care and Use Committee, and were performed in accordance with the USPHS Policy on Humane Care and Use of Laboratory Animals. C57Bl/6J mice were raised on a 10 kCal% (control) or 60 kCal% (obese) fat diet (Research Diets, New Brunswick, NJ) at the Jackson Laboratory (Bar Harbor, ME). Mice were euthanized at 20 weeks of age by cardiac perfusion with PBS/Heparin under anesthesia. Various tissues were removed rapidly and washed in cold PBS. Small pieces of each tissue (100 mg) were washed twice with RPMI plus 10% FBS, and cultured in the same medium. One day later, the culture medium was changed with fresh medium. Tissue explants were then cultured for an additional two days without media change before use for migration or chemotherapy protection assay.

2.3. Cell lines

Murine pre-B 8093-ALL and GFP positive 8093-ALL cells have been previously described [8,16]. Human leukemia cell lines RS4;11, BV173, SD1 and K562, and Murine fibroblastic 3T3-L1 and OP-9 cells were purchased from ATCC. The human primary leukemia cell strains ICN13, BLQ1, UCSF02, US.7 and TXL-2 [17,18] were kindly provided by Yong-mi Kim and Markus Müschen. ChubS7 cell line was described before [19]. 3T3-L1, OP-9, and ChubS7 cells were cultured at confluence in a 24-well plate and differentiated as previously described [8,19]. Pre-adipocyte (FCM) and adipocyte conditioned media (ACM) were collected after 48 hour conditioning of 3T3-L1, OP-9 and ChubS7 pre-adipocytes and adipocytes.

2.4. Migration assay

For migration of mouse ALL, FCM and ACM were made by culturing 3T3-L1 cells in RPMI medium containing 10% FBS for 2 days. Migration of mouse ALL toward feeder layers were set up with a confluent monolayer of 3T3-L1 and OP9 pre-adipocytes and adipocytes. RPMI medium containing 10% FBS was incubated with the feeder layers for 48 h prior to the assays. For migration of human ALL cells, we generated serum-free ACM in Opti-MEM medium (Invitrogen, Carlsbad, CA) with differentiated 3T3-L1 adipocytes. Migration assays were all performed in 24-well tissue culture plates, using TransWell inserts with 5 μm (for 8093-ALL cells) or 8 μm (for human cells) pores (Millipore, Billerica, MA). ALL cells in RPMI with 10% FBS were seeded into the top chambers. The bottom chambers contained cultured tissue explants (held down by a 1 mm pore size nylon mesh), pre-adipocyte or adipocyte monolayers, or conditioned media. After 1.5 h (8093-ALL cells) or 3 h (human leukemia cells), viable cells in each chamber were quantified by trypan blue exclusion. The number of cells (% migration) that migrated to the bottom chamber was calculated from the total cell count in top and bottom chambers. In some experiments where differentiated adipocytes were used, leukemia cells were collected from the bottom chambers by vigorous pipetting, and counted using trypan blue exclusion.

SDF-1α concentrations in plasma, obese and control mice tissue explant conditioned media, and 3T3-L1 and OP9 FCM and ACM, were measured in duplicate by ELISA (RayBiotech, Norcross, GA).

2.5. Western blot

Total protein was extracted from murine or human leukemia cells with protein isolation buffer [8]. Lysates were sonicated briefly and centrifuged for 10 min at 14,000 rpm. The supernatant was used for protein measurement. Twenty micrograms of protein was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in 5% milk and probed simultaneously with CXCR4 and actin antibody (Cell Signaling Technology, Danvers, MA) because CXCR4 and actin have clearly distinct molecular weights.

2.6. In vivo leukemia cell implantation

To determine whether leukemia cell migration into adipose tissue is an early event *in vivo*, GFP positive 8093-ALL cells were implanted retro-orbitally into 20-week old syngeneic obese and lean C57Bl/6J mice (10,000 cells per mouse, [8], 6 mice per group). Ten days after engraftment, blood was collected from the submandibular plexus by cheek bleeds, and then mice were sacrificed by cardiac perfusion.

For FACS analysis of leukemia cells, various tissues (brain, lung, liver, muscle, spleen, and kidney), were removed and washed once with cold PBS then digested with Liberase (Roche) as per their instruction manual to prepare stromal vascular fraction (SVF). Blood was processed with BD Pharm Lyse (BD Biosciences) according to the instruction manual. Bone marrow cells were collected from femurs by flushing with PBS and then pelleted at 300 g for 5 min. Processed blood, bone marrow and SVF were subjected to FACS analysis in a FACScan (BD Bioscience) machine. DAPI was added to each sample to distinguish live cells. Tissues from a non-transplanted mouse were used as negative controls, respectively, for setting up GFP+ gating.

2.7. Statistical analyses

All statistical tests were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Two-sided Student's *t*-tests were used to compare control and ACM groups, and tissue explants. Square-root transformation was used when data were not normally distributed. The data are presented as mean ± SD. A p value equal or less than 0.05 was taken as statistically significant.

3. Results

3.1. ALL cells migrate into adipose tissue

To investigate whether leukemia cells actively migrate into adipose tissue *in vivo*, we implanted 20 week-old obese C57Bl/6 mice syngeneically with GFP positive 8093 ALL cells. We collected tissues at an early time point, ten days after implantation, to measure homing to different locations. The presence of 8093 cells in these tissues was analyzed by FACS. At this early time point, there were few circulating ALL cells, with all 6 obese mice and 4 of 6 control mice having detectible leukemia, though at <0.1% of events. Half the mice from each group had detectible leukemia in the bone marrow and 2 obese and 3 control mice had detectible leukemia in the spleen, all at <11% of events (Fig. 1A). Thus, the leukemia burden in these mice was similar to one would expect in a patient prior to clinical symptoms.

At this time point, all mice also had detectible leukemia in their visceral fat pads, and most in other fat pads as well. When leukemia cells were present in marrow, spleen and liver, they were present in large numbers (>0.5% of events on average), while the burdens in kidney, lung, and brain were lower (Fig. 1B). Leukemia burden was also high in both visceral and perirenal fat depots. There was a significantly higher burden of leukemia cells in visceral fat from obese compared to control mice. Although diet group had no effect on leukemia burden in any other tissue, obese mice had significantly more fat in all four depots compared to lean mice (Fig. 1C), and therefore the absolute number of leukemia cells in these depots would be expected to be higher in the obese mice.

The leukemia burden in the adipose tissue apparently increased over time; in two additional mice, we found that adipose tissue leukemia burden was substantially higher when mice were sacrificed 21 days after leukemia transplantation (not shown). Therefore, leukemia cells appear to migrate into adipose tissue early after implantation, when the leukemia burden is still relatively low.

To test whether adipose tissue attracts leukemia cells, we performed *ex vivo* migration assays with mouse adipose tissue explants. Murine preB ALL cells migrated through TransWells toward various fat depots (3–15% within 90 min; $n = 3$, Fig. 1D). Consistent with the *in vivo* findings, very few cells migrated toward muscle or control media.

3.2. Leukemia cells exhibit chemotaxis towards adipocytes

Adipose tissue is comprised of multiple cell types, including adipocytes, endothelial cells, stromal cells, and immune cells. Since adipocytes occupy the most volume in adipose tissue, we next tested whether the chemotaxis of leukemia cells was specifically toward adipocytes. Murine preB ALL cells migrated toward differentiated 3T3-L1 and OP9 adipocytes, and to media