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Cellular elements of the subarachnoid space promote ALL survival during chemotherapy

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Abstract

CNS infiltration by leukemic cells remains a problematic disease manifestation of acute lymphoblastic leukemia (ALL). Prophylactic regimens for CNS leukemia including intrathecal chemotherapeutics have decreased CNS involvement in ALL, but are not without toxicities. Using co-culture models, we show that astrocytes, choroid plexus epithelial cells, and meningeal cells protect ALL cells from chemotherapy-induced cell death using drugs included in prophylactic regimens—cytarabine, dexamethasone, and methotrexate. Understanding how ALL cells survive in the CNS remains invaluable for designing strategies to prevent CNS leukemia and minimizing the need for treatment in this sensitive anatomical site where treatment-induced toxicity is of significant concern.

Keywords

CNS; ALL; subarachnoid space; chemotherapy; survival

Introduction

Infiltration of the central nervous system (CNS) by acute lymphoblastic leukemia (ALL) contributes to relapse of disease and predicts poor disease outcome.[1;2] Risk factors associated with the development of CNS leukemia include young age, high leukocyte

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Conflict of Interest

The authors declare no conflicts of interest.

Authors' Contributions

SMA provided concept design, data collection/assembly, data analysis/interpretation, manuscript writing; SLR and JEF supplied data collection/assembly and data analysis/interpretation and LFG provided concept design, financial support, data interpretation, manuscript writing and final approval.

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counts, and the presence of high-risk cytogenetics.[1;2] At diagnosis, less than 10% of patients with ALL have CNS involvement and using prophylactic regimens, less than 15% will relapse with CNS complications.[1;2] Standard prophylaxis of CNS leukemia consists of intrathecal chemotherapy, high-dose systemic chemotherapy, and cranial or craniospinal irradiation.[3] Intrathecal chemotherapeutic regimens often include the use of methotrexate, cytarabine, and a steroid, such as dexamethasone.[3–5] The use of prophylaxis has decreased the rates of CNS involvement, but treatments targeted for the CNS result in toxicities including seizure, dementia, intellectual dysfunction, leukoencephalopathy, and growth retardations.[1;2] These adverse effects are even more detrimental given the preponderance of pediatric ALL cases. While prophylaxis reduces the rate of CNS involvement, the implications of CNS directed therapeutic toxicities, the persistence of CNS relapse in some patients despite prophylaxis, and the poor prognosis surrounding CNS relapse highlight the need to understand the biology involved in the communication between ALL cells and the CNS.

Leukemic meningitis, a diffuse infiltration of the meninges and subarachnoid space by leukemic cells, is the most common form of CNS involvement in ALL.[1] The unique cerebrospinal fluid-filled microenvironment of the subarchnoid space is generated by cell types including astrocytes, choroid plexus epithelial cells, and meningeal cells.[6] We investigated the interactions between ALL cells and three cell types present in the subarachnoid space—human astrocytes (NHA), human choroid plexus epithelial cells (HCPEpiC), and human meningeal cells (HMC). We demonstrate that ALL cells migrate towards NHA, HCPEpiC, and HMC, which is in part mediated through CXCL12/CXCR4 signaling. Additionally, we document the physical interaction of ALL cells with these three CNS-derived cell types and through the use of *in vitro* co-culture models, we show that NHA, HCPEpiC, and HMC confer protection to ALL cells from chemotherapy-induced cell death. This novel model provides the framework for studies that are necessary to develop innovative therapeutic strategies for eradication of leukemia resident in the CNS.

Materials and Methods

Cell culture and reagents

The ALL cell lines JM-1 (CRL-10423), REH (CRL-8286), RS4;11 (CRL-1873) and SUP-B15 (CRL-1929) were obtained from ATCC (Manassas, VA). Leukemic cells were maintained at a density of 1×10^6 cells/mL in Iscove's DMEM (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM l-glutamine (Mediatech), 0.05 μ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri), 100 U/mL penicillin (Sigma-Aldrich), and 0.1 mg/mL streptomycin (Sigma-Aldrich). Primary human meningeal cells (HMC) and human choroid plexus epithelial cells (HCPEpiC) were obtained from ScienCell (Carlsbad, CA) and maintained in complete fibroblast media and epithelial cell media, respectively (ScienCell). Normal human astrocytes (NHA) were obtained from Lonza (Basel, Switzerland) and maintained in astrocyte growth media (Lonza). Primary ALL cells were isolated from de-identified leukapheresis products following separation using Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ). The chemotherapeutics cytosine β -D-arabinofuranoside (cytarabine, Ara-C, Sigma-Aldrich), dexamethasone (DEX, Sigma-Aldrich), and methotrexate (MTX, Parenta Pharmaceuticals Yardley, PA) were maintained as 10mM stock solutions in Iscove's DMEM complete media.

Leukemic cell co-culture with HMC, HCPEpiC, and NHA

HMC, HCPEpiC, or NHA were grown to confluence in 96-well plates (Corning, Lowell, MA). The media was removed and leukemia cells (1×10^6 cells/mL, 190 μ L) were added. Leukemia cells were co-cultured with the adherent cell population for 24 h prior to addition

of chemotherapeutics. Co-culture continued throughout the course of chemotherapy treatment.

Production of HMC, HCPEpiC, and NHA conditioned media

HMC, HCPEpiC, or NHA were grown to confluence on 10 cm petri dishes (Corning). The media was removed and replaced with Iscove's DMEM complete media (7 mL). This media was conditioned with soluble factors from the adherent cell population for 48 h. For experiments investigating the contribution of HMC, HCPEpiC, or NHA soluble factors to ALL survival during chemotherapy treatment, ALL cells were pelleted by centrifugation and resuspended in Iscove's DMEM complete media control or HMC-, HCPEpiC-, or NHA-conditioned media at a density of 1×10^6 cells/mL.

Glutaraldehyde fixation of HMC, HCPEpiC, and NHA

To block the metabolic activity, and thereby secretion of soluble factors, of HMC, HCPEpiC, or NHA, while leaving surface proteins intact, HMC, HCPEpiC, or NHA were grown to confluence in 96-well plates and fixed with glutaraldehyde. The culture media was removed and cells were fixed for 5 minutes in glutaraldehyde (2% in PBS). After washing the cells three times in 1X PBS and twice with complete growth media, the cultures were returned to the incubator overnight in complete growth media for thorough rinsing of glutaraldehyde.

Chemotaxis assay

REH and SUP-B15 leukemic cells (1×10^6 cells/mL in 150 μ L media) were added to the top chamber of the transwell system (5 μ m pore size, Corning) and allowed to migrate toward media; HMC-, HCPEpiC-, or NHA-CM; or media supplemented with 100 ng/mL CXCL12 (R&D Systems, Minneapolis, MN) for 4 h. Samples were collected from the bottom chamber and were evaluated by flow cytometry. Migration is expressed as the number of events acquired during 30 s of high flow rate. Samples were evaluated in triplicate.

For experiments evaluating the contribution of CXCL12 to ALL cell migration, HMC, HCPEpiC, or NHA were grown to confluence in the bottom chamber of a transwell system and were allowed to condition media for 24h. SUP-B15 cells were left untreated or were treated with AMD-3100 (50 μ M, Sigma-Aldrich) for 20 min. The leukemic cells were then used as described above.

ELISA

To complete the CXCL12 ELISA (R&D Systems), HMC, HCPEpiC, or NHA were plated at 100% confluence in a 96-well plate. Following 24h of incubation supernatants were collected and analyzed for CXCL12 following the manufacturer's instructions.

MTT viability assay

MTT substrate (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich) was added to tumor cells or tumor cell co-cultures growing in 96-well plates (190 μ L/well) at a final concentration of 0.5 mg/mL and allowed to incubate at 37°C for 3 h. Formed formazan crystals were dissolved by adding 100 μ L of a solubilization solution to each well. The solubilization solution contained N, N-Dimethylformamide (DMF, 50% v/v, Sigma-Aldrich) and sodium dodecyl sulfate (SDS, 20% w/v, Sigma-Aldrich). The 96-well plates were analyzed by measuring optical density at a wavelength of 562 nm using a μ Quant Scanning Microplate Spectrophotometer (Biotek, Winooski, VT). Data were analyzed using KC Junior software (version 1.41.8, Biotek). Average optical densities were obtained from three technical replicates. In each culture condition (i.e. media, NHA, HCPEpiC, or HMC) the

optical density for each chemotherapy treated well was normalized to the average optical density of the media treated group. To perform statistical analysis, $\log_2$ of each optical density was calculated. From these values, mean $\log_2$ (optical density) and standard error of the mean was calculated. Paired Student t-tests were used to compare the effect of each culture condition on ALL survival during chemotherapy treatment to the media control culture condition. Significance denoted by (*) indicates $p < 0.05$.

Results

ALL cell line response to Ara-C, DEX, and MTX is time and cell line dependent

To evaluate the effect of chemotherapeutics used in the prophylaxis of CNS leukemia on ALL cell viability, REH and SUP-B15 cells were treated with Ara-C, DEX, and MTX (1 μ M to 1 mM). Viability was determined by trypan blue exclusion following 24 h, 48 h, and 72 h of treatment. Figure 1 summarizes the response of REH and SUP-B15 cells to Ara-C and MTX, which is time dependent. In contrast to the ability of Ara-C and MTX to induce cell death in both the REH and SUP-B15 cells, DEX only induced death in SUP-B15 cells. This finding is consistent with other reports of REH insensitivity to DEX treatment.[7] Based on these data, all subsequent viability-based experiments utilized chemotherapeutics at a final concentration of 1 μ M and viability was determined following 48 h of treatment.

ALL cells migrate toward NHA-, HCPEpiC-, and HMC-derived soluble factors

Migration of ALL cells to supportive niches is required for ALL cells to be protected from the effects of chemotherapy by unique microenvironment cues. To determine whether ALL cells could migrate toward chemotactic stimuli provided by cells of the CNS, REH and SUP-B15 cells were allowed to migrate through a transwell system toward NHA-, HCPEpiC-, or HMC-CM. Media with no defined chemotactic stimulus served as a control for random migration. Following 4h of migration, samples were collected from the bottom chamber of the transwell system and the number of migrated cells was enumerated by flow cytometry. As is shown in Figure 2a, REH and SUP-B15 cells migrate more efficiently towards NHA-, HCPEpiC-, and HMC-CM than towards the media control.

Our laboratory and others have demonstrated that CXCR4/CXCL12 signaling promotes chemotaxis of ALL cells. To evaluate whether this signaling axis was involved in the migration of ALL towards cells derived from the CNS, we performed ELISA to determine the production of CXCL12 by NHA, HCPEpiC, and HMC. We also evaluated CXCL12 production by human bone marrow stromal cells and human osteoblasts as positive controls. Figure 2b demonstrates that all three CNS-derived cell lines produce CXCL12 comparable to bone marrow stromal cells and human osteoblasts. To directly evaluate the contribution of CXCL12 to mediating the migration of ALL towards NHA, HCPEpiC, and HMC, SUP-B15 cells were treated with the CXCR4 antagonist, AMD-3100, prior to use in a chemotaxis assay. As is shown in Figure 2c, treatment with AMD-3100 effectively blocked CXCL12/CXCR4 signaling as is demonstrated by the abrogated movement of SUP-B15 cells toward CXCL12 alone and significantly reduced the migration of SUP-B15 cells toward HCPEpiC-CM and HMC-CM. Treatment with AMD-3100 did not, however, effect the migration of SUP-B15 cells toward NHA-CM. Together these data demonstrate that while CXCL12 plays a role in the migration of ALL cells towards HCPEpiC and HMC, other soluble factors also must be involved in the migration of ALL cells towards the CNS-derived cells.

ALL cells physically interact with cellular elements of the CNS

Once in a supportive niche, physical interactions between ALL cells and other cell types in that microenvironment can mediate ALL response to chemotherapy. Short-term co-cultures of REH and SUP-B15 cells were established with NHA, HCPEpiC, and HMC to visually

inspect ALL adhesion to the CNS-derived cells. Figure 3 demonstrates that in contrast to the media control group, in which the leukemic cells are in suspension, both REH and SUP-B15 cells robustly adhere to the NHA, HCPEpiC, and HMC layers.

ALL cell co-culture with NHA, HCPEpiC, and HMC blunts chemotherapy-induced cell death

We next determined whether interaction of ALL cells with constituents of the subarachnoid space altered the response of ALL cells to chemotherapy. Co-cultures between the ALL cell lines, JM-1, REH, RS4;11, and SUP-15, and NHA, HCPEpiC, and HMC were established and subsequently treated with Ara-C, DEX, or MTX. Figure 4A summarizes data suggesting that culture of ALL cells with NHA, HCPEpiC, or HMC promotes leukemic cell survival during treatment with Ara-C and MTX. In addition, SUP-B15 cells, which are sensitive to DEX, have higher viability following DEX treatment when cultured in the presence of NHA, HCPEpiC, or HMC. As an additional control, NHA, HCPEpiC, and HMC in culture alone were treated with Ara-C, DEX, and MTX. Treatment with these chemotherapeutics does not decrease NHA, HCPEpiC, or HMC viability at the doses we used to treat the leukemia cells *in vitro* (data not shown).

We subsequently evaluated the individual contribution of adhesion-mediated signaling and soluble factor-mediated signaling to leukemic cell survival. To separate the physical-mediated interactions from the soluble factor derived cues, NHA, HCPEpiC, and HMC were fixed with glutaraldehyde. To evaluate the contribution of soluble factors alone, NHA-, HCPEpiC-, and HMC-CM was produced. Cultures between leukemia cells, and either glutaraldehyde-fixed NHA, HCPEpiC, and HMC or NHA-, HCPEpiC-, and HMC-CM were established for 24 h then treated with Ara-C, DEX, or MTX. Figure 4B demonstrates that while culture of ALL cells with glutaraldehyde-fixed NHA, HCPEpiC, or HMC produces an increase in leukemic cell survival following treatment with Ara-C and MTX, the effect is modest and does not account for the degree of protection seen during culture of ALL cells with viable adherent cells. Likewise, SUP-B15 cells cultured with glutaraldehyde-fixed NHA, HCPEpiC, and HMC have modestly higher viabilities than cells cultured in media alone following DEX treatment. As is shown in Figure 4C, culture of ALL cells in NHA-, HCPEpiC-, or HMC-CM promotes leukemia survival during treatment with Ara-C and MTX. Furthermore, SUP-B15 cells are more viable following DEX treatment when cultured in the presence of NHA-, HCPEpiC-, or HMC-derived soluble factors compared to treatment in media alone. Again, however, the protection conferred by soluble factors alone is less than that which is offered by direct co-culture of ALL with cellular elements of the subarachnoid space.

To evaluate this protective effect on primary cells, ALL cells isolated from de-identified leukapheresis products were used. Co-cultures between the primary ALL cells and NHA, HCPEpiC and HMC were established and treated with Ara-C, DEX or MTX as described above for 24 h. Figure 5 summarizes data suggesting that culture of primary ALL cells with NHA, HCPEpiC or HMC promotes leukemic cell survival during chemotherapy treatment consistent with that observed for leukemic cell lines.

Discussion

Our findings provide rationale for further examination of soluble and physical mediators of ALL interaction with cells present in the CNS. Through the study of ALL in other sanctuary sites, such as the bone marrow, specific physical and soluble cues have been identified that promote functions of ALL cells observed in the current study.[8;9] It is well documented that the chemokine CXCL12 is a potent chemoattractant and survival factor for hematopoietic and leukemia cells.[10;11] While CXCL12 is produced by cells of the bone marrow, its expression by astrocytes, choroid plexus epithelial cells, and meningeal cells has

also been documented in the CNS.[12–14] In this setting, CXCL12 has been demonstrated to promote B-cell survival in the pathogenesis of the experimental autoimmune encephalomyelitis model of human multiple sclerosis.[12] While we observed that CXCL12 was produced by NHA, HCPEpiC, and HMC and in part mediated the migration of ALL cells towards the CNS-derived cells, using AMD-3100 to interrupt CXCL12/CXCR4 signaling we did not observe CXCL12 mediating the pro-survival effect of CNS-derived soluble factors on ALL cells (data not shown). Other than CXCL12, cellular elements of the CNS secrete numerous growth factors and cytokines including IL-6, IL-10, BAFF, and the neurotrophins NGF, BDNF, and NT-3, which have been shown to promote B-cell survival.[15–20] Of note, while the ALL cell lines used expressed the neurotrophin receptors TrkA, TrkB, TrkC, and p75NTR, addition of rNGF, rBDNF, and rNT-3 during chemotherapy treatment did not elicit protection of ALL cells (data not shown), suggesting other soluble cues are important in the CNS microenvironment. In addition to the soluble factor-mediated migration and protection of ALL that we observed, proteins responsible for physical interactions mediated adhesion and protection of ALL. Just as studies from the bone marrow help us choose targets for further investigation of soluble factors, they also inform us as to which proteins may mediate adhesion. Work done by our lab, as well as others has demonstrated the importance of VCAM-1/VLA-4 interactions in mediating adhesion of ALL to cells in the bone marrow that leads to enhanced ALL survival following chemotherapy treatment.[21] These interactions may be important in the CNS, as well being that VCAM-1 expression has been documented on constituents of the CNS.[22;23]

While *in vitro* models have long been used to understand the impact of microenvironments on ALL survival, to our knowledge, this is the first report documenting protection of ALL cells by elements of the subarachnoid space from chemotherapy. Our data demonstrate that ALL cells physically interact with and migrate towards three cellular constituents of the CNS—astrocytes, choroid plexus epithelial cells, and meningeal cells. Furthermore, interactions between these cells promote ALL cell line and primary cell survival following exposure to chemotherapeutics used in the prophylaxis of CNS leukemia. While adhesion-mediated signaling and soluble factors individually contribute to ALL viability, protection reaches maximal levels during direct co-culture. Cancers having CNS involvement generally place patients at high risk for poor disease outcomes, which defines the broad impact of pre-clinical models which investigate the CNS microenvironment specifically. Many recent studies have aimed to identify risk-factors associated the development of CNS leukemia in efforts to tailor patient specific prophylactic regimens to prevent over-treatment or under-treatment.[24;25] Therefore, understanding the molecular mechanisms through which ALL cells interact with the unique environment of the subarachnoid space is critical for designing strategies to prevent CNS leukemia and may have relevance to other malignancies with propensity for CNS metastasis.

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Figure 1. ALL cell line response to Ara-C, DEX, and MTX is time and cell line dependent
 REH and SUP-B15 cells were treated with Ara-C, DEX, or MTX at doses ranging from 1 μM to 1 mM. Cell viability was determined by trypan blue exclusion counting following 24 h, 48 h, and 72 h of treatment. Data are expressed as percent viable cells (mean + SEM, N=3). *p<0.05

Figure 2. ALL cells migrate toward NHA-, HCPEpiC-, and HMC-derived soluble factors

(A) Chemotaxis assays were performed allowing REH (top) and SUP-B15 (bottom) cells to migrate towards media control or NHA-, HCPEpiC-, or HMC-CM for 4 h. Cells that had migrated through the transwell system were collected and enumerated using flow cytometry. Data are expressed as number of migrated cells collected during 30 sec high flow rate (mean + SEM, N=3). *p<0.05.

(B) ELISA was performed on supernatants from NHA, HCPEpiC, HMC, bone marrow stromal cells (BMSC), and human osteoblasts (HOB) to detect CXCL12. Data are expressed as mean CXCL12 concentration (pg/mL) + SEM.

(C) Chemotaxis assays were performed by allowing SUP-15 cells treated with AMD-3100 (50 mM) or media control to migrate toward media; NHA, HCPEpiC, or HMC; or CXCL12 for 4h. Cells that had migrated through the transwell system were collected and enumerated using flow cytometry. Data are expressed as number of migrated cells collected during 30 sec high flow rate (mean + SEM, N=3). *p<0.05

Figure 3. ALL cells physically interact with NHA, HCPEpiC, and HMC
 REH and SUP-B15 cells were cultured in media or were co-cultured with NHA, HCPEpiC, or HMC for 24 h. Photomicrographs were taken of each culture condition at 100X magnification.

Figure 4. ALL cell co-culture with NHA, HCPEpiC, and HMC blunts chemotherapy-induced death

JM-1, REH, RS4;11, and SUP-B15 cells were cultured in media or were co-cultured with (A) NHA, HCPEpiC, or HMC; (B) glutaraldehyde-fixed NHA, HCPEpiC, or HMC; or (C) NHA-, HCPEpiC-, or HMC-CM for 24 h. Cultures were then treated with Ara-C, DEX, or MTX at a final concentration of 1 μ M or media control for 48 h. Following treatment, MTT assay was performed and optical densities were determined using a microplate reader. The optical density measurements for drug treatment groups were normalized to the media control for each culture condition. Data are reported as relative viability (mean $\pm$ SEM, N=3) compared to untreated control. *p<0.05

Figure 5. Primary patient leukapheresis ALL cell co-culture with NHA, HCPEpiC, and HMC blunts chemotherapy-induced death

Two different primary leukapheresis ALL samples (**A**, **B**) were cultured in media or were co-cultured with NHA, HCPEpiC or HMC for 24 h. Cultures were then treated with Ara-C, DEX, or MTX at a final concentration of 1 μ M or media control for 24 h. Following treatment, MTT assay was performed and optical densities were determined using a microplate reader. The optical density measurements for drug treatment groups were normalized to the media control for each culture condition. Data are reported as relative viability (mean + SEM, N=3) compared to untreated control. * $p < 0.001$