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Research paper

A novel extracellular matrix-based leukemia model supports leukemia cells with stem cell-like characteristics

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ABSTRACT

Acute myeloid leukemia (AML) relapse results from the survival of chemotherapy-resistant and quiescent leukemia stem cells (LSC). These LSCs reside in the bone marrow microenvironment, comprised of other cells and extracellular matrix (ECM), which facilitates LSC quiescence through expression of cell adhesion molecules. We used decellularized Wharton's jelly matrix (DWJM), the gelatinous material in the umbilical cord, as a scaffolding material to culture leukemia cells, because it contains many components of the bone marrow extracellular matrix, including collagen, fibronectin, lumican, and hyaluronic acid (HA). Leukemia cells cultured in DWJM demonstrated decreased proliferation without undergoing significant differentiation. After culture in DWJM, these cells also exhibited changes in morphology, acquiring a spindle-shaped appearance, and an increase in the $ALDH^+$ cell population. When treated with a high-dose of doxorubicin, leukemia cells in DWJM demonstrated less apoptosis compared with cells in suspension. Serial colony forming unit (CFU) assays indicated that leukemia cells cultured in DWJM showed increased colony-forming ability after both primary and secondary plating. Leukemia cell culture in DWJM was associated with increased N-cadherin expression by flow cytometry. Our data suggest that DWJM could serve as an ECM-based model to study AML stem cell-like cell behavior and chemotherapy sensitivity.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematopoietic malignancy characterized by an aberrant clonal expansion of undifferentiated myeloid blasts. Studies have shown that leukemia stem cells (LSCs) contribute to relapse after chemotherapeutic treatment. [1] Like normal hematopoietic stem cells (HSCs), LSCs maintain their self-renewal ability while generating clonogenic leukemic progenitors capable of producing leukemic cells [2]. Anti-proliferative chemotherapeutic agents commonly target the rapidly cycling leukemic cells, but they generally are ineffective against the quiescent LSCs, partly because of enhanced drug efflux in LSCs [3]. Therefore, it is important to develop therapeutic strategies which eliminate the LSCs in the bone marrow, where they share the "hematopoietic niche" along with normal HSCs [4]. The LSC niche, similar to the hematopoietic niche, is a 3D microenvironment composed of bone marrow stromal cells and ECM components like collagen, fibronectin and tenascin [4]. These components create compartments that not only provide structural support to the cells in the bone marrow, but also provide chemokines and cytokines that are important in regulating LSC self-renewal, trafficking, proliferation and differentiation [5].

Currently, most leukemia *in vitro* studies are based on conventional two-dimensional (2D) cultures in tissue culture polystyrene (TCP) dishes/ flasks and stromal co-cultures. These models are useful in elucidating some of the molecular mechanisms of leukemia initiation and progression. However, 2D culture systems lack the leukemia-microenvironment interaction present in the 3D bone marrow microenvironment. Therefore, the LSCs in 2D culture frequently differentiate and lose their "stem-ness". The development of a 3D model that replicates the *in vivo* mechanical and biochemical properties of bone

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marrow could allow maintenance of a true LSC-state.

Our laboratory has been focused on characterizing decellularized Wharton's jelly matrix (DWJM) and examining its potential for regenerative medicine applications. [6–8], We hypothesized that DWJM would provide a similar environment to the bone marrow ECM, because its components, such as collagen, fibronectin, hyaluronic acid, and sulphated proteoglycan [8], also exist in the bone marrow hematopoietic niche. Moreover, because cell-ECM interactions play an important role in chemoresistance in leukemia cells [9], the environment provided by DWJM could support the maintenance of LSCs.

We therefore used DWJM as an ECM to examine leukemia cell-ECM interactions, hypothesizing that DWJM would support leukemia cells with LSC-like characteristics. In this model, we investigated the growth pattern of 3 human leukemia cell lines (HL60, Kasumi-1, and MV411), with a focus on proliferation, viability, morphology and myeloid differentiation. We also studied the drug resistance and stem cell characteristics of leukemia cells cultured in this model, compared to leukemia cells cultured in suspension. We found that leukemia cells cultured in our DWJM-based ECM model had LSC characteristics, suggesting that DWJM may prove useful in LSC characterization and in developing therapeutic interventions that target LSCs.

2. Materials and methods

2.1. Cell culture

Human AML cell lines HL60, Kasumi I and MV 411 (ATCC, Manassas, VA) were maintained in T 75 tissue culture flasks with Advanced Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco), supplemented with 5% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (pen/strep) (Life Technologies). Cells were maintained at 37 °C in a fully humidified 5% CO2 incubator.

2.2. DWJM scaffold preparation

The preparation of DWJM was previously described. [8,10], Briefly, to prepare DWJM scaffolds, fresh human umbilical cords were dissected after removing surrounding membranes and blood vessels. Then they were subjected to two cycles of osmotic shock in hypertonic and hypotonic solutions, followed by immersion in a non-ionic detergent Triton-x, an anionic detergent sodium lauryl succinate; finally, they underwent enzymatic digestion with recombinant endonuclease. The resulting DWJM pieces were cut into thin wafers (3 mm thick) as previously described [10] (Supplementary Figure-01A). Cartoon depiction of leukemia cells interacting with DWJM fibers (Supplementary Figure-01B).

2.3. Seeding DWJM wafers with AML cell lines

Before seeding, cryopreserved DWJM wafers were thawed, washed three times in phosphate buffered saline (PBS), and pre-incubated with Advanced RPMI overnight. AML cells ($2*10^5$ cells/well) were seeded into DWJM wafers in 24-well non-tissue culture treated plates with 60% area of each well covered by DWJM. Culture plates were then placed in an incubator at 37 °C with 5% CO2 and maintained in Advanced RPMI with 5% FBS for 7 days; half of the medium was changed every other day. AML cells in suspension ($2*10^5$ cells/well), cultured under the same conditions, were used as controls. In AlamarBlue assay, CellTrace proliferation and Ki67 immunohistology sample preparation, cells were maintained in RPMI 1640 (Sigma-Aldrich) with 10% FBS for HL60 and MV411 cells, and 20% FBS for Kasumi I cells.

2.4. AlamarBlue assay

To assess the proliferation of AML cells, DWJM wafers with cells were transferred to new 24 well plates and washed with PBS three times, 4 h prior to proliferation assessment. 10% alamarBlue (Biocentric) was added into each well. After 4 h, 100 µl of supernatant from each well was aspirated to a new well of a 96 well plate and fluorescence was measured by micro-plate reader, with excitation wavelength at 530 nm and emission wavelength at 590 nm.

2.5. CellTrace proliferation assay

To monitor the cell division of leukemia cells in suspension and in DWJM, cells were labeled with CellTrace Violet (Life Technology) before seeding. Briefly, cells were washed and resuspended with PBS at the concentration of 10^6cells/ml, and CellTrace Violet stock solution was added in a final concentration of 1 µl/ml. After incubation at room temperature for 20 min, 5 ml of PBS with 10% FBS were added and incubated for 5 min, followed by centrifugation to obtain pellets. Cells were resuspended in culture medium and cultured in either suspension or DWJM. Cell division was measured by flow cytometry soon after seeding and after 7 days of culture. To isolate cells from DWJM, we washed wafers in PBS, and then used collagenase II (0.05 *g* collagenase II in 50 ml DMEM for 1–2 hours) to digest DWJM at 37 °C.

2.6. Cell viability

Cell survival in DWJM was measured by Vi-CELL Series Cell Viability Analyzer (Beckman Coulter), which is based on Trypan Blue dye exclusion. Cells in each DWJM wafer were released by treating with 1 ml 0.002 g/ml collagenase II (Worthington) for about 2 h at 37 °C. The released cells were assessed for viability according to the manufacturer's recommendations.

2.7. Histology and immunohistochemistry

For morphological analysis, wafers were washed with PBS three times, fixed in 4% PFA, embedded in paraffin, sectioned and stained with hematoxylin and eosin, and visualized under the microscope using an Olympus BX40 microscope; pictures were obtained using a DP72 digital camera.

2.8. Treatment with chemotherapeutic agents

After 7 days, cells cultured in suspension and cells in DWJM were treated with 50µM of doxorubicin hydrochloride (Sigma-Aldrich) for 48 h. For cells in suspension, culture medium was removed, and chemotherapeutic agents were added in fresh medium. For cells in DWJM, scaffolds were transferred into new 24-well plates and washed with PBS three times; then a chemotherapeutic agent was added to the culture medium.

2.9. Apoptosis assay

Apoptosis in leukemia cells was measured by flow cytometry using Annexin V-Alexa 568 (Invitrogen, USA) and DAPI (Invitrogen, USA) staining. Prior to flow cytometry analysis, cells in DWJM wafers were released as described previously and 10^5 released cells as well as cells in suspension were stained with DAPI and Annexin V according to manufacturer's recommendations. Data were acquired within 1 h, using LSR II (BD Biosciences), and analyzed by FlowJo software.

2.10. Aldefluor assay

Aldehyde dehydrogenase (ALDH) activity was examined by using Aldefluor reagent (Stem Cell Technologies) according to the manufacturer's protocol, followed by flow cytometry. Cells negative for propidium iodide (PI) staining were considered positive for ALDH, based on a negative control using the ALDH inhibitor diethylaminobenzaldehyde (DEAB). Data were analyzed within 1 h, using LSR II (BD