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3D tissue-engineered bone marrow as a novel model to study pathophysiology and drug resistance in multiple myeloma

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ABSTRACT

Purpose: Multiple myeloma (MM) is the second most prevalent hematological malignancy and it remains incurable despite the introduction of several novel drugs. The discrepancy between preclinical and clinical outcomes can be attributed to the failure of classic two-dimensional (2D) culture models to accurately recapitulate the complex biology of MM and drug responses observed in patients. Experimental design: We developed 3D tissue engineered bone marrow (3DTEBM) cultures derived from the BM supernatant of MM patients to incorporate different BM components including MM cells, stromal cells, and endothelial cells. Distribution and growth were analyzed by confocal imaging, and cell proliferation of cell lines and primary MM cells was tested by flow cytometry. Oxygen and drug gradients were evaluated by immunohistochemistry and flow cytometry, and drug resistance was studied by flow cytometry.
Results: 3DTEBM cultures allowed proliferation of MM cells, recapitulated their interaction with the microenvironment, recreated 3D aspects observed in the bone marrow niche (such as oxygen and drug gradients), and induced drug resistance in MM cells more than 2D or commercial 3D tissue culture systems.
Conclusions: 3DTEBM cultures not only provide a better model for investigating the pathophysiology of MM, but also serve as a tool for drug development and screening in MM. In the future, we will use the 3DTEBM cultures for developing personalized therapeutic strategies for individual MM patients.

1. Introduction

Multiple myeloma (MM) is the second most prevalent hematological malignancy and remains incurable with a median survival time of 3–5 years [1,2]. Despite the introduction of several novel drugs and their high efficacy in vitro, only about 60% of patients initially respond to therapy, and among relapsed patients more than 90% develop drug resistance [3–6].

The discrepancy between in vitro efficacy and clinical outcomes can be attributed to limitations of classic two-dimensional (2D) tissue culture and drug screening models. First, despite the fact that the interactions of MM cells with bone marrow (BM) microenvironment components was shown to induce resistance [7–10], most of the in vitro models use MM cell line mono-cultures and neglect the vital role of the microenvironment. Second, the BM niche is a three-dimensional (3D) structure which induces oxygen and drug concentration gradients as a function of distance from blood vessels known to significantly affect drug efficacy [11–14]. 2D tissue culture systems cannot reproduce the oxygen and drug gradients found in the BM niche, which limits the ability of 2D cultures to accurately predict drug sensitivity. Therefore, there is an urgent need to develop a model that addresses these limitations to investigate biological mechanisms and drug resistance in MM that are relevant and translatable to improved patient response.

Previous models have been developed to recreate the 3D microenvironment of the BM using collagen [15,16], Matrigel [17],

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acrylic polymers [18], silk [19], hyaluronic acid [20], and ossified tissues [21]. These models have probed the importance of using 3D rather than 2D models to recreate myeloma growth; however, each has its limitations. For example, although hydrogel systems (such as collagen, Matrigel or synthetic polymers) [15–19] are simple and reproducible, these materials are not physiologically found in the BM and may cause significant changes in the culture milieu. Solid systems (such as ossified tissues) mimic BM physiological conditions [21,22]; however, these are technically challenging due to reproducibility and adaptability problems, and rely on a normal BM microenvironment for the growth of MM cells, which was previously proven to be significantly different (in some cases opposite) from the effect of the MM microenvironment [23,24].

In this study, we developed a 3D scaffold derived from the BM supernatant of MM patients to incorporate different BM components including MM cells, stromal cells, and endothelial cells. This model was defined as a 3D tissue engineered bone marrow (3DTEBM) culture, and we hypothesized that it will promote better growth of MM cells and provide a more patient relevant model for evaluating drug efficacy in MM (Fig. 1A).

2. Materials and methods

2.1. Reagents

Calcium chloride ($CaCl_2$), tranexamic acid, type I collagenase, dimethyl sulfoxide (DMSO), propidium iodide (PI, excitation, 488 nm; emission, 655–730 nm), and doxorubicin (excitation, 488 nm; emission, 585/40 nm) were purchased from Sigma–Aldrich (Saint Louis, MO). Cell trackers including DiO (excitation, 488 nm; emission, 525/50 nm), DiD (excitation, 635 nm; emission, 655–730 nm), DiI (excitation, 488 nm; emission, 585/40 nm) and Calcein violet (excitation, 405 nm; emission, 450/50 nm) were purchased from Invitrogen (Carlsbad, CA). Drugs including bortezomib and carfilzomib were purchased from Selleck Chemicals (Houston, TX).

2.2. Cell lines

The MM cell lines (MM1s, H929, RPMI8226, and MM1s-GFP-Luc) were a kind gift from Dr. Irene Ghobrial (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Human umbilical vein endothelial cells were purchased from Lonza (Walkersville, MD). All cells were cultured at 37 °C, 5% CO_2; MM cells in RPMI-1640 media (Corning CellGro, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life technologies, Grand island, NY), 2 mmol/l of L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Corning CellGro), and endothelial cells in EGM-2 completed media (Lonza). Before experiments, MM cells and endothelial cells (1×10^6 cells/ml) were pre-labeled with Calcein violet (1 μg/ml) or DiI (10 μg/ml) for 1 h, respectively.

2.3. Primary cells

Primary $CD138^+$ and $CD138^-$ cells were isolated from BM aspirates of MM patients from the Siteman Cancer Center, Washington University in Saint Louis, by magnetic-bead sorting as previously described [7]. Informed consent was obtained from all patients with an approval from the Washington University Medical School IRB committee and in accord with the Declaration of Helsinki. For frozen samples, cells were viably frozen at −80 °C in FBS with 10% DMSO (v/v). Plasma samples were extracted from peripheral blood (PB) and BM aspirates by centrifugation at 1620 g for 10 min, and finally frozen at −80 °C. Primary $CD138^+$ cells were cultured in RPMI-1640 media supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin. Primary MM-derived stromal cells ($CD138^-$) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning CellGro) supplemented with 20% FBS, L-glutamine, penicillin, and streptomycin. MM-derived stromal cells were cultured for three weeks and monitored for the development of spindle-shaped cells. Before experiments $CD138^+$ and MM-derived stromal cells (1×10^6 cells/ml) were pre-labeled with DiO (10 μg/ml) and DiD (10 μg/ml) for 1 h, respectively.

2.4. Development of 3DTEBM cultures

3DTEBM cultures were developed through cross-linking of fibrinogen (naturally found in the plasma of the PB and BM supernatant) with $CaCl_2$ concentrations ranging from 0 to 4 mg/ml, as previously described [25,26]. Gelification time was measured and the $CaCl_2$ concentration that induced the fastest gelification time was selected for further studies (Suppl Fig. 1A). The effect of tranexamic acid addition at various concentrations (0–10 mg/ml) was studied for preventing the degradation of fibrin fibers and improving scaffold stability. The scaffold stability was measured at 3 weeks and assessed by comparing the tranexamic acid containing scaffold weight to the weight of non-stabilized scaffolds, and the tranexamic acid concentration that induced the maximal stabilization was selected for further studies (Suppl Fig. 1B). The impact of tranexamic acid on the viability of myeloma cell lines was tested by MTT. No effect on viability was found at any concentration tested (1–10 mg/ml). The concentration of 4 mg/ml has no impact on MM cell lines (MM1s and H929) (Suppl Fig. 1C). Briefly, 3DTEBM cultures were formed by mixing the following components: 40 μl of plasma (from PB and BM supernatants) diluted in RPMI-1640 completed media to form total volume of 100 μl, with a final concentration of 1 mg/ml $CaCl_2$, and 4 mg/ml tranexamic acid. Scaffolds were allowed to gelify for 2 h in a 96-well plate in incubator at 37 °C, 5% CO_2, before being covered with additional RPMI-1640 completed media. The medium was changed every 3 days during the culture period. Cells were added to the solution before fibrin clotting agents ($CaCl_2$ and tranexamic acid). Fig. 1A shows the strategy used for the development of 3DTEBM from MM patients. Morphology of the 3DTEBM scaffolds was analyzed with light microscopy (Axiovert 35, Zeiss) and size measurements were taken based on diameter and height (Image J software, NIH, Bethesda, MD) (Suppl Fig. 1D). The structure of 3DTEBM with and without MM1s cells was studied using scanning electron microscopy (SEM) (Suppl Fig. 1E), as previously described [26].

2.5. Confocal imaging

Distribution and growth of mono-cultures and multi-cultures of MM1s-GFP, endothelial cells-DiI, and MM-derived stromal cells-DiD through the 3DTEBM scaffolds were tested using confocal microcopy at days 1, 2, 3, 5 and 7. The 3DTEBM was glued to a glass slide. A ring was placed around the culture and filled with phenol red free DMEM. The cultures were imaged using a FV1000 confocal microscope with an XLUMPLFLN 20XW/1.0 immersion objective lens (Olympus, PA, USA). The cultures were excited at 488 nm (GFP/DiO), 543 nm (DiI), and 633 nm (DiD) and the emission light was collected at 500–530 nm, 555–625 nm, 650 long pass, for each channel respectively. Z-stack images of approximately 1 mm thickness were taken of each sample at 2 μm step sizes. Each frame consisted of a 520 × 520 pixel image, taken at a rate of 1 μs/pixel.

2.6. Cell proliferation using flow cytometry

Cell proliferation assays were performed by digestion of 3DTEBM cultures with type I collagenase (25 mg/ml for 2–3 h at