Characterization of Multiple Myeloma Clonal Cell Expansion and Stromal Wnt/β-catenin Signaling in Hyaluronic Acid-based 3D Hydrogel

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Abstract. Background: Emerging interest on three-dimensional (3D) cell culture models to replace two-dimensional cultures of cancer cells and their xenografts in immunocompromised animal hosts prompted us to investigate the use of new biodegradable gels to recapitulate the physiological conditions of the microenvironment of multiple myeloma (MM) cells. Materials and Methods: In the present study, for the first time, we used a new 3D model of hyaluronic acid (HA)-based hydrogels with difference in their matrix composition and stiffness. Results: We demonstrated that hyaluronic acid (HA)-based hydrogels perfectly accommodate MM cells; confirmed by cell survival, migration, colony forming units and expression of cell adhesion proteins of the Wnt signaling pathways over a period of time. Conclusion: This study provides the first 3D microenvironment data that HA-based hydrogels could provide with a suitable 3D substratum for MM cells to comprehensively analyze phenotypic changes and the influence of bone marrow stromal stem cells on Wnt/β-catenin signaling in response to targeted drug treatments.

Two-dimensional (2D) cell culture has been routinely and diligently undertaken in thousands of laboratories, however, the 2D cell cultures does not reproduce the anatomy or physiology of the respective tissue microenvironment.

Therefore, creating a three-dimensional (3D) cell culture is clearly more relevant, but requires for a multidisciplinary approach. When using 3D cell culture models, it is important to consider the design of scaffolds or the substratum that could support the organization of cells for controlling nutrient exchange (1). Interestingly, tumor cells grow perfectly in a 3D environment in which intercellular cross-talk exists between differentiated cancer cell sub-populations and non-transformed neighboring, partly normal host cells. Recently, 3D cell culture models have been used in cancer research as a compromise between two-dimensional (2D) cultures of isolated cancer cells and xenografts of human cancers in immunocompromised animal hosts (2). Consequently, 3D cell culture models have become more attractive pre-clinical testing tools for novel therapeutic approaches. However, 3D cell culture models to recapitulate the physiological conditions of the bone marrow microenvironment of multiple myeloma are very limited.

Multiple myeloma (MM), an incurable cancer with 3- to 5-year survival is characterized by monoclonal immunoglobulin (Ig), lytic bone lesions (3-5), and monoclonal plasma cells (PCs) in the bone marrow (BM). Because clonal expansion of primary MM cells outside their BM microenvironment has been unsuccessful (6), most pre-clinical studies have used MM cell lines derived from leukemic-phase cells that have escaped BM dependence. Therefore it is likely that BM niches maintain MM cancer stem cells (MM-CSCs) in a quiescent, drug-resistant state. To date, pre-clinical models do not take into account adhesion-mediated drug resistance (7), and none allows for testing of drug efficacy on MM-CSC populations. Mouse models of MM are inadequate for pre-clinical use because they cannot faithfully recapitulate human disease (8, 9). Recently, a stromal spheroid co-culture model and various scaffolds (10, 11) have been developed to recreate the 3D environment of the BM, but these models are not suitable to

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recapitulate the physiologic conditions of the BM with the exception of a model that was used to evaluate multiple myeloma therapy (12, 13).

To adequately investigate B-cell development and pathogenesis, there is a great need for a novel 3D cell culture system. Studies are indicating that migration of tumor cells in 3D matrices is partly governed by matrix stiffness along with cell-matrix adhesion and proteolysis (14-16). This is consistent with our current findings on the use of hyaluronic acid (HA)-based 3D matrix of varying stiffness. In the present study, surface seeding and encapsulation of human multiple myeloma (BMMCs) and the bone marrow stromal cells (BMSCs) showed migration and cellular changes associated with Wnt/β-catenin signaling. Based on these findings, for the first time, we predict that HA-based 3D hydrogels with difference in the matrix composition and stiffness could provide a suitable substratum for MM/BMSC to comprehensively analyze phenotypic and molecular changes of a progressive disease or in response to treatments over a period of time.

Materials and Methods

Suitability of 3D substratum for cell culture. Based on the emerging interest on 3D substratum for cell culture, numerous efforts are being made to investigate the suitability of 3D microenvironments on cell survival and behavior, particularly by testing the effects of varying stiffness of the substratum, matrix components, and chemical stimuli (17-22). In the present study we used photocrosslinkable-methacrylated hyaluronic acid (Me-HA) with methacrylated gelatin (Me-Gel) to examine their suitability as a substratum to grow human primary multiple myeloma cells (MM) and the matching bone marrow stromal cells (BMSCs).

Preparation of Me-HA-based 3D hydrogels. Photocrosslinkable-methacrylated hyaluronic acid (Me-HA) with methacrylated gelatin (Me-Gel) was prepared as previously described (15). The Me-HA precursor and Me-Gel solutions were kindly provided by Dr. Jonathan Butcher, Department of biomedical engineering, Cornell University, Ithaca, NY, USA. Briefly, HA with different molecular weight was first prepared by reacting HA (Novozymes) with sodium periodate (NaIO₄, 0.1 mol/ml, Sigma). Then the methacrylated HA and OHA (Me-HA and MOHA) were synthesized by the addition of a methacrylate functional group to the HA at 40˚C for 6 h and the pH of mixture during reaction was maintained at 8.5. Me-HA was fabricated with varying molecular weight (high ~1000 kDa, medium ~500 kDa and low ~300 kDa) and degree of methacrylation (~22.5%, ~30.7% and ~32.5%) as described earlier (15). Me-Gel was prepared by reacting gelatin with methacrylic anhydride at 40˚C for 1 h. Three different types of hybrid hydrogels with tunable stiffness were fabricated by using Me-HA with different molecular weights with the addition of Me-Gel. The stiffness of Me-HA/Me-Gel hydrogels were measured to be 4.53±0.24 kPa, 5.67±0.43 kPa and 7.31±0.35 kPa, and denoted as Stiffness 1, 2 or 3.

BMMC and BMSC samples. Human serum, BMMCs (bone marrow mononuclear cells isolated from bone marrow aspirates) were obtained from patients with active late-stage multiple myeloma. Informed consent for the human samples was approved by New York University School of Medicine, Institutional Review Board to Dr. Mazumder MD, (Director of Myeloma Program).

2D and 3D cultures of BMMC and BMSCs. Survival and migratory potential of human BMMC and BMSCs were assessed firstly by performing assays for cell proliferation. The studies were performed via two approaches (a), by surface seeding of cells (2D) and (b), homogenous hydrogel encapsulation of cells (3D), as described earlier (15). Briefly, for surface seeding, the hydrogel discs were gently placed on to a 48-well plate containing BMCC culture medium (23). After stabilization of the discs at 37˚C overnight, cells (5x10⁵ cells/ml) were overlaid onto the hydrogel discs (2D) and incubated at 37˚C for a period of up to 5 weeks, or more if needed. Similar, but parallel independent experiments were conducted to measure the cell proliferation by performing an MTT assay as directed by the manufacturer (Promega) in triplicate wells. Next, we performed a homogenous encapsulation of cells (3D) throughout the hydrogel discs by re-suspending 20 μl of the cell suspension at a density of 5x10⁵ in the hydrogel-polymer mix plus cell culture medium (400 μl/well) in to the 48-well plate and the cells were grown at a 37˚C incubator. The above hydrogels containing a surface with coated or encapsulated cells were monitored for survival, migration and colony forming units (CFUs) over a period 21 days.

Cell viability and proliferation. The cell viability was determined using live/dead cell viability assays (Invitrogen) determined by counting live (green) and dead (red) cells or trypan blue exclusion assays for live (no color) and dead (blue). The average cell count in a total of 10 (×20) fields was calculated for the percentage (%) of survival. Cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

Colony forming units (CFU). Progenitors that give rise to colonies containing a heterogeneous population of macrophages and granulocytes were recorded on a weekly basis using phase-contrast microscopic observations at ×20 (Leica). Cell aggregates containing 40 or more cells were scored as colonies.

Immunofluorescence detection of β-catenin. To view the nuclear localization of β-catenin, cells collected from hydrogel surface seeding and/or from encapsulation were transferred to two-well chamber slides and incubated in the culture medium for 24 h and then fixed with 10% neutral-buffered formalin (NBF) at RT for 30 min. The cells were washed gently with 1x PBS, permeabilized in 1% triton-X and followed by incubated in 5% FBS at RT for 30 min. After gently removing the blocking solution, the cells were incubated with mouse anti-β-catenin antibody (Invitrogen, lot # 940535A) for 1 h followed by staining with phallolidin dye Alexaflour 488 goat anti-rabbit vs. isotype control. Nuclear staining with 4’,6-diamidino-2-phenylindole (DAPI) was performed before the cells were imaged for localization of β-catenin. Green fluorescence signal for β-catenin over DAPI was viewed at ×40 using an Olympus AX-70 epi-fluorescence microscope (Olympus America, Melville, NY, USA) equipped with a computer-controlled digital camera (Spot) for imaging. The positively-stained cells were quantified with Image Pro plus software (Media Cybernetics, Silver Spring, MD) as described earlier (24). Phase-contrast images of unstained cells were captured using Leitz-LABOVERT microscope.
Statistical analysis. Data on cell viability, MTT, IL-6 and other findings are statistically analyzed and expressed as mean±standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) using Graphpad Prism 5 software (San Diego, CA). Three independent sets of experiments were performed for every analysis simultaneously to confirm reproducibility.

Results

Survival, proliferation and colony formation of BMMCs in Me-HA-based 3D hydrogels. To test whether bioactive hydrogels support BMMCs survival and proliferation, first, we performed cell proliferation assays, in addition to regular microscopical observations. Findings from cell viability assays in hydrogel-encapsulated BMMCs of human primary multiple myeloma cells showed a higher % survival in the matrix of medium stiffness (MW 500kDa) compared to other lower or higher stiffness during the indicated time periods (Figure 1); however, the observed difference on cell viability between three different stiffness in the matrix was found to be statistically insignificant. Interestingly, data from MTT assays (O.D. values) showed an increase in the rate of cell proliferation (Figure 2) at the indicated time points.

BMMCs encapsulated in 3D hydrogels showed an increase in the CFUs. Since colony forming units (CFU) are the basis of an in vivo clonal expansion, we examined the rate of CFUs in the Me-HA-based 3D hydrogels. Homogenous encapsulation of $5 \times 10^5$ BMMCs showed small colonies identified as multiple thin, flat cells emanating from a central cluster of rounded cells as described earlier (25, 26). As presented in Figure 3 (phase-contrast view of colonies) and Table 1, we observed an increase in CFUs in hydrogel with medium stiffness of Me-HA after 7 (ranging from 16 to 17%, $p<0.05$) and 21 days (ranging from 26 to 27%, $p<0.01$); and from 20% to >65% increase ($p<0.01$) in the 3D gels respectively, in contrast to that in 2D or in a commercial matrigel, where the total number of CFUs was smaller.
Table I. Hydrogel composition, stiffness, molecular weight and the rate (%) of CFUs.

<table>
<thead>
<tr>
<th>3D Hydrogel Composition</th>
<th>Stiffness</th>
<th>Me-HA Molecular Wt.</th>
<th>Percentage of CFUs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day-4</td>
</tr>
<tr>
<td>Hybrid Me-HA/Me-Gel*</td>
<td>1</td>
<td>high molecular weight (~1000 kDa)</td>
<td>3.00±0.98</td>
</tr>
<tr>
<td>Hybrid Me-HA/Me-Gel*</td>
<td>2</td>
<td>Medium molecular weight (~500 kDa)</td>
<td>7.00±2.01</td>
</tr>
<tr>
<td>Hybrid Me-HA/Me-Gel*</td>
<td>3</td>
<td>Low molecular weight (~300 kDa)</td>
<td>3.33±1.53</td>
</tr>
<tr>
<td>Me-HA</td>
<td>-</td>
<td></td>
<td>2.33±0.58</td>
</tr>
<tr>
<td>Hybrid Me-HA/Me-Gel + Collagen-1</td>
<td>2</td>
<td>Medium molecular weight (~500 kDa)</td>
<td>8.33±2.89</td>
</tr>
<tr>
<td>Hybrid Me-HA/Me-Gel + Coll+Fib</td>
<td>2</td>
<td>Medium molecular weight (~500 kDa)</td>
<td>10.33±5.51</td>
</tr>
<tr>
<td>Matrigel (BD Biosciences)</td>
<td>-</td>
<td></td>
<td>6.67±2.89</td>
</tr>
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*Supports both surface seeding and encapsulation: Duan B et al. (15). Statistically significant after 7 days (ranging from 16 to 17%, **p<0.05) and 21 days (ranging from 26 to 27%, *p<0.01), respectively. Me-HA: Methacrylated hyaluronic acid; Me-Gel: methacrylated gelatin; Coll: collagen; Fib: fibronectin.
suggesting that the Me-HA-based 3D hydrogels are suitable for BMMCs survival and clonal expansion.

**Wnt-β-catenin signaling in BMSC encapsulated in Me-HA-based 3D hydrogels.** Recent advances in osteogenesis and osteolytic activities of the bone-related multiple myelomas indicate a critical role of Wnt pathways in the differentiation of bone marrow progenitor cells into osteoblasts or osteoclasts. Based on this, we attempted to detect a Wnt/β-catenin protein expression in hydrogel-encapsulated BMSCs after 21 days in culture. By performing immunofluorescence detection, we localized the β-catenin expression in the differentiating fibroblast-like cells viewed under high and lower magnifications. The cytoplasmic expression level of β-catenin was found to be significant in the differentiating encapsulated cells compared to that in the non-differentiating single cells (Figure 4A and 4B). It is important to mention

![Figure 4.](image)

Figure 4. Wnt-β-catenin signaling in human BMSC encapsulated in Me-HA-based 3D hydrogels: Wnt/β-catenin protein expression detected after 21 days in culture. A, Hydrogel encapsulation of BMSCs observed at lower magnification (20×), showing the spreading of cells inside the hydrogels, green signal for β-catenin stained with anti-β-catenin antibody. B, Immunofluorescence detection (as described in the methods section) of β-catenin (green) and nuclear staining with DAPI (blue), cellular localization of β-catenin protein in the differentiating fibroblast-like cells were captured by epifluorescence/confocal microscope (40×).

![Figure 5.](image)

Figure 5. IL-6 production of BMSC cells encapsulated in Me-HA-based 3D hydrogel: Production of interleukin-6 by bone marrow-derived stromal cells encapsulated in the hydrogel was measured in the cell culture medium collected at different time points. Bar graph represents mean±SD of the IL-6 level; Overall, the data indicate a gradual increase in IL-6 with a maximum after 21 days (p<0.01).
that β-catenin is a dual-function protein involved in osteogenesis, bone remodeling, regulation of the cell-cell adhesion and the transcription of the downstream targets of the Wnt signaling pathways in multiple myeloma.

**IL-6 production of BMSC cells encapsulated in Me-HA-based 3D hydrogel.** Since undifferentiated mesenchymal stem cells are reported (27, 28) to produce interleukin-6 (IL-6) supporting myeloma cell proliferation, we examined the levels of IL-6 in the cell culture medium collected at different time points after BMSC encapsulation in the hydrogels. Our findings from IL-6 ELISA assays showed a gradual increase in IL-6 with a maximum after 21 days (Figure 5) \( (p<0.001) \), suggesting the possible influence of stromal BMSCs on IL-6 production. The present findings are consistent with earlier reports in that when MM cells were adhered to BMSCs, there was a significant increase in IL-6 (1.9- to 56-fold) secretion and thus support our findings in that IL-6 probably was exclusively produced by BMSCs, rather than MM cells (27).

**Discussion**

Spheroid co-culture model and various scaffolds (10, 11) have been developed to recreate a 3D environment of the BM, but these models fail to recapitulate the physiological conditions of the BM with the exception of a model that was used to evaluate multiple myeloma therapy (12, 13). In the present study, for the first time, we used methacyrlated hyaluronic acid (Me-HA)-based hybrid hydrogels, to characterize multiple myeloma-related BMMCs and BMSC clonal expansion, differentiation and Wnt/β-catenin signaling. A novel aspect of this study is that we used Me-HA/Me-Gel hydrogels which are photocrosslinkable hydrogels with tunable physical properties, based on the oxidation and methacyrlation of HA and gelatin. We believe that these are promising scaffolds and are suitable to investigate bone marrow-derived stromal influence on the invasive and migratory pathobiology of myeloma disease. In the present study, we found that the hydrogels with medium mechanical stiffness (~500 kDA) could support the encapsulated BMMCs stimulating proliferation, spreading and the development of CFUs. Introducing Me-Gel enabled cells to interact with the hybrid hydrogels and stimulate cell spreading, proliferation, and migration is a unique observation reported for the first time. Our findings clearly showed the accommodation of the BMMC and BMSC cells in to the Me-HA hydrogels of medium stiffness that allowed us to further investigate survival, proliferation and clonal expansion (CFU formation) over a period of time ranging from 2 to 5 weeks similar to that of short-term animal studies. Based on these findings, we believe that 3D hydrogels with difference in the matrix composition and stiffness could provide a suitable substratum to comprehensively analyze molecular changes and to test drug efficacy. To date, to adequately investigate B-cell development and pathogenesis, there is a great need for a novel new 3D culture system. Our findings on BMMCs using Me-HA/Me-gel-hydrogels are consistent with the findings from our collaborators in that hydrogels are shown to be bioactive and biodegradable that mimic the extracellular matrix and accommodate cell survival (29). Overall, the new three-dimensional hybrid hydrogels can be tailored to be biomimetic and accurately recapitulate the native in vivo scenario and are expected to provide an important alternative to complex in vivo whole-organism approaches.

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**References**


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