Original Study

Nonadherent Spheres With Multiple Myeloma Surface Markers Contain Cells that Contribute to Sphere Formation and Are Capable of Internalizing Extracellular Double-Stranded DNA

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Abstract

We present phenotypic analysis of free-floating spheres derived from the bone marrow clonogenic aspirate of a multiple myeloma patient. Besides CD73/CD20$^+$/CD45$^+$/CD19^{dim} B cells, these spheres encompass a special subpopulation of cells that can natively internalize extracellular double-stranded DNA and that contribute to the colony assembly. Various cell types found in the free-floating spheres communicate with each other by secreting distinct sets of cytokines. These spheres are capable of forming xenotransplants upon grafting to immunodeficient NOD/SCID mice.

Background: The most prominent features of cancer stem cells are asymmetric cell division, tumorigenicity, and clonogenicity. Recently one more feature of poorly differentiated cell types of various origin, including cancer stem cells, has been described. Namely, these cells can internalize extracellular DNA natively, without additional transfection procedures. **Patients and Methods:** Using our approach to trace internalization of a TAMRA (carboxy tetramethylrhodamine [fluorescent dye])-DNA labeled probe by poorly differentiated cell types, we isolated and characterized the cells from free-floating spheres derived from the bone marrow clonogenic aspirate of a multiple myeloma patient. **Results:** Nonadherent spheres display a B-cell phenotype (CD73/CD20$^+$/CD45$^+$/CD19^{dim}). Further, free-floating spheres contain 1% to 3% cells with a clonogenic potential, and these cells display a marker of poorly differentiated cell types (TAMRA$^+$). Upon association with a group of ~ 10 free-floating TAMRA$^-$ cells, this peculiar cell type forms a sphere-forming cluster that initiates secondary aggregation of cells into a spheric structure. TAMRA$^+$ and TAMRA$^-$ cells secrete distinct sets of cytokines indicative of the paracrine regulation. Grafting experiments of intact whole spheres versus cell suspensions prepared from dispersed spheres indicate that successful engraftment only occurs in the former case. **Conclusion:** Nonadherent 3-D cell colonies (spheres) encompass B cells with CD73/CD20$^+$/CD45$^+$/CD19^{dim} phenotype, as well as double-stranded DNA-internalizing cells. The latter cell type appears to function as a sphere-forming center. Different cells in the spheres communicate with each other by secreting specific sets of cytokines. For successful engraftment and tumor growth in mice, intact spheres containing ~ 10^6 cells must be used.

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Introduction

Multiple myeloma (MM) is a malignant disease characterized by uncontrolled proliferation of plasma cells in the bone marrow. MM frequently manifests as lytic lesions in the bones, osteoporosis, and diffuse plasmacytosis in the bone marrow. Blood and urine tests typically show significant levels of "myeloma protein" or M-component (monoclonal immunoglobulin). Infiltration of bone marrow with plasma cells results in anemias, leucopenia, and/or thrombocytopenia, which leads to higher susceptibility to infections.[1-3]

MM is hypothesized to originate from a malignant transformation of a B cell followed by its clonal expansion in the bone marrow. All B cells are derived from a single lymphoid progenitor cell. After a process of maturation in the bone marrow, naive B cells are released into circulation and reach secondary lymphoid organs. Upon antigen encounter, immunoglobulin loci in B cells undergo somatic recombination. Such B cells clonally proliferate and form germinal centers, where memory B cells, short-lived plasmablasts, and long-lived plasma cells are produced. The cell clone obtained is characterized by a unique antibody idiotype, which in turn dictates the specific affinity for the antigen.

Much as normal B cells, MM cells also have their V(D)J loci rearranged, and they produce monoclonal immunoglobulin throughout the disease. This observation is consistent with the idea that postgerminal B cell is the likely candidate for MM cancer stem cell.[2] At the post-GC stage, only memory B cells are known to undergo symmetric (self-renewing) division, which is one of the prominent features of cancer stem cells and which is required for instant reactivation of the B cell clone upon antigen reexposure. Cumulative experimental evidence suggests that myeloma is organized in a hierarchy of cells, wherein a memory B cell that has undergone malignant transformation and that functions as an MM stem cell is found at the top of the hierarchy.[1-4]

Early mouse studies demonstrated that only a minor fraction of myeloma cells are clonogenic, ie, those responsible for the serial engraftment of MM, with later reports estimating their frequency as 1 in 1000 to 100,000 cells.[5,6] Significant efforts were made to identify and characterize specific cell surface markers of MM cancer stem cells. Clonogenic cells were shown to share several features of a stem cell: they display multiple drug resistance, can efflux lipophilic dyes, and produce large amount of aldehyde dehydrogenase.[1-3] Different studies have reported distinct and sometimes mutually exclusive combinations of cell surface markers for putative MM cancer stem cells. For instance, $CD19^{-}/CD45^{low-}/CD38^{high}/CD138^{+}$, $CD138^{+}$, $CD138^{neg}CD20^{+}CD27^{+}$, $CD19^{-}/CD38^{++}$, $CD45^{-}/CD38^{++}$, $CD19^{+}/CD20^{+}/CD27^{+}/CD138^{-}$, and $CD229^{+}$ phenotypes have been ascribed to MM stem cells.[1,7-11] These differences are likely attributable to the source of cells and the procedure used for establishing the MM cell population. An alternative explanation relies on the plasticity of cancer stem cells in terms of the markers of stemness.[2,3,10]

The salient feature of cancer stem cells, including those of MM, is their clonogenicity. There are 2 major ways to functionally assay this important property. First is the in vitro test for the presence of poorly differentiated cell types measured by the formation of primary and, upon serial passages, secondary spheres in a semiliquid medium. Nonetheless, the tumorigenic potential of these cells can only be unequivocally established in vivo, and serial engraftment into NOD/SCID (nonobese diabetic/severe combined immunodeficiency) mice is used for this purpose.[6,12] This latter approach is gaining popularity, as it is much more informative: not only it allows tumorigenicity estimates to be made, but also to perform phenotypic analysis of the cell clone, as well as to characterize the potential of serially passaged tumorigenic cells for self-renewal.

Several studies have reported on the analysis of MM colonies produced ex vivo.[1,6,13] Primary tumorospheres typically take several weeks to form.[6] Several cell surface markers are associated with sphere-forming cells: $CD138^{-}$, $CD34^{-}$, $CD45^{+}$, $CD19^{+}$, $CD22^{+}$, $CD138^{neg}$, $CD20^{+}$, $CD27^{+}$.[1,13] Thus, the spheres are composed of poorly differentiated plasmoblasts and mature plasma cells, with up to 80% of cells in the colony producing immunoglobulin of a single idiotype.[6]

Previously we discovered and comprehensively analyzed an important biological phenomenon, an ability of poorly differentiated cells of various origin to natively internalize the fragments of extracellular double-stranded DNA.[14-16] Using dUTP-carboxy tetramethyl-rhodamine (fluorescent dye) (TAMRA)-labeled *Alu* DNA as a probe, we showed that mouse Krebs 2 ascites cells and human glioblastoma neurospheres that internalize such extracellular DNA (and so become TAMRA positive) are the cells that are largely responsible for the graft tumorigenicity. Interestingly, $TAMRA^{+}$ cells show distinct spatial arrangement in different tumors. In the solid form of the Krebs 2 tumor model, meristemic cells appear as multilayered sheets of up to 500 cells randomly scattered throughout the tumor. In the context of glioblastoma transplants, $TAMRA^{+}$ cells are chaotically scattered in groups of up 50 cells or are present as individual cells.[15]

In the present study, we characterize spheroid-like 3-D cell colonies that were formed during long-term incubation of adherent cell fraction of a bone marrow aspirate from a MM patient. Our analysis suggests that colony-forming cells are of the B-cell lineage and are clonogenic. Using a TAMRA DNA internalization assay, we isolated and characterized the cells that can natively internalize DNA. These cells display a number of relevant cell surface markers and participate in the initial steps of assembly of secondary colonies, thereby functioning as clonogenic cells. Clonogenic properties of cells from nonadherent spheres were also confirmed in vivo. Transplanting intact 3-D cell aggregates resulted in successful grafting and tumor growth in NOD/SCID mice.

Materials and Methods

Patient Report

A 60-year-old man was enrolled onto the study with a diagnosis of MM (C90.0 World Health Organization 10.0). The disease had spread to the bones of skull, thoracic and lumbar spine, soft-tissue parts of the sternum, and the right scapula. Complete remission was achieved after treatment (4 PAD [bortezomib, doxorubicin and dexamethasone] and 5 VCD [bortezomib, cyclophosphamide and dexamethasone] courses).

The patient was admitted to the hematology department of the immunopathology clinics of the Institute of Fundamental and Clinical Immunology to undergo the procedure of autologous transplantation of peripheral hematopoietic stem cells combined with bone marrow mesenchymal stromal cells (authorized by the