Myeloma plasma cells alter the bone marrow microenvironment by stimulating the proliferation of mesenchymal stromal cells

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Methods (supplementary):

Colony Forming Unit-Fibroblast (CFU-F) Assay

Unfractionated or immune-selected BMMNC were plated at densities ranging from 0.1 to 1 x 10^4 per cm^2 and grown in α-MEM supplemented with 20% fetal calf serum, 2 mM L-glutamine and 100 μM L-ascorbate-2-phosphate, 50 U/ml Penicillin, 50 μg/ml Streptomycin in 5% CO_2 at 37˚C humidified atmosphere, as previously described (1). Colonies (cell clusters of >50 cells) were counted following fixation with 4% paraformaldehyde and staining with 0.1% toluidine blue. Primary BMSSC cultures were established by plating 1 to 5 x 10^4 unfractionated or STRO-1+ immune-selected BMMNC per cm^2 and then grown in α-MEM as described above.

Enzymatic Digestion of BM Stromal Cultures

Single cell suspensions were obtained from confluent primary stromal cultures by enzymatic digestion, as previously described (2). The cultures were washed twice in phosphate buffered saline pH 7.4 and then digested in a solution of collagenase (3 mg/ml) (Collagenase Type I; Worthington Biochemical Co., Freehold, NJ) and dispase (4 mg/ml) (Neutral Protease Grade II; Boehringer Mannheim, GMBH, Germany) for 90 minutes at 37˚C. Cell suspensions were then washed with growth medium before being passed through a Falcon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to obtain a single cell suspension.

Flow Cytometric Analysis and Cell Sorting

Stromal cells were resuspended in blocking buffer (HBSS + 20 mM Hepes, 1% normal human AB serum, 1% bovine serum albumin (BSA: Cohn fraction V, Sigma Aldrich Pty Ltd, NSW, Australia), and 5% FCS for 20 minutes on ice. Approximately 1-3 x 10^7 cells were pelleted in 14 ml polypropylene tubes (Falcon; Becton Dickinson, Franklin Lakes, NJ) and resuspended in 200 ml of saturating concentrations of B4-7827 (mouse IgG1 anti-human bone/liver/kidney alkaline phosphatase; Developmental Studies Hybridoma Bank, Iowa University, IA) and STRO-1 for 45 minutes on ice. The isotype matched negative control antibodies, IgG1 (3D3) and IgM (1A6.12) were used under identical conditions. Cells were washed in HBSS with 5% FCS and incubated with a goat anti-mouse IgG-PE (1/50) and a goat anti-mouse IgM-FITC (1/30) (Southern Biotechnology Associates, Birmingham, AL) for 45 minutes on ice. Cells were washed a further two times and resuspended to approximately 10^6 cells/ml prior to analysis with a Beckman Coulter Cytomics FC500 (Beckman Coulter, Miama, FL) using CXP Cytometry List Mode Data Acquisition and Analysis Software version 2.2, or sorting with a Becton Dickinson Aria flow cytometer (BD Biosciences, San Diego, CA), using FACS Diva Software version 6.1.3 and re-analysis using FCS Express for Flow Cytometry version 4 (De Novo Software, Los Angeles, CA). Positivity for each antibody was defined as the level of fluorescence greater than 99% of the isotype matched control antibodies. Following the initial sort each STRO-1/AP subpopulation was re-sorted and analysed to ensure a purity of >99%.

Isolation of Compact Bone-Derived Cells

Mice were humanely killed 4-weeks post tumour inoculation. Tibiae and femora were extracted and cleaned thoroughly. Bones were gently crushed with a mortar and

pestle and washed with 2% FCS/2 mM EDTA/PBS solution to remove marrow. Bones were incubated in 3 mg/mL collagenase solution containing 0.2% DNase for 5 minutes and cut finely using a #22 scalpel. Bone fragments were incubated in collagenase solution for 45 minutes, shaking at 37°C. Supernatant was collected through a 70 um nylon cell strainer and centrifuged at 400 x g for 10 minutes to collect cells.

RNA Isolation and Real-time PCR

For human samples, total cellular RNA was routinely prepared from 2 x 10^4 FACS double-sorted stromal cell subpopulations using Trizol extraction method (Life Technologies), as per the manufacturer's protocol. 1-2 µg of RNA was reverse transcribed using Superscript III (Life Technologies) as per the manufacturer's protocol. Real-time PCR was conducted on the Corbett Rotorgene using the primers detailed in Table 1. For mouse studies, RNA was extracted using the RNeasy Micro Kit, including DNase I treatment (Qiagen, Hilden, Germany) according to manufacturer's protocol. cDNA was synthesised with random primers using the AffinityScript QPCR cDNA synthesis kit (Agilent Technologies, Victoria, Australia) according to manufacturer's protocol. Real-time PCR was conducted using Brilliant II SYBR Green QPCR master mix (Agilent Technologies) in 20 µl reactions using 1 µl each forward and reverse primers (5 µM) on an Agilent Technologies, Stratagene MX3000P machine using primers detailed in Table 1.

Table 1. Real-time PCR primers

Gene	Species	Direction	Sequence (5'-3')
β-actin	Human	Forward	GATCATTGCTCCTCCTGAGC
		Reverse	GTCATAGTCCGCCTAGAAGCAT
IL-6	Human	Forward	ACAGACAGCCACTCACCTCTT
		Reverse	TTTCACCAGGCAAGTCTCCT
RankL	Human	Forward	TCAGCCTTTTGCTCATCTCACTAT
		Reverse	CCACCCCCGATCATGGT
OPG	Human	Forward	CGCTCGTGTTTCTGGACAT
		Reverse	ACACGGTCTTCCACTTTGCT
β2M	Mouse	Forward	TTCACCCCACTGAGACTGAT
		Reverse	GTCTTGGGCTCGGCCATA
IL-6	Mouse	Forward	TAGTCCTTCCTACCCCAATTTCC
		Reverse	TTGGTCCTTAGCCACTCCTTC
RankL	Mouse	Forward	AACATTTGCTTTCGGCATC
		Reverse	TTTCGTGCTCCCTCCTTTC
OPG	Mouse	Forward	TGTCCAGATGGGTTCTTCTCA
		Reverse	CGTTGTCATGTGTTGCATTTCC

References

1. Gronthos S, Fitter S, Diamond P, Simmons PJ, Itescu S, Zannettino AC. A novel monoclonal antibody (STRO-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. Stem Cells Dev. 2007 Dec;16(6):953-63.
2. Gronthos S, Zannettino AC, Graves SE, Ohta S, Hay SJ, Simmons PJ. Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 1999 Jan;14(1):47-56.