A Peculiar Molecular Profile of Umbilical Cord-Mesenchymal Stromal Cells Drives Their Inhibitory Effects on Multiple Myeloma Cell Growth and Tumor Progression

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Bone marrow-derived mesenchymal stromal cells (BM-MSCs) are under intensive investigation in preclinical models of cytotherapies against cancer, including multiple myeloma (MM). However, the therapeutic use of stromal progenitors holds critical safety concerns due to their potential MM-supporting activity *in vivo*. Here, we explored whether MSCs from sources other than BM, such as adipose tissue (AD-MSCs) and umbilical cord (UC-MSCs), affect MM cell growth in comparison to either normal (nBM-MSCs) or myelomatous marrow MSCs (MM-BM-MSCs). Results from both proliferation and clonogenic assays indicated that, in contrast to nBM- and MM-BM-MSCs, both AD and particularly UC-MSCs significantly inhibit MM cell clonogenicity and growth *in vitro*. Furthermore, when co-injected with UC-MSCs into mice, RPMI-8226 MM cells formed smaller subcutaneous tumor masses, while peritumoral injections of the same MSC subtype significantly delayed the tumor burden growing in subcutaneous plasmocytoma-bearing mice. Finally, both microarrays and ELISA revealed different expression of several genes and soluble factors in UC-MSCs as compared with other MSCs. Our data suggest that UC-MSCs have a distinct molecular profile that correlates with their intrinsic anti-MM activity and emphasize the UCs as ideal sources of MSCs for future cell-based therapies against MM.

Introduction

MESENCHYMAL STROMAL CELLS (MSCs) constitute the stroma of organs and tissues, and they contain a subset of stem cells with self-renewal and differentiation potential [1]. Besides the bone marrow (BM), MSCs are abundant in fat as adipose (AD) MSCs and in perivascular connective tissues such as the umbilical cord (UC) Wharton's jelly, as well as in other fetal or adult tissues where they act as dynamic cells for tissue repair and regeneration [2–4]. Extensive studies in xenogenic tumors have described that MSCs are chemoattracted toward the tumor microenvironment where they exert controversial effects as supporters or inhibitors of the tumor progression [5], whereas major data exploring the role of BM-MSCs in multiple myeloma (MM) definitely support their stimulatory activity on MM cell growth [6,7].

The expansion of MM cell clones within the BM is basically sustained by BM-MSCs that, once stimulated by malignant plasma cells, upgrade their secretion of interleu-

kin (IL)-6, a major growth factor for MM cells [8–10]. Moreover, direct molecular interactions of MSCs with other molecules such as CD44, very late antigen -4 and -5, vascular cell adhesion-1, and syndecan-1 on MM cells [11], in association to inflammatory cytokines, pro-angiogenic and pro-osteoclastogenic molecules secreted in response to the cell-to-cell cross-talk, contribute to tumor expansion [12]. Nevertheless, a suppressive activity of MSCs on MM cell growth has also been reported, both *in vitro* and in animal models of the human disease [13]. We have recently demonstrated that AD-MSCs stably engineered to express the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) efficiently migrate toward MM cells and exert anti-MM cytotoxicity *in vitro* [14], while others showed that MM-bearing SCID-rab mice injected with placenta-derived MSCs underwent dramatic inhibition of tumor growth within the bone [15].

Despite these encouraging data and successful MSC-based approaches in different solid tumors [16–18], the therapeutic potential of MSCs in MM is debated and largely dismissed in

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view of their supportive role in MM cell growth. Molecular studies of BM-MSCs from MM patients compared with healthy controls have, indeed, revealed recurrent genomic imbalances as deregulation of several genes [19], chromosomal gains and losses [20], and upregulation of factors implicated in MM progression and bone disease [21]. It has also been demonstrated that even normal MSCs co-cultured with MM cells undergo the genomic and phenotype alterations typical of MSCs derived from BM of MM patients [22]. Thus, the environment permissive for MM growth is attributable to genomic and secretory aberrations induced in quiescent MSCs by malignant plasma cells, which generate an inflamed marrow milieu where different soluble factors support the clonal expansion of MM cells [23].

Although the genomic conditioning of BM-MSCs in MM patients is apparently correlated to the extent of marrow and skeletal involvement, recent studies suggest that fetal MSCs, as those from placenta, are resistant to genomic aberrations induced by MM cells and exert a tumor-restraining effect in a mouse model of MM [24]. The suppression of Burkitt's lymphoma cell proliferation by UC-MSCs, indeed, emphasizes the native tumoricidal property of fetal MSCs in hematological malignancies [25].

Here, we investigated the effects of UC-MSCs as compared with AD-MSCs, as well as with normal and myelomatous BM-MSCs in co-cultures with MM cells. We found that healthy UC-MSCs definitely suppress myeloma cell growth both in vitro and in MM-bearing mice. Genomic and proteomic analyses of fetal MSCs revealed a variable content of anti-inflammatory and anti-proliferative factors that largely explain their inherited, general anti-myeloma activity both in vitro and in vivo. Our findings emphasize previous in vivo evidence [24] and support the use of fetal MSCs in planning novel cell-based strategies against MM.

Materials and Methods

MSCs, MM cell preparations, and phenotyping

MSCs were derived from different human tissues of healthy donors and MM patients: We obtained normal (n) and MM-BM-MSCs from eight healthy subjects undergoing orthopedic surgery and three patients with active, stage III MM. AD-MSCs were provided by Dr. Massimo Dominici (University of Modena and Reggio Emilia), and from aesthetic liposuction samples of three healthy individuals. Finally, UC-MSCs were isolated from ten UCs. All subjects gave informed consent to the study, which was approved by the local ethics committees.

Both nBM- and MM-BM-MSCs were isolated from marrow mononuclear cells (MNC) by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient centrifugation. Briefly, BM-MNC were plated in alpha-MEM medium (PAA Labs, Pasching, Austria) at a density of $8 \times 10^5/\text{cm}^2$ and incubated for 10 days. Then, nonadherent cells were removed and adherent cells were further expanded and replaced until the passage five at a density of $6,000/\text{cm}^2$. After the second passage, BM-MSCs from both control donors and MM patients were ready for further studies. AD-MSCs were similarly cultured in alpha-MEM medium, whereas UC-MSCs were obtained from fresh UCs as described [4]. Both RPMI-8226 and U266 MM cell lines (DSMZ, Braunschweig, Germany) were cultured in RPMI-

1640 medium (PAA Labs, GmbH), whereas primary myeloma cells were isolated from BM aspirates of two MM patients by adsorption on anti-CD138 immunomagnetic columns and incubated in RPMI 1640 medium.

Phenotype assessment of all MSC preparations was completed by a FACScanto cytometer (Becton-Dickinson, San Diego, CA) using MoAbs for mesodermal antigens such as CD73, CD90, and CD105 (BD Biosciences - Pharmingen, San Diego, CA) and hematologic markers, including CD14, CD45, and CD34. Experiments were performed in triplicate and included relative isotype controls.

Conditioning MM cell growth by MSCs

To assess the effect of MSCs on MM in vitro cell growth, two proliferation assays were performed. Briefly, MM cells were first stained with $1 \mu\text{L/mL}$, equal to 5 mM as working concentration, of CFSE (carboxy-fluorescein succinimidyl ester) (Molecular Probes, Life Technologies, Carlsbad, CA; CellTrace CFSE kit, Cat. No. C34554); then seeded at $50,000$ cells/well either in six-well co-culture plates; and supplemented with MSCs from each derivation that were positioned in $0.4 \mu\text{m}$ pored inserts (Corning, New York, NY) at 1:2 (MM cells: MSCs) cell ratio, or in culture using separately the conditioned media (CM) from the different MSC populations. These were obtained as follows: All MSC populations were grown in alpha-MEM, 10% FBS (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine (Lonza, Verviers, Belgium), and 100 U penicillin/streptomycin (PAA Laboratories) at $6,000$ cells/ cm^2 . At confluence, the cells were starved for 48 h at 37°C and the media were then removed, diluted with supernatants from MM cultured cells (RPMI 1640; PAA Laboratories) at different ratios (1:1 or 2:1), and used for CFSE-stained MM cell culture. The CFSE signal was calculated in living cells as mean fluorescence intensity (MFI) by the FACScanto at the first time point (T_0 , 1 h) and after 24, 48, and 72 h of co-culture. A live/dead stain (7-AAD) together with FSC/SSC analysis was used to gate live cells. At each time point, the decrease of MFI reflected the degree of MM cell proliferation as compared with controls. Data were analyzed by both FlowJo and FACSDiva software (BD Biosciences), and relative differences were interpreted as proliferation fold increase or MFI fold decrease. MM cells stained with $1 \mu\text{L/mL}$ CFSE and cultured in RPMI1640 served as controls for each time point. To assess the clonogenic effect of conditioned medium (CM) from each MSC preparation on MM cells, myeloma cells were seeded at $2,500$ cells/ cm^2 in 24-well plates using methyl-cellulose-based medium (MethoCultTM; Stem Cell Technologies, San Diego, CA) supplemented with each CM from the MSC preparations diluted in 1:1 volumes with MM cell medium. After 10 days, clumped colonies in wells were inspected by independent investigators, then arbitrarily selected in relation to their cell number higher than 50 cells, and subsequently counted on 10 microscopy fields by accurate optical microscopy measurement.

Moreover, we tested the capacity of UC-MSC conditioned medium to revert the clonogenic stimuli induced by BM-MM-MSCs on MM cells after 10 days of culture. Briefly, MM cells were harvested and plated at a clonal density of $2,500$ cells/ cm^2 using MethoCult separately supplemented with CMs from both starved UC-MSCs and

TABLE 1. MEAN LEVELS AS PG/ML OF PROTEINS MEASURED BY ELISA IN CONDITIONED MEDIA FROM MSCs FROM NORMAL BONE MARROW, MULTIPLE MYELOMA BM, AND UMBILICAL CORDS

PROTEIN	nBM-MSCs	MM-BM-MSCs	UC-MSCs
BLC	0.6	1.2	4.5
EOTAXIN	19	17.7	20.9
EOTAXIN-2	31.5	24.1	28.8
G-CSF	1	1	107.6
GM-CSF	33.4	33.9	36
I-309	128	281.1	707.9
ICAM-1	147.4	110.4	142.6
IFN- γ	130.7	122	119
IL-1 α	71.2	61.6	72.2
IL-1 β	13.8	19.3	46
IL-1ra	21.8	26	21.9
IL-2	17.4	14.4	17.9
IL-4	20.6	19.7	22.8
IL-5	66.6	56.3	62.8
IL-6	3594.7	6359.1	1808.8
IL-6Sr	28.3	73	20.1
IL-7	9.6	4.6	13.6
IL-8	221.5	578.7	1023.8
IL-10	31.8	24.9	26.4
IL-11	66.3	62.8	265.5
IL-12p40	62.7	70.2	62.9
IL-12p70	8.3	8.3	8.1
IL-13	16.4	16.5	15.8
IL-15	133.7	184.7	117.6
IL-16	54.4	41.4	39.9
IL-17	65.5	68.9	99.6
MCP-1	1156.5	4230.3	3945.4
M-CSF	2.7	4.7	2.9
MIG	496.6	372.4	392.3
MIP-1 α	7.8	41	9.9
MIP-1 β	0.4	18.3	0.1
MIP-1 δ	4.1	2.1	1
PDGF-BB	4.8	2.7	4.4
RANTES	2.4	14.3	1
TIMP-1	25957.8	22794.1	24905.7
TIMP-2	3298.1	1267.9	14436.9
TNF- α	176.3	150.3	144
TNF- β	61.4	23.5	22.5
TNF-R1	57.6	18.5	71.1
TNF-R2	1	1	1
AR	76.2	104	88.8
BDNF	5.5	4.2	16.7
β -FGF	105.9	50.3	86.2
BMP-4	145.3	84.7	167.7
BMP-5	98.1	374.3	360.5
BMP-7	35.2	75.1	72
β -NGF	3.6	5.4	8.5
EGF	2.8	0.6	0.1
EGFR	28.7	10.1	25.8
EG-VEGF	1	0	8.1
FGF-4	5671.5	4036.2	2573.6
FGF-7	438	286	319.4
GDF-15	6.2	41.4	19.1
GDNF	8.9	13	9.4
GH	367.2	424.4	391.6
HB-EGF	15.2	14.9	21.7
HGF	61.2	20.4	233.4
IGFBP-1	3.7	17.1	0.3
IGFBP-2	3518.2	649.7	134.3

(continued)

TABLE 1. (CONTINUED)

PROTEIN	nBM-MSCs	MM-BM-MSCs	UC-MSCs
IGFBP-3	14997.6	2613.3	1908.1
IGFBP-4	3684.4	2138.7	3391.9
IGFBP-6	619.3	207.8	124.3
IGF-1	254	104.3	22
Insulin	896.7	1055.7	1141.7
MCF-R	1810	1676.2	1968.9
NGF-R	175.4	148.3	166.4
NT-3	26.4	30.3	22.5
NT-4	28.3	13.6	0.2
OPG	2162.3	3980	1449.8
PDGF	6.6	2.5	7.1
PIGF	46.1	42.8	20.6
SCF	1	33.2	1
SCF-R	215.5	224.4	240.1
TGF- α	1	1	1
TGF- β 1	881.1	805.8	841.8
TGF- β 3	62.8	1	1
VEGF	1205.9	484	1
VEGF-R2	51.5	98.6	85.8
VEGF-R3	1070.5	1125.9	1441.6
VEGF-D	510.7	588.8	628.1

n, normal; BM, bone marrow; MM, multiple myeloma; UC, umbilical cords; MSC, mesenchymal stromal cell.

BM-MM-MSCs at a 1:1 ratio with MM cell CM. MM cells in normal CM were the controls.

In vivo effects of MSCs on MM cell growth—6–8-week-old NOD.CB17-Prkdc^{scid}/J mice (Charles River, Milan, I) were housed under pathogen-free conditions according to the guidelines, protocols, and permission terms of the Animal Care and Use Committee of the University of Bari. The animals were subcutaneously injected in the abdomen with different cell preparations and the tumor masses were measured at 10, 20, and 30 days after inoculation. Briefly, five groups of 10 mice were injected as follows: (a) 1×10^6 RPMI-8226 cells as control group; (b) 1×10^6 RPMI-8226 cells plus UC-MSCs at a 1:2 ratio; (c) 1×10^6 RPMI-8226 cells plus MM-BM-MSCs at a 1:2 ratio; and (d) 1×10^6 RPMI-8226 cells plus nBM-MSCs at a 1:2 ratio. Finally, additional mice (e) were similarly injected with RPMI-8226 cells and after 7 days, they were treated with peritumoral injections of 2×10^6 UC-MSCs in comparison with control animals treated with PBS.

Analysis of soluble factors in MSC CM

By using the Multiplex ELISA (Quantibody Human Cytokine Antibody Array; RayBiotech, Inc., Norcross, GA), we measured approximately 80 soluble proteins (Table 1) in UC-, nBM-, and MM-BM-MSC supernatants. Assays were performed according to the manufacturer's instructions, and data were obtained from experiments in triplicate.

Microarray analysis

Based on the different content of cytokines and soluble factors in conditioned media from MSC types, we investigated their RNA by multivariate microarray analysis. mRNAs were extracted by the RNeasy Kit (Quiagen,

Hilden, Germany) from ten UC- and eight nBM-MSC preparations at the second culture passage. Total RNA integrity was assessed using the Agilent 2100 Bioanalyzer® (Agilent Technologies, Santa Clara, CA), and samples with an RNA Integrity Number (RIN) less than 7.0 were excluded from the analyses. Labeled cRNA was obtained following the Low Input Quick Amp Labeling (LIQA) protocol (Agilent Technologies) and hybridized for 17 h at 65°C on Whole Human Genome Microarray 4×44 K slides that contain 44,000 probe sets representing all human genes. After hybridization, slides were washed according to the manufacturer's protocols and scanned using the High-Resolution Microarray C Scanner (Agilent). The Microarray assay was completed on triplicates of cell preparations and the gene expression analysis was assessed by GeneSpring GX 12.5 software that selected genes using a modified *t*-test, choosing 0.05 as *P*-value cut-off and ± 1.5 and ± 2.5 as fold change cut-offs. The *in silico* functional analysis was performed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity System, Redwood City, CA; www.ingenuity.com), to highlight main biological activities and functional networks connecting the genes of interest.

Statistical analysis

Data were analyzed by two-tailed Student's *t*-test for mean comparisons or one-way ANOVA for multiple comparisons. Statistical analyses were performed using Microsoft Excel 2010 (Microsoft, Redmond, WA) or Prism, Version 4 (GraphPad, Inc., San Diego, CA). Significance was set at $P < 0.05$.

Results

Morphology and immunophenotype of MSCs

Microscopy inspection showed the typical fibroblast-like, spindle-shaped morphology in all MSC preparations at the second culture passage (Fig. 1- upper section). Although deriving from different sources, all preparations showed a similar morphology, with a small increase in cell size for UC-MSCs. The expression of phenotype markers is also shown in representative cytometric panels (lower section). Mesodermal markers, including CD73, CD90, and CD105, occurred at the highest intensity in all MSC preparations; whereas those of hematopoietic lineage, such as CD45, CD34, and CD14, were absent. Mean values of both

FIG. 1. Morphologic and immunophenotypic features of mesenchymal stromal cells (MSCs) from different sources. Representative optical microscopy images (scale bars: 30 μ m) of adherent MSCs at the second culture passage. MSCs from different tissues sources show typical fibroblast-like morphology, co-express the mesodermal surface antigens CD73, CD90, and CD105 at high levels, while lacking the hematopoietic marker CD45, as indicated in the relative representative flow cytometry panels.

expression and mean fluorescence intensity ratio (MIFR) are detailed in Tables 2 and 3. This first assessment showed an apparent similarity in both morphologic and phenotype peculiarities among all MSC preparations regardless of the tissue sources and subjects.

Effect of MSCs on myeloma cell proliferation and clonogenicity

MM cells were co-cultured with each MSC population and the proliferation was measured at different time points (24, 48, and 72 h), as illustrated in Fig. 2A that representatively shows the variations of CFSE in RPMI 8226 cells, whereas Fig. 2B shows the results obtained in both primary plasma cells and MM cell lines. Similar to findings by others [26,27] on B-cell proliferation, CFSE peaks of MM cells underwent uniform variations. In fact, we found that when co-cultured till 72 h, myeloma cells maintained their proliferative ability with nBM-MSCs and this was apparently reinforced by MM-BM-MSCs as compared with control cell samples, although this increase was not significant ($P > 0.05$). By contrast, they underwent a significant decrease of proliferation rates when cultured with either AD- or UC-MSCs ($P < 0.05$ in all instances). The proliferation rate was significantly increased in RPMI-8226 cells in the presence of BM-MSCs and particularly with MM-BM-MSCs, whereas it was suppressed in co-cultures with either AD- or UC-MSCs ($P < 0.05$ in both instances). U266 cells were slightly reinforced in their growth by nBM- and MM-BM-MSCs, whereas again, they were suppressed by both AD- and UC-MSCs as compared with controls ($P < 0.05$). These data were corroborated by additional assays using CMs from different MSC populations. Supplementary Figure S1A (Supplementary Data are available online at www.liebertpub.com/scd) shows the time course of MFI decrease of CFSE, measured at different time points (24, 48 and 72 h) of MM cell growth. As illustrated, when cultured in AD- or UC-MSC CMs, both primary MM cells and established MM cell lines were suppressed in their cell proliferation (Supplementary Fig. S1B, $P < 0.05$); whereas when cultured in CM from BM-MM-MSCs, they were slightly reinforced with respect to controls ($P < 0.05$). These results suggest that, in relation to their tissue derivation, MSCs exert different effects on MM cell proliferation since AD- and particularly UC-MSCs significantly suppressed the cell growth compared with nBM-MSCs.

The clonogenic assays also supported differential proliferative effects in relation to the MSC subset. Figure 3 (up)

TABLE 2. EXPRESSION LEVELS AS PERCENTAGE OF POSITIVE CELLS OF MAJOR MESENCHYMAL AND HEMATOPOIETIC MARKERS OF MSCs FROM DIFFERENT SOURCES

	MM-BM-MSCs	nBM-MSCs	AD-MSCs	UC-MSCs
CD73	98.8±0.8	97.9±1.7	87.6±1.3	97.2±0.8
CD90	96.4±0.7	92.3±2.0	87.3±1.9	98.4±1.3
CD105	95.5±1.8	96.8±2.2	91.0±2.6	98.8±0.7
CD34	0.3±0.1	0.4±0.1	0.6±0.2	0.6±0.2
CD45	0.7±0.2	0.6±0.2	1.6±0.3	0.6±0.3
CD14	0.8±0.1	0.9±0.4	0.7±0.1	0.8±0.1

Values as M±SD of biological triplicates.

TABLE 3. MEAN FLUORESCENCE INTENSITY RATIO (MIFR) OF EXPRESSED MARKERS CALCULATED AS RATIO BETWEEN THE MEDIAN FLUORESCENCE OF EACH CELL POPULATION AND RELATIVE ISOTYPE CONTROL

	MM-BM-MSCs	nBM-MSCs	AD-MSCs	UC-MSCs
CD73	66±1.8	29±2.0	30±2.4	27±0.8
CD90	50±2.1	32±1.2	30±1.7	33±1.7
CD105	20±0.9	76±3.5	23±2.3	28±1.5
CD34	n.d.	n.d.	n.d.	n.d.
CD45	n.d.	n.d.	n.d.	n.d.
CD14	n.d.	n.d.	n.d.	n.d.

Values as M±SD of biological triplicates.
n.d., not done.

shows the RPMI colony formation in the presence of normal CM (Fig. 3A), CM from MM-BM-MSCs (Fig. 3B), and CM from UC-MSCs (Fig. 3C) that induced a generally higher suppressive effect. By counting the colonies in the presence of different CMs, we found that both primary myeloma cells and MM cell lines underwent a significant reduction equal to a three-fold decrease of the number of colonies formed in both AD- and UC-MSC CMs ($P < 0.05$) as compared with controls.

Variable effects were recorded in clonogenicity of MM cells by CMs. In fact, when cultured in the presence of CMs from either nBM- or MM-BM-MSC, all commercial MM cells displayed a significant two-fold increase of the colony number compared with controls; whereas with the exception of the MM cells from patient #1, whose colony formation was significantly inhibited by the CM from nBM-MSCs, the majority of MM cell preparations generated a number of colonies similar to controls in the presence of CMs from both nBM- and MM-BM-MSCs. Remarkably, both RPMI-8226 and U266 cells showed a high sensitivity to all MSC supernatants and alternatively underwent an increase or decrease of their clonogenic behavior in the presence of CMs from nBM- and MM-BM-MSCs or AD- and UC-MSCs, respectively ($P < 0.05$ in all instances). These data support the fact that CM from individual MSC populations contains soluble factors exerting variable effects on MM cell growth.

We also completed an additional clonogenic assay, proving that among factors released by UC-MSCs there are specific molecules that are capable of restraining, and probably reverting, the proliferation stimuli induced by BM-MM-MSCs. In fact, when cultured in 48 or 72 h CM from UC-MSCs after 10 days of exposure to BM-MM-MSCs, both primary and commercial MM cells showed a clonogenic potential similar ($P > 0.05$) or 50% lower ($P < 0.05$) than controls. By contrast, when cultured in the presence of CM from BM-MM-MSCs, MM cells dramatically increased till 2.5 fold the number of colonies, thus suggesting that factors released by BM-MM-MSCs reinforced the clonogenic behavior (data not shown).

In vivo effect of MSCs on MM growth—We next addressed the question as to whether MSCs derived from sources other than BM, in particular UC-MSCs, inhibit MM cell growth in vivo as compared with both nBM- and MM-BM-MSCs. We treated SCID mice with abdominal subcutaneous co-injections of RPMI-8226 cells and UC-MSCs at a 1:2 ratio, and we monitored the growth of plasmacytomas by measuring their volume at different time points (10, 20, and 30 days). As

FIG. 2. Effect of MSCs from different sources on multiple myeloma (MM) cell proliferation. **(A)** Representative flow cytometry panels of proliferation assays showing changes in fluorescence intensity between RPMI-8226 cells cultured alone (red peaks) and with MSCs (blue peaks). RPMI-8226 myeloma cells were labeled with fluorescence dye (CFSE) and analyzed by flow cytometry for decreased fluorescent signals as a measure of their proliferative rate after 24, 48, and 72 h as compared with T0 control (left box). Similar analyses were performed on parallel MM cell cultures supplemented with nBM-MSCs, MM-BM MSCs, UC-MSCs, and AD-MSCs. Analyses were performed by FlowJo software and calculated as the mean fluorescence intensity for each sample. **(B)** Histograms illustrating the impact of differentially sourced MSCs on the proliferation of both primary and commercial MM cells after 72 h of co-culture. Analysis of CFSE signals was performed with FlowJo software; MFI obtained from MM cells were normalized to T0 controls; and differences were expressed as proliferation fold increase. Data are reported as mean $\pm$ SD from three different experiments (* $P < 0.05$).

shown in Fig. 4, tumor masses showed a different growth and size in relation to the source of MSCs co-injected with the RPMI-8226 cells since although nBM- and MM-BM-MSCs exerted a mild tumor-supporting activity, co-injections of RPMI-8226 with UC-MSCs resulted in evident inhibition of tumor growth. These mice showed, indeed, tumor

masses with a 50% average size smaller than controls ($P < 0.05$). Figure 4 illustrates representative images of MM masses excised from mice 30 days after injection with either RPMI-8226 alone and with each MSC preparation.

Finally, we further verified the UC-MSC potential to suppress the tumor progression by peritumoral inoculations

FIG. 3. Effect of MSCs on MM cell clonogenicity. (Up)–Images of colonies of RPMI 8226 cells formed in the presence of normal (A) complete medium (CM), CM from MM-BM-MSCs (B), and CM from UC-MSCs (C) showing general higher suppressive effect on MM cell clonogenicity by CM from UC-MSCs. (Down)–Numbers of colonies formed by different MM cell lines after exposure to culture media from nBM-, MM-BM-, UC-, and AD-MSCs. Colonies with more than 50 cells were counted by optical microscopy inspection of 10 fields. A major suppressive effect on clonogenicity was uniformly recorded by CM from AD- and UC-MSCs in all instances ($*P < 0.05$). Data are expressed as mean $\pm$ SD from three independent experiments.

on 1-week established subcutaneous plasmacytomas in mice. Figure 5 shows the dynamic effect obtained by UC-MSCs 30 days after the injections when the suppressive effect resulted in significant delay of the cell growth with an approximately 20-fold decreased size of the tumor burden as compared with control animals injected with PBS ($P < 0.05$).

MSCs display peculiar secretome patterns

Based on our in vitro and in vivo findings, we investigated the paracrine anti-proliferative activity of UC-MSCs by analyzing their secretome in comparison to other MSC preparations. CM from UC-, nBM-, and MM-BM-MSCs were measured by quantitative ELISA screening of 80 different proteins whose mean levels are listed in Table 1. We then performed comparative analyses to verify differences between MSC populations and Fig. 6 (left) shows that, compared with UC-MSCs, both MM-BM- and nBM-MSCs secreted significantly higher levels (fold-change ≥ 1.5) of 19 and 16 cytokines, respectively. In detail, these cells over-secreted nine factors that are critically involved in MM cell growth, namely IL-6, RANTES, EGF, FGF-4, IGF-1, IGFBP-1, IGFBP-2, IGFBP-6, and VEGF. We also observed that IL-15, MIP-1 α , OPG, GDF-15, and SCF, which promote the MM progression through both direct and indirect mechanisms, appeared significantly over-secreted by MM-BM-MSCs, whereas other factors such as VEGF, TGF- β 3, NT4, and IGFBP1-3 were also

produced by nBM-MSCs. On the other hand, additional analyses revealed that UC-MSCs significantly overexpressed seven and nine cytokines compared with MM-BM- and nBM-MSCs, respectively (Fig. 6 right). Among these, BMP-4, BMP-5, BMP-6, BMP-7, I-309, and TIMP-2 are known to exert an inhibitory activity on MM growth.

Taken together, these data suggest that, compared with fetal MSCs as those from UCs, the stromal cells from both healthy and diseased BM predominantly possess a constitutive MM-supporting activity sustained by specific secretory profiles. The peculiar pattern of soluble molecules expressed by UC-MSCs thus explains, at least in part, the paracrine inhibition on MM cell growth in relation to their physical separation from MSCs in co-cultures.

MSC functional genes are differentially regulated according to the tissue origin

The gene expression profile of UC- and nBM-MSCs was assessed in triplicates by the Whole Human Genome microarray (44,000 probesets). Then, data obtained by an unsupervised analysis were filtered according to the ratio of the mean expression values in each sample group. A total of 353 distinct genes were found to be differentially expressed among these MSC populations, with a typical profile of 265 upregulated and, vice versa, 88 downregulated genes in UC- as compared with nBM-MSCs.

FIG. 4. Effects of MSCs on tumor growth in a subcutaneous model of MM. Measurement of the subcutaneous plasmacytoma growth in SCID mice at day 10, 20, and 30 after injection of RPMI-8226 cells alone or with MSC preparations from different sources at a 1:2 ratio. A significant reduction of the tumor burden was detected in mice treated with UC-MSCs in comparison with differently marrow-derived MSCs after 30 days of observation (* $P < 0.05$). Images are representative for each experiment completed in triplicate.

Using the Ingenuity platform, we also assessed the functional role of these genes, which are clustered in Table 4, according to their activity. As shown, we arbitrarily defined and grouped 191 genes into seven functional categories, correlated to major functions such as cell-to-cell

interaction, morphology, movement, inflammatory response, and molecular transport. In line with our ELISA data, several genes of soluble factors involved in MM biology, such as IL-6, IGF-1, and VEGF, were downregulated in UC- as compared with nBM-MSCs (data not shown).

FIG. 5. Effect of peritumoral injection of UC-MSCs in MM-bearing mice. SCID mice were subcutaneously injected with RPMI-8226 cells to produce plasmacytoma masses and after 1 week, they were peritumorally inoculated with UC-MSCs. The tumor growth was suppressed in mice treated with these cells compared with controls injected with PBS. Images of myeloma masses, excised from a representative animal (SCID mouse # 3) at 30 days after MM cell injection, proved the significant difference between the tumor masses developed (* $P < 0.05$), in terms of size and vascularization.

FIG. 6. Cytokine expression profile of MSCs from different sources. Comparison of ELISA measurement of 80 different proteins in supernatants of nBM-, MM-BM-, and UC-MSCs revealed that (A) both MM-BM- and nBM-MSCs released higher amounts of several cytokines and growth factors than UC-MSCs (fold change > 1.5). Most of them, as referred in the list later, stimulate MM cell growth, survival, and angiogenesis both in vitro and in vivo. (B) Vice versa, as compared with marrow-derived MSCs, UC-MSCs overexpressed several factors implicated in inhibition of the MM cell growth due to their anti-proliferative and anti-inflammatory properties. Data are expressed as mean fold-change values $\pm$ SD from biological triplicated measurements of each cytokine.

TABLE 4. FUNCTIONAL CATEGORIES OF 176 GENES DIFFERENTIALLY EXPRESSED BY UC-MSCS AS COMPARED WITH NBM-MSCS

Functional category (N. of differentially expressed genes)	Gene symbol
Cell death and survival [44]	ADAM, APMAP, ATG12, BCAR, BCL2, CCT2, CDIP1, CNL2, CREBBP, CTCF, DNAJB, EIF4, GAPDH, HRPN, HSPA, IRAK1, KEAP, LZTS, MAP3K, MBD, MCL, MED, MLH, MPG, MSH, MSR, MXD1, NFY, ODC, OGT, PDLIM, PIK3CP, PTPN, RABG, SIRT, SMAD, SMCO, STK, SUPT6, TASP, TCF3, THOC2, UHRF, VDAC
Cellular organization and molecular transport [37]	ADAMTS14, ATG12, ATG12, BCAR, BCL2, CROCC, CTCF, DNAJB1, DNAJC5, EIF4E, FTL, GAPDH, GGA1, HGS, HMBOX1, HNRNPA1, HPRT1, HSPA1A/1B, KIA1598, LZTS2, MCL1, MFF, MPG, NRF1, ODC1, P2RX4, PAFAH1B, PIK3C3, PLOD3, PMS2, RALA, RDH11, SLC25A29, SPTLC1, STARD3NL, STAU2, TUBB
Cellular function and maintenance [27]	ADAMTS14, ATP6AP1, ATP6V1D, BCAR1, BCL2, CREBBP, DNAJC5, ELK4, GAPDH, GRB2, HMBOX1, HNRNPA1, HSPA1A/1B, KIAA1598, MAP3K7, MCL1, MLH1, MSH2, P2RX4, PAFAH1B1, PIK3C3, PLOD3, PMS2, PTPN2, RALA, STAU2, TUBB
Cell-cell interaction and inflammatory response [18]	ADAM17, BCL2, ELK4, GRB2, HSPA1A/1B, IFNGR2, IRAK1, LRBA, MAP3K7, MCL1, MPG, MSH2, OGT, PAFAH1B1, PTPN2, RALA, SMAD4, TWF2
Cell growth [25]	ADAM17, BCL2, CNOT7, CREBBP, CTCF, DYRK3, FAH, GRB2, HSPA1A/1B, LZTS2, MCL1, MLH1, MSH2, MXD1, MYCBP, ODC1, PIK3C3, PMS2, RALA, SCLT1, SEL1L, SENP1, SMAD4, TCF3, TNRC6B
Cell morphology and movement [17]	BCAR2, BCL2, CREBBP, EIF4E, GAPDH, GRB2, HGS, LZTS2, PAFAH1B1, PIK3C3, PLOD3, RALA, STAU2, STK4, TCF3, TWF2, WASF2
Lymphoid tissue development [8]	BCL2, CREBBP, ELK4, GRB2, MCL1, NFYA, PTPN2, TCF3

FIG. 7. Differential gene expression profile between UC- and nBM-MSCs. Among the 353 differentially expressed genes observed by microarray analysis, 32 genes were arbitrarily selected according to their highly significant up- (black) or downregulation (gray) in UC- compared with nBM-MSCs (fold change > 2.5). Based on their main biological role, as indicated by Ingenuity Pathway Analysis software, these genes were then grouped into different functional categories potentially implicated in the cross-talk between MSCs and other cellular elements, including tumor cells. The columns express the mean fold-change $\pm$ SD of values from biological triplicates of cell preparations.

To emphasize differences in this group of deregulated genes, we arbitrarily extended the cut-off to a 2.5-fold change to select a restricted number of genes differentially expressed between UC-MSCs and nBM-MSCs. Figure 7 depicts the differential expression profile of 32 genes belonging to various functional clusters. As can be seen, UC-MSCs significantly overexpressed genes involved in cell-to-cell communication such as *RALA*, *MPG*, *SMAD-4*, *OGT*, *GRB-2*, and *MAP3K7*, while others that were functionally implicated in cell responses to inflammatory stimuli such as *ELK-4*, *MCL-1*, *BCL-2*, and *MSH-2* were significantly downregulated ($P < 0.05$ in all instances). Furthermore, other genes related to cell motility and chemotactic functions, such as *HGS*, *BCAR-1*, *PLOD-3*, and *PAFAH1B1*, were also overexpressed till three-fold ($P < 0.05$) in fetal MSCs as compared with nBM-MSCs. We also recorded upregulated genes till three-fold ($P < 0.05$) *GAPDH*, *HGS*, *ATG13*, and *STARD3NL*, which are known to drive the intracellular formation of exosomes and microvesicles [28], as well as a variable modulation of genes involved in the secretion of dense granules and exocytosis of soluble molecules such as *DNAJB-1*, *DNAJC-5*, *HPRT-1*, *HSPA1A*, and *BCL-2*.

Discussion

Based on their native homing property toward tumor sites and delivery of anti-tumor drugs after genetic engineering, MSCs from human tissue sources have been successfully explored in preclinical models of cytotherapies against cancer [29]. With the aim of investigating the application of this cell-based approach to treat MM, we examined possible molecular and functional discrepancies among MSCs from healthy adult and fetal tissues, and BM-MSCs from normal donors and MM patients, since these cells act as supporters of MM cell growth [7]. In fact, a major translational drawback of MSC-cytotherapies in MM was that marrow stromal progenitors sustain the survival of malignant plasma cells and contribute to the disease progression [30]. Using comparative assays, we found that UC-MSCs, in particular,

definitely exert a suppressive effect on MM progression, and they are therefore candidates for future cytotherapies to combat this malignancy.

We derived MSCs from UCs, adult fat, and BM from both normal donors and MM patients and found that freshly isolated cells from all these sources showed a similar fibroblast-like morphology with a typical mesenchymal immunophenotype and homogeneity in early culture passages. However, while UC-, AD-, and nBM-MSCs preserved their morphology during their culture passages, BM-MSCs from MM patients failed to maintain their shape till the third passage and underwent early senescence, as shown by their larger, flattened morphology and intense cytoplasmic granularity. This aspect of BM-MSCs has recently been correlated to the pathophysiology of MM, since senescent MM-conditioned MSCs are more likely to secrete factors, such as IL-6, IGF-1, and OPG, promoting oncogenic modifications of the MM microenvironment [31]. We observed that myelomatous BM-MSCs clearly reinforce the clonogenicity of most MM cell strains and hypothesized that this effect was due to an increased release of growth factors such as effect of the original influence of MM cells [22]. In fact, it has been reported that MM-derived BM-MSCs maintain their paracrine tumor-supporting activity even when removed from their microenvironment and after multiple culture passages [19].

BM-MSCs have been described to exert immunosuppression on T cells [32]. In our study, we found that nBM-MSCs were permissive for MM cell proliferation and clonal expansion, implying an inherited commitment to support the cell growth also based on marrow immunosuppressive properties that probably favor both survival and marrow localization of malignant plasma cells [33]. On the contrary, our in vitro studies revealed that healthy MSCs from sites other than BM, such as UC and fat, restrained the MM cell proliferation and colony-forming capacity and the repeated clonogenic assay proved that among the factors released by UC-MSCs there are specific molecules that are capable of restraining and perhaps reverting the stimuli given by the

exposure to BM-MM-MSCs. These results suggest that, depending on both source and physiological role, each MSC population generates peculiar sets of intercellular signals producing a general pro- or anti-tumor effect, and that the use of autologous or allogeneic BM-MSCs in future models of anti-MM cytotherapies still holds critical safety concerns, including the risk of long-term tumor-supporting effects in vivo.

To better explore these diversities, we investigated secretomes from all MSC preparations. Our analyses confirmed that, compared with UC-MSCs, both nBM- and MM-BM-MSCs produce higher amounts of pro-MM cytokines, namely IL-6, IL-15, IGF-1, MIP-1 α , OPG, and RANTES [34–38]. These results appear in line with most studies reporting that BM-MSCs from MM patients mediate the formation of an intense marrow gradient of soluble [39,40] or exosome-incorporated cytokines with a biological impact on the survival of myeloma plasma cells [6]. A striking result was that both MM-BM-MSCs and nBM-MSCs secrete VEGF at much higher levels than UC-MSCs. This strongly supports the notion that elevations of VEGF are detectable in marrow plasma of MM patients [41] and implies a powerful tumorigenic potential of BM-MSCs due to the pathogenic role of pro-angiogenic factors in marrow MM expansion and progression [42]. Similarly, our observations parallel the results from other studies showing that MM-BM-MSCs produce greater amounts of GDF-15 [20], a microenvironment-derived factor belonging to the TGF superfamily, with a known role in supporting both proliferation and chemo-resistance of myeloma cells [43].

On the other hand, UC-MSCs were found to release higher amounts of BMP-5, BMP-6, TIMP-2, and I-309, which exert a defined antiproliferative effect on cell growth by inhibiting the cell cycle [44–46]. This intrinsic paracrine anti-tumor activity of UC-MSCs has been further confirmed by recent in vivo cytotherapy strategies against breast cancer, in which fetal MSCs successfully control the tumor growth by inhibiting both PI3K and AKT protein kinase activities [47]. Besides these results, other differences were reported in secretomes between UC- and adult BM-MSCs describing a higher content of trophic factors in BM-MSCs [48] and emphasizing the immunosuppressive properties of UC-MSCs [32,49] in relation to their peculiar paracrine profiles.

To verify whether the apparently MM-suppressive profile of UC-MSC secretomes was paralleled by consistent gene expression, we compared the global gene profiles of UC- with nBM-MSCs, namely the MSC population commonly used in experimental anti-cancer cytotherapies in humans, and found 353 differentially expressed genes. In particular, mRNAs of many soluble factors implicated in both direct and indirect stimulation of MM cell growth, such as *IL-6*, *IGF-1*, and *VEGF*, were significantly overexpressed in nBM-MSCs, whereas UC-MSCs were downregulated in others that participate in controlling both apoptosis and pro-inflammatory responses, namely *ELK-4*, *MCL-1*, *BCL-2*, and *MSH-2* [45,48]. The observed levels of these genes suggested a weaker pro-inflammatory profile of UC-MSCs as compared with nBM-MSCs, which reflected the lower levels of pro-inflammatory and tumorigenic cytokines detected in the proteomic assay. In addition, we identified several genes implicated in cell-to-cell communication such as *RALA*, *MPG*, *SMAD-4*, *GRB-2*, *OGT*, and *MAP3K7* [50–52], which are significantly overexpressed in UC- compared

with nBM-MSCs. It is thus conceivable that they mediate the expression of specific signals from UC-MSCs to tumor cells and drive, both directly and indirectly, the inhibitory effect that fetal MSCs exert on MM cell growth in comparison to marrow MSCs.

These effects may also be supported by the significant differential expression of other genes such as *DNAJC-5*, *STARD3NL*, *HPRT-1*, and *HGS*, which play a role in the assembly, accumulation, and exocytosis of secretory granules and exosomes [53,54]. These, in turn, may confer a peculiar paracrine ability to each MSC population in relation of their protein content. Finally, we found that, compared with nBM-, UC-MSCs significantly overexpressed *GRB-2*, a pivotal gene involved in cell motility that controls cellular movement in response to inflammatory stimuli and tumor-derived chemokines [55]. Although worthy of further investigation, this finding supports previous evidence attributing an efficient tumor-homing capacity to UC-MSCs and further highlights their potential for use in applicative studies of translation to the clinical setting of anti-tumor cytotherapies. Our microarray data thus provide evidence supporting the molecular, genomic, and functional diversities of MSCs depending on their tissue derivation. On the other hand, fetal MSCs have been shown to express a peculiar tumoricidal pattern against hematologic malignancies [25], rather than a profile committed to generating inflammatory responses.

Such a putative anti-inflammatory and generally anti-tumor activity of UC-MSCs was also evaluated in vivo in MM-bearing SCID mice. In our subcutaneous model of myeloma, UC-MSCs significantly inhibited tumor growth after both concomitant and delayed co-injections with MM cells. This effect was particularly impressive when comparing the effect of UC- with either nBM- or MM-BM-MSCs that, on the contrary, promoted the development of voluminous tumor masses in mice. As compared with other animal models of intra-bone MM-bearing mice [24], our subcutaneous MM prototype supported the concept that the tumor suppression was directly related to the UC-MSC paracrine activity. Even when injected in established plasmocytomas, in fact, UC-MSCs exerted a dramatic inhibition of the malignant cell growth, thus highlighting the anti-tumor activity of their secretome.

Taken together, our findings emphasize the potential of naïve stromal cells from fetal healthy tissues in the design of cell-based treatments against MM. To date, adult MSCs “armed” by genetic engineering have been used to target MM cells in a few pioneering cytotherapy approaches [29]. BM-MSCs induced to overexpress Fas-L or IFN- α have been successfully applied to induce apoptosis in MM cells [13,56], while we have recently proved that AD-MSCs, engineered to stably overexpress the membrane form TRAIL, trigger a remarkable apoptosis of MM cells in vitro [14], thus improving the therapeutic efficacy of TRAIL in MM. This study, however, indicates that UC-MSCs possess a constitutive ability to control malignant plasma cell growth both in vitro and in vivo by paracrine mechanisms. Therefore, they can be cellular vectors for delivering MM-killing agents. In fact, beyond their intrinsic tumor tropism [57], these cells possess further advantages for cytotherapies such as their virtually unlimited availability, low immunogenicity [58], anti-inflammatory potential [59], absence of

spontaneous malignant transformation [60], and suitability to genetic manipulation [61]. However, further studies are needed to optimize their therapeutic use in preclinical models of MM as well as to define their efficacy and safety profiles for future cell-based gene therapies for the clinical setting.

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