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ARTICLE

Inappropriate Notch Activity and Limited Mesenchymal Stem Cell Plasticity in the Bone Marrow of Patients with Myelodysplastic Syndromes

Gergely VARGA,[1] Judit KISS,[2] Judit VÁRKONYI,[1] Virág VAS,[2] Péter FARKAS,[1] Katalin PÁLÓCZI,[3] Ferenc UHER[2*]

[1]3rd Department of Internal Medicine, Semmelweis University, [2]Stem Cell Biology, National Medical Center, [3]Department of Immunology, National Medical Center, Budapest, Hungary

Myelodysplastic syndromes (MDSs) are a heterogeneous group of hematological disorders characterized by ineffective hematopoiesis, enhanced bone marrow apoptosis and frequent progression to acute myeloid leukemia. Several recent studies suggested that, besides the abnormal development of stem cells, microenvironmental alterations are also present in the MDS bone marrow. In this study, we have examined the relative frequencies of stem and progenitor cell subsets of MDS and normal hematopoietic cells growing on stromal cell layers established from MDS patients and from normal donors. When hematopoietic cells from MDS patients were co-cultured with normal stromal cells, the frequency of either early or late cobblestone area-forming cells (CAFC) was significantly lower compared to the corresponding normal control values in 4 out of 8 patients. In the opposite situation, when normal hematopoietic cells were incubated on MDS stromal cells, the CAFC frequencies were decreased in 5 out of 6 patients, compared to normal stromal layer-containing control cultures. Moreover, a soluble Notch ligand (Jagged-1 protein) was an inhibitor of day-35-42 CAFC when normal hematopoietic cells were cultured with normal or MDS stromal cells, but was unable to inhibit MDS stem and early progenitor cell growth (day-35-42 CAFC) on pre-established stromal layers. These findings suggest that in early hematopoietic cells isolated from MDS patients the Notch signal transduction pathway is disrupted. Furthermore, there was a marked reduction in the plasticity of mesenchymal stem cells of MDS patients compared with those of normal marrow donors, in neurogenic and adipogenic differentiation ability and hematopoiesis supporting capacity *in vitro*. These results are consistent with the hypothesis that when alterations are present in the myelodysplastic stroma environment along with intrinsic changes in a hematopoietic stem/progenitor cell clone, both factors might equally contribute to the abnormal hematopoiesis in MDS. (Pathology Oncology Research Vol 13, No 4, 311–319)

Key words: cobblestone area-forming cells, Jagged-1, myelodysplastic syndromes, Notch signaling, stem cell plasticity

Introduction

Notch signaling plays a key role in the development and differentiation of various hematopoietic lineages. In the hematopoietic system, Notch receptors are expressed in early hematopoietic cells, whereas Notch ligands are found in bone marrow stroma that provides the microenvironment necessary for stem and progenitor cell survival, proliferation and differentiation.[3,11-13] Recently we have shown that Jagged-1 immobilization on stromal layer or on Sepharose-4B beads are required for the induction of self-renewing divisions of days 28-35 murine cobblestone area-forming cell (CAFC). On the other hand, the free, soluble Jagged-1 has a dominant-negative effect on self-renewal in the early progenitor cell compartment, presumably by sequestering the Notch receptor and preventing Notch interaction with the immobilized ligand.[30] While there is evidence indicating that Notch signaling is involved in the pathogenesis of human bone marrow-derived neoplasms,[31] no data are available regarding the role of Notch receptors and ligands in myelodysplastic

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Correspondence: Ferenc UHER, PhD, National Medical Center, Stem Cell Biology, Diószegi út 64., Budapest, H-1113, Hungary; Tel: +36 1 372 4334, fax: +36 1 372 4352, e-mail: uher@kkk.org.hu

Abbreviations: AML, acute myeloid leukemia; BFU-E, burst-forming units erythroid; BM, bone marrow; CAFC, cobblestone area-forming cells; CFU-GEMM colony-forming units granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM); CFU-GM, colony-forming units granulocyte-macrophage; MDS, myelodysplastic syndromes; MSC, mesenchymal stem cells; RA, refractory anemia; RAEB, RA with excess blasts; RARS, RA with ringed sideroblasts

syndromes (MDSs). The only possible exception is a delta-like protein (Dlk) which might belong to the Notch family of signal transducers. This protein was upregulated in low-grade MDS as demonstrated by DNA array screening and flow cytofluorometry.[10,14,16]

The MDSs are a heterogeneous group of hematologic disorders characterized by cytopenias in one or more hematopoietic cell lineages despite marrows that demonstrate hypercellularity and evidence for ineffective and dyspoietic hematopoiesis. Patients with these disorders are afflicted with complications related to cytopenias and progression to acute leukemia.[15,22] The pathogenesis of MDS is complex, since blood-forming cells and the hematopoietic bone marrow microenvironment are both involved in the establishment and progression of the disease. Earlier studies examining the stromal microenvironment in MDS were unable to identify stromal anomalies co-existing with stem cell abnormalities.[2,7] Recently, however, cultures of normal marrow and umbilical cord blood hematopoietic precursor cells on MDS-derived stroma have highlighted the inability of MDS stroma to support hematopoiesis, compared with normal stromal layers.[1,6,23-25] Significant differences in terms of cytokine production by microenvironmental cells from normal and MDS stroma have also been observed.[8,32]

In the present study, we have obtained serially passaged populations of bone marrow stromal cells, i.e. mesenchymal stem cell (MSC) cultures, from normal donors and MDS patients. We investigated their growth pattern and differentiation ability (plasticity) *in vitro*. We also examined their capacity to sustain and control growth and differentiation of hematopoietic progenitors of the same bone marrow sample or of normal blood-forming cells. We studied this both in the presence and in the absence of soluble Jagged-1 protein. We have found that adherent cell layers from MDS marrow are usually defective in their ability to support normal hematopoiesis *in vitro*, showing poor maintenance of stem and early progenitor cells. We have also shown evidence of limited MSC plasticity and defective Notch regulation of myelodysplastic hematopoiesis.

Materials and Methods

Patients

Bone marrow samples were obtained from 10 untreated MDS patients and 15 healthy donors. Clinical and hematological data have been classified according to the World Health Organization (WHO) subtype[28] and listed in *Table 1*. None of the patients had received cytotoxic drugs during the preceding 3 months. The control subjects were hematologically normal, predominantly surgery patients of the same age range as the MDS patients. Not all assays were performed in all cases because of the limited availability of specimens collected from the patients (*Tables 1-3*).

Isolation and cultures of human bone marrow cells

For the isolation of human BM cells, bone marrow aspirates were taken from normal adult donors and MDS patients after having their informed consent, according to a protocol approved by the local Ethics Committee (No: 2003-1018EKU). Nucleated cells were isolated with a density gradient (Lymphoprep, Nycomed Pharma, Oslo, Norway) and resuspended in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO). All nucleated cells were plated in 25-cm^2 flasks (BD Falcon, Bedford, MA) at 37°C in humidified atmosphere containing 5% CO_2. After 24 hours, nonadherent cells were removed and cryopreserved in liquid nitrogen until use. The remaining adherent cells were thoroughly washed with Hanks balanced salt solution (HBSS) (GIBCO). Fresh complete culture medium was added and replaced every 3 or 4 days.

Table 1. **Clinical and hematological characteristics of the patients**

Patient	*Age*	*Sex*	*WHO classification*	*Cytogenetics*	*WBC ($x10^9$/l)*	*Hemoglobin (g/dl)*	*Platelet ($x10^9$/l)*
MDS-1	71	Male	RA	46, XY	5.2	13.0	114
MDS-2	80	Male	RARS	49, XY, +8, +12, +13	6.6	5.8	127
MDS-3	90	Male	RAEB II.	ND	1.6	9.4	26
MDS-5	84	Female	RA	46, XX, del 16q	9.5	7.7	355
MDS-7	68	Female	RA	45, XX, del 5q, -8, -18, -20	2.9	8.2	191
MDS-11	78	Male	RARS	43, XY, del 5q, -14, -18, -20	4.0	8.4	40
MDS-12	51	Male	RAEB I.	45, XY, -7	2.7	12.6	25
MDS-24	66	Female	RAEB I.	47, XX, +10	5.5	10.0	10
MDS-25	58	Male	Post-MDS AML	46, XY	50.0	8.1	50
MDS-26	68	Male	RARS	46, XY	1.4	9.0	80

AML, acute myeloid leukemia; RA, refractory anemia; RAEB, RA with excess blasts; RARS, RA with ringed sideroblasts; WHO, World Health Organization

Table 2. **Colony-forming ability of patient's mononuclear bone marrow cells**

Patient	*Number of colonies per 10^5 mononuclear cells (mean ± SD)*		
	CFU-GM	*BFU-E*	*CFU-GEMM*
MDS-1	62.3 ± 5.1	22.5 ± 2.5	2.5 ± 1.5
MDS-2	1.7 ± 1.1	0	0
MDS-3	1.7 ± 1.1	0	0
MDS-5	15.7 ± 1.2	0	0
MDS-7	79.3 ± 5.1	17.5 ± 1.7	2.5 ± 0.7
MDS-11	96.0 ± 19.0	0	0
MDS-12	58.7 ± 7.5	12.5 ± 0.7	0
MDS-24	18.7 ± 3.1	12.5 ± 2.1	2.5 ± 0.7
MDS-25	>300	NT	NT
MDS-26	3.3 ± 1.5	0	0

Normal values were 52-128 for CFU-GM, 23-91 for BFU-E, and 2-11 for CFU-GEMM, respectively (n=15). NT, not tested

When cells grew to 80% confluence, they were harvested with 0.25% trypsin and 1 μM EDTA (Sigma Chemical Co., St. Louis, MO) for 5 minutes at 37°C and designated as passage 1. These cells were further expanded with splitting 1:3-1:5 in 175-cm^2 flasks (BD Falcon). In this study, cells between passage 3 and 6 were used as mesenchymal stem cells (MSC).

The total numbers of nucleated and viable cells were determined with a hemocytometer, using Turck's solution and trypan blue stain, respectively. The morphology of MSC was examined every week under an inverted microscope (Olympus CK2, Tokyo, Japan) to identify possible structural differences between MDS and normal MSC layers.

Table 3. **Effect of soluble Jagged-1 on CAFC frequencies of patients' bone marrow mononuclear cells in the presence of normal stromal cells***

Patient	*Day-7 CAFC frequency per 10^5 mononuclear cells*		*Day-42 CAFC frequency per 10^5 mononuclear cells*	
	Medium	*Soluble Jagged-1 (5 μg/ml)*	*Medium*	*Soluble Jagged-1 (5 μg/ml)*
MDS-7	17	25	15	8
MDS-1	16	33	13	21
MDS-11	29	41	7	11
MDS-12	14	29	5	9
MDS-26	12	22	5	9
MDS-5	3	1	4	5
MDS-3	0	1	0	1
MDS-2	3	1	0	2

*Cultures initiated with non-adherent mononuclear cells from MDS patients' bone marrow in the presence of established irradiated stromal cell layers from normal individuals

Recombinant Jagged-1 protein

Human recombinant Jagged-1 protein was cloned and purified as previously described.[30] Briefly, COS7 cells were transfected with the pUSEamp vector containing cDNA coding for the Jagged-1 extracellular domain (Upstate Biotechnology, Lake Placid, NY). Conditioned medium samples were collected and absorbed on, and eluted from Sepharose-4B (Amersham Pharmacia Biotech, Uppsala, Sweden) column coated with goat anti-Jagged-1 antibody (Sigma).

Progenitor (colony-forming cell) assays

Quantification of the number of colony-forming units granulocyte-macrophage (CFU-GM), burst-forming units erythroid (BFU-E) and CFU granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM) was performed using a semisolid colony-forming cell assay. Nonadherent bone marrow cells were suspended in Iscove's modified Dulbecco's medium supplemented with 1% methylcellulose, 30% FCS, 10% v/v 5637 (ATCC, HTB-9) human bladder carcinoma-conditioned medium, 5 IU/ml erythropoietin, 4 x 10^{-3} M/l L-glutamine, 2.5 x 10^{-4} a-thioglycerol, 1% deionized bovine serum albumin (Sigma), and antibiotics. They were plated in triplicate in 35-mm culture dishes (BD Falcon) and incubated in humidified 5% CO_2 incubator at 37°C. Colonies with more than 50 cells were counted on days 9 and 14 of culture in the same dishes.

Stromal ability to support hematopoiesis (cobblestone area-forming cell assay)

Hematopoiesis-supporting stromal cell layers were established in CAFC medium (α-modified DMEM supplemented with 12.5% FCS, 12.5% horse serum, HEPES (3.5 mM), L-glutamine (2 mM), β-mercaptoethanol (10^{-4} M) (all from GIBCO), hydrocortisone 21-hemisuccinate (10^{-6}) and antibiotics from normal or MDS MSCs. Confluent stromal layers were irradiated with a dose of 15 Gy and washed with CAFC medium.

Determination of hematopoietic stem and progenitor cell frequencies was preformed by limiting dilution analysis of CAFC in micro-cultures according to methods previously described by Rob E. Plomacher's group.[5] Nonadherent bone marrow cells were washed with CAFC medium before the CAFC assay. For each sample, 6 to 8 serial two-fold dilutions of cells were prepared and plated in flat-bottom 96-well plates (BD Falcon) over a pre-established confluent layer of stromal cells. Twenty-four wells per

dilution were plated for each group of cells and maintained at 33°C and 5% CO_2. Once a week, half of the medium (100 μl) was carefully removed from each well, and an equal volume of fresh medium was added. Wells were evaluated for cobblestone areas weekly from day 7 to 42. The proportion of negative wells at each dilution was used in a Poisson-based limiting dilution analysis calculation to determine the frequency of CAFC using the L-Calc software (Stem Cell Technologies, Vancouver, BC, Canada).

Adipogenic and osteogenic differentiation

To induce adipogenic differentiation, MSC monolayers were treated with adipogenic medium for two weeks. Medium changes were carried out twice weekly and adipogenesis was assessed by Oil Red O (Sigma) staining. Adipogenic medium consisted of DMEM supplemented with 10% FCS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), and 1 μM dexamethasone (Sigma).

For osteogenic differentiation, confluent MSC monolayers were treated with osteogenic medium for two weeks with medium changes twice weekly. Osteogenic differentiation was evaluated by Alizarin Red S (Sigma) staining. Osteogenic medium consisted of DMEM supplemented with 10% FCS, 10 mM β-glycerophosphate (Sigma), 50 μg/ml ascorbic acid (Sigma), and 0.1 μM hydrocortisone (Sigma).[19]

Photomicrographs were taken with an inverted microscope (Olympus CK2) and a digital camera (Nikon Coolpix 4500, Tokyo, Japan).

Neuronal induction

For neuronal induction, 50-60% confluent cultures of MSC were washed with HBSS and transferred to induction media consisting of 2.5 mM β-mercaptoethanol and antibiotics in DMEM, according to the method of Woodbury et al.[33]

Immunofluorescence

Cultured cells were rinsed briefly in PBS, fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 minutes, and treated with 5% normal mouse sera in PBS for 30 minutes, all at room temperature. They then were incubated with the primary antibodies – monoclonal anti-neuron-specific enolase (NSE) IgG1 (Chemicon, Temecula CA, MAB314), monoclonal anti-neuron-specific nuclear protein (NeuN) IgG1 (Chemicon, MAB377), and monoclonal anti-glial fibrillary acidic protein (GFAP) IgG1 (Chemicon, MAB360) – for 18 hours at 4°C, rinsed three times with PBS, and incubated for a further 4 hours with a secondary antibody (fluorescein isothiocyanate-labeled rabbit anti-mouse IgG). Control staining without primary antibody was used as negative control.

Statistical analysis

Statistical analysis was performed by using the Student's *t*-test. A *p*-value of <0.05 was considered statistically significant.

Results

Hematopoietic progenitor cells in the initial bone marrow

All bone marrow samples from MDS patients and normal donors were assayed for *in vitro* colony formation. In MDS patients, the number of colonies varied from patient to patient (*Table 2*). In these 10 studies, BM mononuclear cells from all patients showed formation of CFU-GM, four formed BFU-E, and three formed CFU-GEMM. In 5 of 10 cases the numbers of CFU-GM deriving from MDS BM mononuclear cells were low or very low compared to that of normal BM mononuclear cells, whereas very high number of GM colonies and clusters were seen in the BM sample from one patient with post-MDS AML. Thus, bone marrow hematopoietic colony-forming cell incidence was generally lower than normal in the MDS patients evaluated.

Establishing adherent stromal cell layers

Colonies of fibroblastic cells appeared in the culture flasks 5 to 7 days after plating of bone marrow mononuclear cells. Stromal cell layers from healthy donors reached confluence earlier (8-14 days) than those from the patients with MDS (10-28 days), indicating decreased proliferative capacity of patient adherent cells. After three or more passages, similar differences could be observed between normal and MDS-derived cell layers in their growth speed but not in their morphology, as seen under the inverted microscope. Healthy MSCs were successfully passaged 20-25 times, whereas MDS MSCs became senescent after 10-15 passages. Spontaneous fat cell formation was absent in the adherent cultures of some MDS patients. After the third passage, in all control and MDS cultures, MSC layers appeared as a well-defined population, consisting of spindle-shaped cells. No macrophages or hematopoietic (CD133-, CD34-, or lineage-positive) cells were observed (data not shown).

Interactions between normal and MDS bone marrow-derived hematopoietic progenitors and stromal cells – effect of soluble Jagged-1 on the development of cobblestone area-forming cells

First, non-adherent fraction of BM mononuclear cells isolated from normal donors were deposited in a limiting dilution setup in 96-well plates containing a pre-established confluent normal MSC cell layer, as described in Materials and methods. It has repeatedly been shown that the time

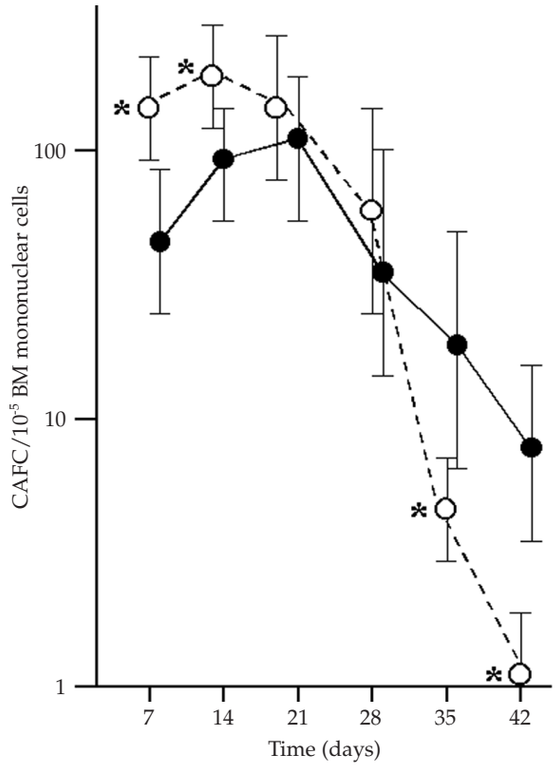

Figure 1. *The effect of soluble Jagged-1 on the development of CAFCs from normal bone marrow cells. Healthy BM mononuclear cells were overlaid on normal MSC-derived stromal cell layers in multiple dilutions for limiting dilution analysis of CAFC in the presence (○- -○) or absence (●—●) of 5 μg/ml soluble recombinant Jagged-1. Wells were evaluated for cobblestone areas weekly from days 7 through 42. Values are mean ± SD (n=9). *Significantly different (p < 0.05) from control*

point of appearance of cobblestone area-like colonies in such long-term bone marrow cultures strongly correlates with the maturity of the hematopoietic cells. In human, committed progenitors form colonies after 7-14 days (hence, they are referred to as day-7-14 CAFC), whereas more primitive stem and early progenitor cells start proliferating only after 5-6 weeks (day-35-42 CAFC).[5] Soluble Jagged-1 had a biphasic effect in this assay system. Day-7-14 CAFC frequencies were increased, whereas day-35-42 CAFC frequencies were significantly decreased in the presence of 2.5-10.0 μg/ml of soluble Jagged-1. Day-21-28 CAFC frequencies were unaffected by soluble Jagged-1 (*Fig. 1*). These data suggest that soluble Jagged-1 is able to inhibit the function of its multivalent form, i.e. transmembrane Jagged-1 molecules expressed by the stromal cells, to induce day-35-42 CAFC formation. On the other hand, it promotes CAFC formation of committed progenitor cells (day-7-14 CAFC).

Next, MDS bone marrow-derived mononuclear cells were deposited in plates containing pre-established confluent normal MSC layers, while normal mononuclear BM cells were seeded on MDS-derived stromal cell layers in CAFC medium. As shown in *Fig. 2a*, when MDS bone marrow-derived mononuclear cells were co-cultured with normal MSC-derived stromal cells, the frequency of either early or late CAFC was significantly lower compared to the corresponding normal control values in 4 out of 8 patients. In the opposite situation, when normal BM mononuclear cells were incubated on MDS MSC-derived stromal cell layers, the CAFC frequencies were decreased in 5 out of 6 patients (markedly in 4 patients), compared to control cultures containing normal stromal cell layers (*Fig. 2b*). Finally, when MDS-derived mononuclear BM cells

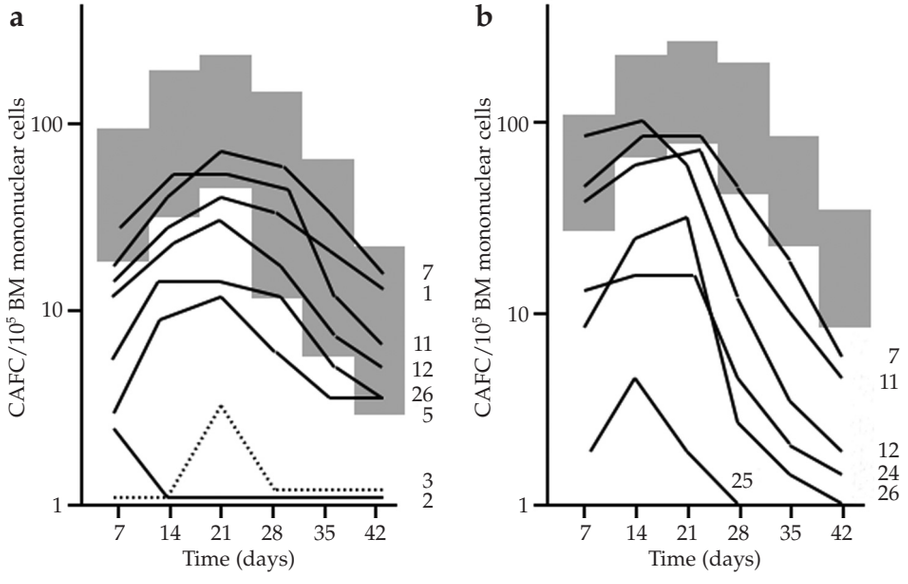

Figure 2. *CAFC frequencies of BM cells isolated from healthy volunteers and from MDS patients. MDS BM-derived mononuclear cells were deposited in plates containing pre-established normal MSC layers* ***(a)*** *and normal mononuclear BM cells were overlaid on MDS-derived stromal cells* ***(b)*** *in CAFC medium. Grey areas show the control ranges, i.e. normal BM mononuclear cells seeded on normal stromal layers (n=8 and n=6, respectively). Lines represent individual patient's data.*

Table 4. **Effect of soluble Jagged-1 on CAFC frequencies of normal bone marrow mononuclear cells in the presence of MDS stromal cells***

Patient	*Day-7 CAFC frequency per 10^5 mononuclear cells*		*Day-35 CAFC frequency per 10^5 mononuclear cells*		*Day-42 CAFC frequency per 10^5 mononuclear cells*	
	Medium	*Soluble Jagged-1 (5 mg/ml)*	*Medium*	*Soluble Jagged-1 (5 μg/ml)*	*Medium*	*Soluble Jagged-1 (5 μg/ml)*
MDS-7	79	142	12	6	7	3
MDS-11	71	123	10	2	6	2
MDS-12	91	134	4	1	2	1
MDS-24	11	22	2	0	1	0
MDS-26	9	15	1	0	0	0
MDS-25	2	1	0	0	0	0

*Cultures established with non-adherent mononuclear cells from normal individuals in the presence of established irradiated stromal cell layers from MDS patients

were incubated with MDS-derived stromal cells, the frequency of day-7 and day-14 CAFC was extremely low and the more primitive hematopoietic cells (day-28-42 CAFC) did not proliferate at all (data not shown). These findings suggest that the hematopoietic microenvironment is frequently co-involved in the disease process of MDS.

Remarkably, a heterogeneous response was observed when the above cultures treated with 5 μg/ml soluble Jagged-1. Day-7 CAFC frequencies were enhanced by the recombinant protein in both co-culture combinations, except in 3 out of 8 and in 1 out of 6 cases, respectively, when the control values were already extremely low (<9 CAFC/10^5 mononuclear cells) (*Tables 3 and 4*). Day-42 CAFC frequencies were unaffected or slightly enhanced by Jagged-1 in cultures containing normal stromal cells and MDS BM mononuclear cells (*Table 3*). In contrast, when normal BM mononuclear cells were incubated on MDS stromal cells, day-35-42 CAFC frequencies were inhibited by soluble Jagged-1 (*Table 4*). Thus, the soluble Notch ligand is (i) a growth factor for committed hematopoietic progenitor cells (day-7 CAFC) isolated from normal, as well as from MDS bone marrow, and (ii) an inhibitor of day-35-42 CAFC for normal hematopoietic cells cultured with normal or with MDS stromal cells, but (iii) is unable to inhibit MDS bone marrow-derived stem and early progenitor cell growth (day-35-42 CAFC) on pre-established stromal cell layers.

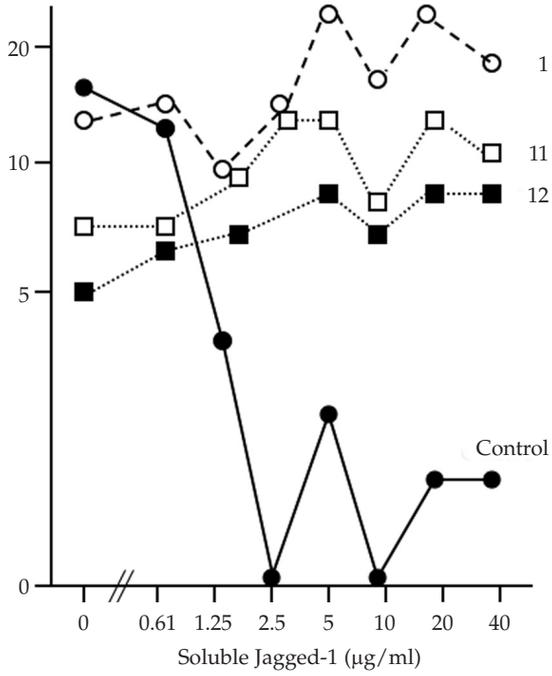

Figure 3. *The effect of different amounts of soluble Jagged-1 on the development of day-35 CAFCs from normal and MDS BM mononuclear cells. BM mononuclear cells isolated from one healthy volunteer and from three MDS patients were incubated on normal MSC-derived stromal cell layers in the presence of 0.6-40.0 μg/ml Jagged-1 protein.*

To confirm the above findings, we performed CAFC assays in which BM mononuclear cells from three MDS patients and from one healthy control donor were seeded on irradiated stromal cell layers in the presence of different amounts of Jagged-1. As shown in *Fig. 3*, the recombinant protein was able to inhibit day-35 CAFC formation in a dose-dependent manner only when the hematopoietic cells were isolated from normal BM.

Plasticity of MSCs derived from MDS bone marrow samples

To evaluate the multipotentiality of patient MSCs, adherent cell layers from 7 patients with MDS and from 8 healthy donors were differentiated along three defined lineage pathways. Neuronal differentiation was assessed by indirect immunofluorescence in cultures maintained for five days under neurogenic conditions. Neural induction medium was converted all MSC cultures from normal

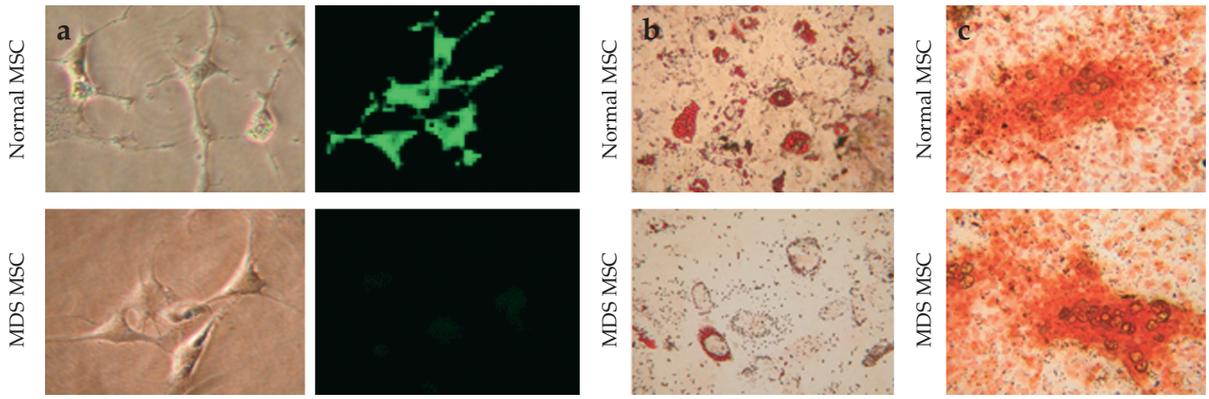

Figure 4. *Plasticity of MSCs isolated from normal donors and from MDS patients.* ***(a)*** *Representative phase contrast (left two panels) and fluorescent micrographs (right two panels) of cells cultured in neurogenic induction media for 5 days.* ***(b)*** *Oil Red O staining of cells incubated in adipogenic induction media for 14 days.* ***(c)*** *Alizarin Red S staining of MSCs cultured in osteogenic induction media for 14 days. (Original magnification x100)*

donors into monolayers containing 50-60% NeuN- and NSE-positive cells. In contrast, none of the MDS bone marrow-derived MSC was able to express neuron-specific proteins after exposure to 2.5 mM β-mercaptoethanol under serum-free conditions (*Fig. 4a*), suggesting a defect in the differentiation potential of patient MSCs.

The capacity of MSCs from MDS patients and from normal donors to undergo adipogenic differentiation was determined by Oil Red O staining. The percentage of cells with Oil Red O-positive vacuoles varied between individual MSC layers, however, the ability of MSCs from MDS patients to undergo adipogenic differentiation was always significantly lower than that of normal MSCs (*Fig. 4b and Fig. 5a*). Osteogenic differentiation was assessed by histologic detection of calcium deposition in cultures maintained for two weeks. All MSC preparations evaluated in this study were capable of formation of a mineralized layer with marked Alizarin Red S-positive staining when cultured in the presence of osteogenic supplements (*Fig. 4c*). Morphometric analysis of the osteogeneic capacity of MSCs from normal donors and from MDS patients also showed that there was no significant difference between these cells (*Fig. 5b*).

Discussion

Over the past several years, there has been intense focus on the function of Notch proteins in hematopoiesis. Notch signaling appears to play a pivotal role at many stages of hematopoiesis, regulating both the self-renewal and differentiation of hematopoietic stem cells and related cell populations, and influencing several downstream differentiation events within the developing myeloid and lymphoid compartments.[18,20] Recently, we have shown that multivalent Jagged-1 ligands are required for the induction of self-renewing divisions of day 28-35 murine CAFC and of long-term repopulating ability of stem cells. The free, soluble Jagged-1 protein, however, has a dominant-negative effect on self-renewal in the stem cell compartment, presumably by sequestering the Notch receptor and preventing Notch interaction with the multivalent, i.e. cell surface expressed or chemically immobilized ligands. In contrast, soluble (monovalent) as well as immobilized (multivalent) Jagged-1 promote growth factor-induced colony formation of committed hematopoietic progenitor cells.[30] The facts that Notch signaling is involved in the pathogenesis of human bone marrow-derived neoplasms,[17,26,31] and that myelodysplastic syndromes originate from dysregulated hematopoietic stem and/or progenitor cells, suggest the existence of some abnormalities in the Notch system. To clarify this, we examined how the cobblestone area formation of human BM mononuclear cells isolated from healthy volunteers and from MDS patients were affected by soluble Jagged-1 protein. We found that the soluble Notch ligand is able to stimulate day-7-14 cobblestone area formation in normal, as well as in MDS BM samples (*Fig. 1, Tables 2 and 3*), suggesting that Jagged-1 might be a growth factor for committed human hematopoietic progenitor cells. These results are consistent with those of Jones *et al.*[11] and Karanu *et al.*,[12] who also found that Jagged-1 represents a novel growth factor of human hematopoietic cells. The committed progenitor cells in MDS patients, when present, appear to be normal in this context (*Tables 2 and 3*). It is noteworthy that a recent report by Tohda *et al.*[27] showed proliferation of a cell line derived from an AML patient with trilineage myelodysplasia in response to a Notch ligand.

Soluble Jagged-1 was able to inhibit the day-35-42 cobblestone area formation of bone marrow mononuclear

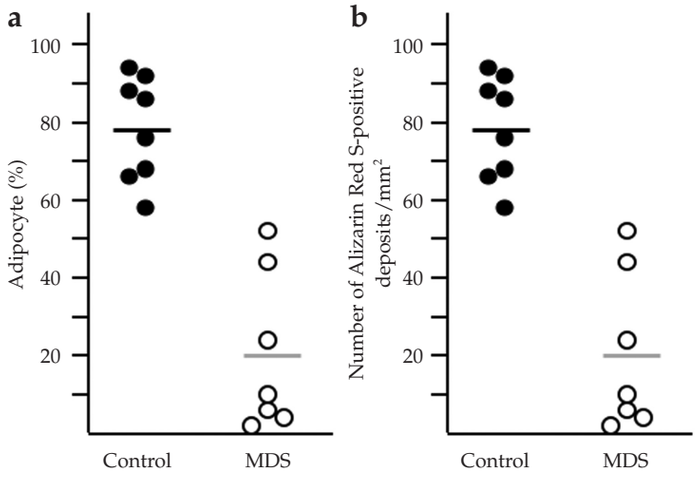

Figure 5. *Adipogenic* ***(a)*** *and osteogenic* ***(b)*** *differentiation of MSCs isolated from normal donors and from MDS patients; individual data and the mean are shown for all cases (n=8).*

cells from normal donors, whereas cells from MDS patients were usually unaffected by the recombinant protein (*Tables 3 and 4, Figs. 1 and 3*). With respect to hematopoietic cell development, our results show that Notch regulation is disturbed in the early stem/progenitor cell compartment of MDS patients. Although the molecular mechanism behind this phenomenon is unclear, such alterations may have an impact on the pathophysiology of myelodysplasia. Data may indicate either that (i) the expression of Notch receptors and/or ligands are dramatically changed in MDS as indicated by some other laboratories,[14] or (ii) the multipotent stem/early progenitor cells from MDS patients are immortalized by constitutive Notch signaling as indicated by Varnum-Finney *et al* [29] for hematopoietic stem cell lines *in vitro*, or (iii) Jagged-1 molecules activate different signal transduction pathways in normal and MDS-derived early hematopoietic cells. According to a recent report,[4] the Dlk molecule overexpressed on CD133+ cells of some MDS patients is a negative regulator of Notch1 activation. Further studies are needed to clarify this point.

The most relevant finding derived from the present study was that in a significant proportion of MDS patients, MSCs showed markedly decreased plasticity as compared to those from normal subjects. In general, there was a marked reduction in *in vitro* hematopoiesis supporting capacity, neurogenic, and adipogenic activity in cultures of MDS MSCs as compared to those normal MSC cultures. Only in osteogenic activity there was no significant difference found between MDS-derived and healthy control MSCs *in vitro* (*Figs. 4 and 5*). Our findings, the decreased hematopoiesis supporting capacity and the limited adipogenic differentiation of MDS stromal cells, are consistent with several previous studies.[1,6,23-25] Shetty *et al*.[21] demonstrated excessive apoptosis, increased phagocytosis and nuclear inclusion bodies in BM stromal cells of MDS patients. Recently, cytogenetic investigations had shown well-defined chromosomal abnormalities in MDS patients' MSCs.[9] Several alterations of the cytokine profile in the BM environment have also been described in MDS.[8,32] In summary, it seems likely that microenvironmental defects are commonly involved in the pathogenesis of MDS. We hypothesize that the myelodysplastic pattern of blood cell formation is the result of the pathologic interaction between the defective stroma and the transformed hematopoietic cell clone. The stromal defect could be based on the accelerated aging and early senescence of the MSCs of MDS patients.

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