

Heterogeneity of mesenchymal stromal cells in lymphoproliferative disorders

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1. ABSTRACT

Accumulating evidence indicates that bone marrow microenvironment plays an important role in the pathogenesis of some myeloid and lymphoid hematological malignancies (HM). Among different environmental associated parameters, those related to functional, cytogenetic and immunological integrity of mesenchymal stromal cells (MSC) are particularly relevant. Functional alterations and immunophenotypic abnormalities have been described in MSC obtained from HM patients. These data seem to confirm the defective biological pattern of MSC especially in myeloid diseases, while MSC cytogenetic profile in HM is still an open question, because it is not clear whether BM stromal cells are “culprit or bystander” displaying or not an abnormal karyotype. Contradictory findings were reported in different HM but the functional implications of altered MSC karyotype need to be further addressed also in light of a clinical use of MSC. A “pathological” *in vivo* supportive function of endogenous MSC, which provide important survival and drug resistance signals to leukemic cells especially in lymphoproliferative disorders, is suggested. Thus, the mechanisms underlying these protective *versus* cytotoxic effects exerted by MSC on leukemic cells need further investigations.

2. INTRODUCTION

The bone marrow (BM) stromal cell compartment refers to a heterogeneous pool of tissue-specific non-hematopoietic cells, which possess different *in vivo* functions and distinct differentiation potential. BM-derived mesenchymal stromal cells (MSC) have been described to form *in vitro* stromal layers also with other adherent non-hematopoietic cells, such as endothelial cells or with the transiently adherent macrophages. The stromal system concept, firstly described by Friedenstein et al. and afterwards by Owen et al. (1-3), was based on the analogy with the hematopoietic system, in which cells of mesenchymal origin reside within the BM and maintain the properties of self-renewal and plasticity, giving rise to a progeny that can differentiate into various connective tissue lineage as postulated by Caplan (4). Among different tissue sources of MSC, the best characterized in terms of biological properties and differentiation potential are BM resident MSC, having long been proposed to provide modulatory signals to hematopoietic progenitor cell turnover, to have multi-lineage differentiation potential and to have immunomodulatory properties. Some *in vitro* and *in vivo* studies showed that MSC could differentiate also into cells of non-mesodermal origin such as neurons, skin and gut epithelial cells, hepatocytes and pneumocytes (5),

but the relevance of this trans-differentiative activity is questionable. Besides, being initially isolated and characterized from the BM, MSC can also be obtained from other sources, such as amniotic membranes, skin, adipose tissue, cord blood, fetal liver, placenta and synovium (6). Whereas MSC have historically been thought to be the same general population of cells regardless of the tissue source of origin, recent data suggest that MSC gene expression profile reflects their tissue origin, indicating that MSC tissue heterogeneity is likely to be biologically relevant (6, 7). Moreover, analysis of MSC transcriptoma has led to the identification of subpopulations of MSC expressing a variety of regulatory proteins that function in angiogenesis, hematopoiesis, neural activities and immunity (8). The MSC biochemical heterogeneity has an obvious impact of the potential therapeutic applications of these cells. Nevertheless, the potential contribution of MSC in regenerating damaged tissues has been limited so far by their limited survival and half-life of transplanted hosts (9). Indeed, in most clinical trials based on the use of MSC, the major therapeutic benefit of these cells does not appear to result from their direct replacement of damaged cells, but rather to the secretion of soluble mediators, which seems to be the predominant mechanism of the therapeutic action after systemic infusion of MSC. Recent evidence sustains the hypothesis of a paracrine role of MSC, which are able to modulate apoptosis as well as the inflammatory responses and also to stimulate the growth of host resident cells (10). The hypothesis of a “trophic activity” of MSC, which may comprise a regenerative microenvironment for tissue-specific stem cells, has been supported by *in vivo* studies on BM models. Based on these studies, MSC are increasingly used in many preclinical as well as clinical settings. Even though the majority of the *in vivo* MSC effects studied in animal models regarded tissue transplantation and autoimmunity, several reports underlined the MSC capability to modulate tumor microenvironment, thus having an impact on the tumor behavior (11). Although human MSC maintained under standard culture conditions are thought to be “non tumorigenic”, recent concerns have been expressed about the potential transformation of MSC during the culture process before infusion. Nevertheless, MSC were found rarely to help tumor development due to their immunosuppressive capacity (12). In our opinion, additional studies are required to better understand the factors affecting malignant cell quiescence *versus* proliferation during MSC-tumor cells interactions (13, 14). The nature and the role of MSC in their micro-environmental niche should be further explored especially in course of lymphoproliferative disorders. For example, the presence of chromosomal translocations within the malignant stem cell is difficult to evaluate, and it is not still clear whether BM stromal cells are “culprit or bystander” displaying or not an abnormal karyotype (15-19). On the other hand, we have also shown that BM MSC can suppress the growth of lymphoma cells in xenograft models (20, 21), a finding confirmed by an independent group in an animal model of brain gliomas (22). Thus, the complex relationship between MSC and tumors should be better clarified to avoid potential risks related to the use of MSC and, on the other hand, in order to take advantage of

potential therapeutic options based on the infusion of *ex-vivo* expanded MSC.

The functional implications of an altered MSC karyotype is another question that needs to be further addressed to strengthen the possibility of using or excluding autologous MSC for clinical purposes. In consideration of the emerging issues about the immunophenotypic and molecular heterogeneity of MSC, it is likely that the specific functions of MSC subpopulations obtained from different tissues will need to be further analyzed and clarified before clinical application. In the next paragraphs of this review, we will focus in particular on the functional, cytogenetic and immunomodulatory properties of BM stromal microenvironment in the BM niche, especially in course of chronic and acute lymphocytic leukemia.

3. MSC IMMUNOPHENOTYPE

The functional and immunophenotypic characteristics of stromal cells have been recently reviewed and redefined by the international society for cellular therapy (ISCT; www.celltherapysociety.org) (Figure 1): they must be defined as MSC (i) if they are adherent to plastic, (ii) if they retain *in vitro* clonogenic potential, defined by the presence of the fibroblast-colony forming unit (CFU-F) (1-3), (iii) if they are capable of supporting hematopoiesis and (iv) if they have a differentiation capacity towards a number of different cell types (osteoblasts, chondrocytes, adipocytes, myocytes) (23, 24). Cultured MSC may be defined only by a combination of non-specific immunophenotypic markers. The methodological isolation of MSC from primary tissues has been difficult to establish because of their exceptionally low frequency and a not universally accepted antigenic determinants for the isolation and phenotypic characterization of MSC. Indeed, only few markers have been developed and proved to be suitable for MSC isolation. Functionally isolated MSC do not provide any information about the antigenic composition of the starting population hampered by the limited selectivity of available markers. Therefore, several questions remain to be solved concerning the origin of MSC and especially their relationship to other stromal cells, such as fibroblasts or other mesenchymal-like cell types sharing the same immunophenotypic markers. Thus, the present definitions of MSC emphasize generic functional properties of these cells and fail to distinguish for example MSC from generic fibroblasts and to detect subsets of MSC with specialized niche functions including the possible support to leukemic cells (25, 26).

3.1. Immunophenotype of non-expanded MSC

Few markers have been developed and proved to be suitable for MSC isolation and, in any case, most of the monoclonal antibodies now available recognize also normal fibroblasts and mesenchymal derived stromal cells (27). Nevertheless, it is noteworthy that a variety of surface markers are exclusively found on freshly isolated MSC but not on their cultured counterparts (such as CD271) (Figure 1). Although no standardized protocols exist, several

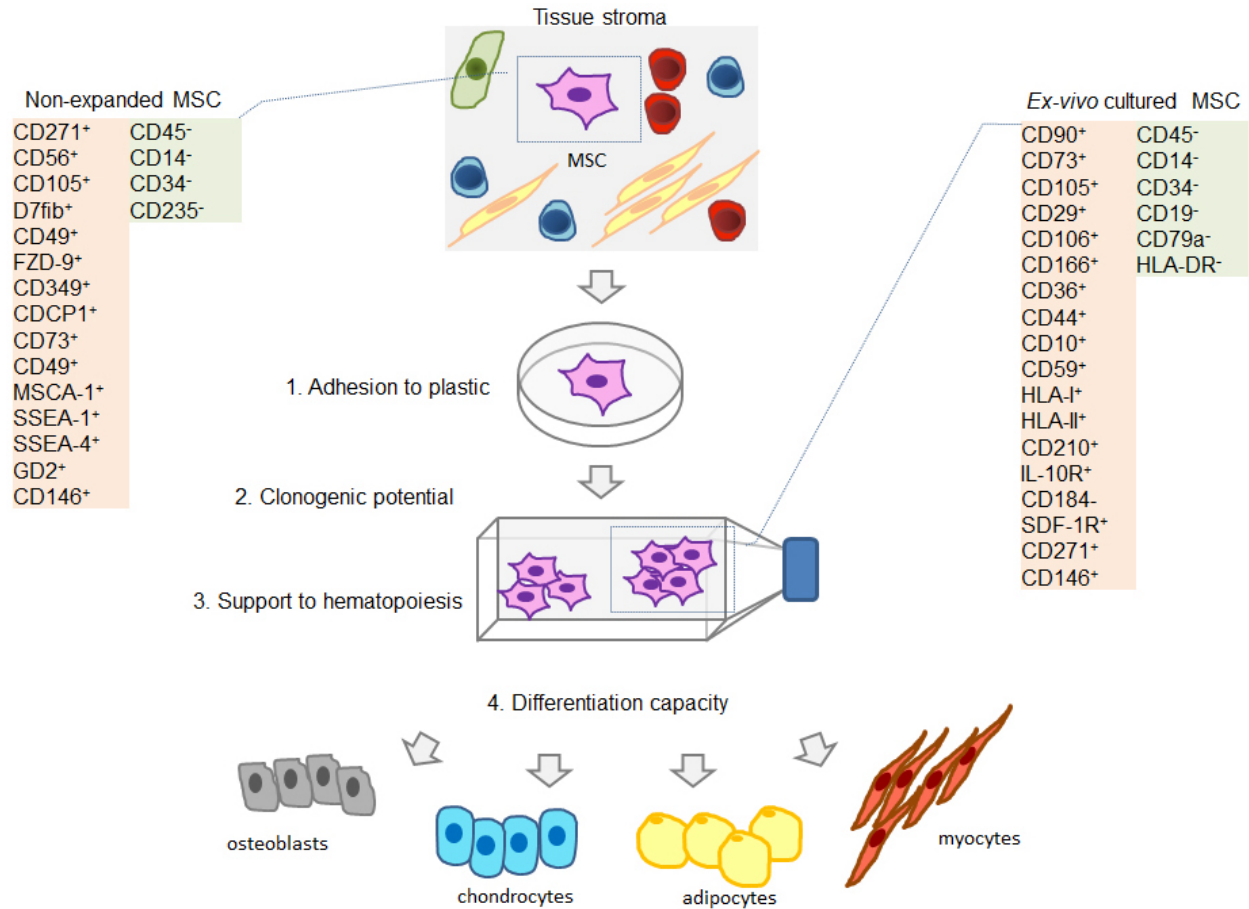


Figure 1. Schematic representation of the MSC immunophenotype and their functional characteristics according to the ISCT guidelines.

protocols have been used to facilitate the isolation of MSC (28, 29). In order to identify “naïve” MSC, positive selection with antibodies against surface molecules, such as CD49, frizzled-9 (FZD-9, Wnt receptor) CD349, CD56, CDCP1, CD73, CD49 and, in particular, CD105 and D7Fib (27, 30) were performed (Figure 1). Other studies showed that MSC may be enriched by negative selection based on the depletion of CD45, CD14, CD34 and/or glycophorin A (CD235) markers. The CD271 seems to be one of the most specific markers (31) especially in combination with the CD56, and MSCA-1 to purify MSC from BM as well as from other tissue sources. Using high sensitive flow cytometry, other authors have demonstrated that SSEA-1 and SSEA-4 could be useful to identify BM-MSC (27), suggesting how these markers may be prospectively used to identify the most primitive progenitors with embryonic stem cell like-features. BM-MSC precursors have also been shown to express a neural ganglioside (GD2), which is not expressed by hematopoietic cells but can be expressed, as recently demonstrated, by some tumor cell types (32). Recent studies characterized stromal cells in their natural BM microenvironment and increased our knowledge of *in situ* immunophenotype of non-expanded MSC. For example, mesenchymal and hematopoietic cells have been shown to form a unique nestin positive bone marrow niche

(33), in which CD146/CD271 expression can distinguish between perivascular and osteoprogenitor non-hematopoietic progenitor cells (34). MSC also express receptors for TNF-related apoptosis inducing ligand (TRAIL) (35) and produce abundant amounts of osteoprotegerin (36, 37), which act as neutralizing receptor for two distinct members of the TNF superfamily, such as TRAIL and RANKL. While emerging data have focused on the immunophenotype of non expanded MSC from normal sources, information related to the immunophenotype of non-expanded MSC isolated from the BM niche of patients with lymphoproliferative disorders are still lacking.

3.2. Immunophenotype of *ex vivo* cultured MSC

Most of the literature findings on MSC concern the immunophenotype of cultured MSC obtained from different tissues. These cells resulted uniformly positive for CD90, CD105, CD73, and negative for stem cell antigen CD34 and other hematopoietic markers, such as CD45 pan-leukocyte marker, CD14, CD19, CD79a, HLA-DR, as now clearly indicated by ISCT guidelines (23), (Figure 1). In addition, other recent studies demonstrated the MSC positivity for other antigens, such as adhesion molecules (CD29, CD106, CD166, CD36), extracellular matrix protein (CD90, CD44), hematopoietic markers (CD10,

Table 1. Summary of relevant studies reporting MSC with a normal cytogenetic pattern, obtained from patients affected by hematological disorders

MSC source	Hematological disorder	Hematopoietic cells alterations	MSC alterations	Reference
BM	MDS and AML	several chromosomal abnormalities	normal	(15)
BM	MDS	5q-; monosomy 7; trisomy 8	normal	(16)
BM	Pediatric ITP and AIN	overexpression of p53, p16, RB, H-RAS. several chromosomal abnormalities	normal	(18)
BM	MDS	several chromosomal abnormalities	normal	(19)
BM	CLL and ALL	several chromosomal abnormalities	normal	(41)
BM	CML	t(9;22)(q34;q11)	normal	(44)
BM	MM	t(4;14); del(13q14); del(17p13)	normal	(45)
BM	MM	t(11;14); t(4;14)	normal	(46)
BM	CML	t(9;22)(q34;q11)	normal	(47)
BM	CML	t(9;22)(q34;q11)	normal	(48)
BM	CLL	several chromosomal abnormalities	normal	(49)

MSC: mesenchymal stromal cells; BM: bone marrow; MDS: myelodysplastic syndrome; AML: acute myeloid leukemia; ITP: immune thrombocytopenic purpura; AIN: autoimmune neutropenia; CLL: chronic lymphocytic leukemia; MM: multiple myeloma.

CD59), histocompatibility antigens (HLA-I and II class), chemokine receptors (CD210, IL-10 receptor, CD184-SDF-1 receptor), neural and endothelial receptors (CD271, CD146). An emerging paradigm is that MSC could have key functional roles in the tissue in which they reside, but different phenotypic and functional characteristics under their *ex vivo* expansion depending on different isolation methods and culture protocols need to be considered. In particular, the effects of serum concentration, addition of platelets lysate, cytokines and growth supplements, cellular density, number of passages, age of cultures and methods for cryopreservation are still not elucidated (8, 25, 38). Moreover, the MSC tissue source origin (normal *versus* pathological, BM versus abdominal fat, donors variability) may be also critically important in determining their biological activity. All these variables might have implications on the selection of functionally and phenotypically distinct MSC subsets and on altering the MSC clonogenic and plasticity potential. Recent data focused on MSC immunophenotype with special regards to the expression of Thy-1 (CD90) molecule and, in a lesser extent, to LNGFR (CD271), CD105, CD44, CD10, CD146 molecules since they could give information about different MSC specific functions (38, 39). For example, differences related to the expression of CD105 and CD106 in cultured BM-derived MSC obtained from patients affected by hematological malignancies (HM-MSC), especially by lymphoproliferative disorders, seem to be correlated to the different plasticity potential and adhesion capacity of MSC as compared to normal samples and skin fibroblasts (SF) (39, 40). In this context, it has been shown that in chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL), malignant cells result sometimes long term strictly adherent to *in vitro* expanded BM-derived MSC, thus rendering difficult the assessment of MSC phenotype (41). From these studies it was also stressed the importance of using a multiparametric flow cytometry protocol to gate out CD45+ cells and other pathologic cell types from MSC analysis. Nevertheless, very few literature findings have focused on the immunophenotype of *in vitro* expanded BM-derived MSC isolated from CLL and ALL patients. In these studies, a normal phenotypic profile of MSC was reported (41). These data may have therapeutic implication, since the use of autologous MSC has been proposed in a wide range of clinical applications including

those in the area of the regenerative medicine and cell therapies.

4. CYTOGENETIC PROFILE OF MSC

To date, the administration of MSC in different clinical trials proved to be safe and with promising therapeutic potential in a variety of disorders. Nevertheless, the potential risks related to the use of MSC in the different clinical setting should be further evaluated. As described above, the emerging functional and immunophenotypical heterogeneity of MSC in relation either to anatomical site, donor type (normal *versus* pathological) and different culture expansion conditions, should be carefully considered for clinical purposes before using *ex-vivo* expanded MSC for reinfusion (42). For example, since the therapeutic use of autologous MSC has been proposed, the evaluation of MSC cytogenetic and functional integrity become necessary, because existing data are controversial. Moreover, whether BM stromal components bear cytogenetic abnormalities in course of the disease still represents a matter of discussion (43, 15-19). In particular, little information is available about the cytogenetic profile of BM-derived MSC in CLL patients, while contradictory findings are reported for ALL patients and overall in myeloid malignancies. Here, we try to summarize the different controversial aspects that are emerging from literature data.

4.1. MSC with a normal karyotype

In general, despite their high proliferation capacity, normal BM-derived MSC maintain stable telomerase and seem not susceptible to transformation after *in vitro* expansion. Nevertheless, a minimal percentage (1-5%) of altered karyotype could be detected only after more than 10-15 *in vitro* passages and in a minimal part of the normal subjects. On the other hand, most of the studies were unable to find specific cytogenetic abnormalities in MSC obtained in prevalence from myeloid malignancies (16, 19, 41, 44). These studies reported that BM stromal component was cytogenetically normal (Table 1), without clonal relationship between BM-derived MSC and the malignant lymphoid clone, thus adding important evidences to the long-standing controversy as to whether a single BM-derived cell can differentiate along both hematopoietic

Table 2. Summary of some relevant studies reporting MSC with altered karyotype obtained from patients affected by hematological disorders

MSC source	Hematological disorder	Hematopoietic cells alterations	MSC alterations	Reference
BM	MDS and AML	t(15;17), t(8;21), inv(16), t(4;11), t(11;19), del(11)(q23)	t(1;6)(p32;p12), del(7)(q11.2q32), t(3;20)(p13;p11.2), inv(X)(q12p22), monosomy 4, monosomy X or Y, trisomy 5*	(15)
BM	CLL and ALL	several abnormal karyotypes	aneuploidy*	(41)
BM	CLL	several chromosomal abnormalities	functional abnormalities*	(49)
BM	MDS	trisomy 8, del(p53), del(CSF1), del(D20S108), del(MLL), other abnormal karyotypes	trisomy 8*	(54)
BM	APL, AML and CML	several abnormal karyotypes	t(3;9)(q25;q31) del(1)(q42) der(13;14)(q10;q10)*	(55)
BM	MDS	hypodiploidy, del(7)(q33); monosomy 5, monosomy 8; other abnormal karyotypes	several abnormalities*	(56)
BM	ALL	MLL-AF4 fusion gene	MLL-AF4 fusion gene [§]	(17)
several normal tissues and tumors	several tumors	several chromosomal abnormalities	trisomy 12; monosomy 13; several isochromosomes [§]	(51)
BM	pediatric ALL	t(12;21), t(1;19), t(11;19), t(11;21), MLL rearrangements	t(12;21), t(1;19), t(11;19), t(11;21), MLL rearrangements [§]	(53)
BM	MDS	syndrome 5q-	syndrome 5q- [§]	(57)

MSC: mesenchymal stromal cells; BM: bone marrow; MDS: myelodysplastic syndrome; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; CLL: chronic lymphocytic leukemia; *: studies on MSC with altered karyotype distinct from the neoplastic clone. [§]: studies on MSC with leukemia associated alterations.

and stromal lineages. As far as lymphoproliferative disorders is concerned, even though BM microenvironment has been demonstrated to play an essential role in promoting the survival and growth of the myeloma plasma cells, the chromosomal analysis revealed that multiple myeloma (MM) patients derived-MSC were devoid of chromosomal clonal markers identified in plasma cells. Nevertheless, Arnulf et al. (45) reported that MSC from MM patients are characterized by abnormal functional features, such as an overexpression of IL-6. These data suggest a paracrine role played by MSC in promoting the survival/growth of malignant plasma cells in MM (46). On the other hand, we showed and confirmed the absence of the Philadelphia chromosome and of other aberrations in MSC obtained from adult ALL patients (41). These observations on ALL samples are in agreement with previous findings on CML (47) and with more recent studies showing that MSC did not harbour Philadelphia chromosome (48) and that MSC did not derive from a common progenitor with hematopoietic stem cells. In a previous study, we also confirmed a normal cytogenetic profile in MSC from patients affected by CLL (41), while intrinsic functional abnormalities, which may be implicated in disease development, has been documented by other authors (49).

4.2. MSC with altered karyotype

Few studies reported adult BM-MSC being characterized by a limited acquisition of chromosomal aberrations, mainly in patients affected by myeloid malignancies. These studies reported that MSC from patients have distinct chromosomal abnormalities compared to the neoplastic clone, suggesting an enhanced genetic susceptibility of MSC to acquire non-specific chromosomal alterations (15, 50-52) (Table 2). These data indirectly confirm the non-hematopoietic origin of BM-MSC (16, 19, 41, 44). The more frequent abnormalities reported in MSC of patients affected by myeloid malignancies are structural alterations, such as

translocation of chromosome 1, deletion of chromosome 7, monosomy of X or Y and trisomy of chromosome 5 and 8 (53, 54). Very interesting is the finding that genetic abnormalities in MSC, again distinct from those of leukemic blasts, have been recently reported also after therapy (55).

4.3. MSC with leukemia associated abnormalities

As above described, most of the available studies reported a normal cytogenetic pattern of BM derived MSC or some non-specific alterations in patients affected by myeloid malignancies, probably as the consequence of the *ex vivo* expansion and of an intrinsic susceptibility. Nevertheless, contradictory findings were reported in myeloid diseases, such as in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) (Table 2). For example, some papers reported that mesenchymal stromal cells from MDS and AML patients have distinct cytogenetic abnormalities compared to leukemic blasts, while other studies demonstrated that both expanded and uncultured MSC from MDS patients show a specific genetic profile, such as the 5q- genomic aberration or other leukemic myeloid alterations (56, 57). Moreover, it has also been reported that cytogenetic abnormalities in MDS-derived MSC are present only in patients with cytogenetic alterations in their hematopoietic cells counterpart (56). It is also noteworthy that we have previously shown that stromal cells obtained from MDS patients produce and release in culture elevated amounts of TRAIL, which might contribute to worsen the degree of anemia in these patients (58).

On the other hand, as far as the lymphoproliferative disorders are concerned, only few studies sustained a MSC leukemia-associated aberration. Among these, leukemia-associated alterations such as TEL-AML1, E2A-PBX1 or MLL, were detected in MSC of pediatric ALL patients (53). In particular, it was also reported that MSC from infants with MLL-AF4+ ALL,

harbour and express the MLL-AF4 fusion gene (17), even though MSC did not show oncogenic properties and were cytogenetically normal as also demonstrated by other Authors (18, 49, 59, 60). In our opinion, such discrepancies could be explained at least in part by substantial differences in the methodological approaches used to purify and expand in culture MSC. For example, it has been demonstrated that the type of culture media could mimic the *in vivo* microenvironment conditions, and appears to be crucial in determining the *in vitro* pathologic lymphocytes protection retention and survival, thus rendering the identification of MSC nuclei more difficult in FISH analysis after *in vitro* expansion and therefore affecting the correct interpretation of the cytogenetic profile of HM-MSC.

4.4. MSC aneuploidy

Since the adequacy of MSC phenotypic and functional characterization could represent a “prerequisite” for future clinical applications in autologous transplantation setting and MSC-based cellular therapy, the study of MSC cellular ploidy (chromosome number set evaluation) is also very important. A single recent study showed that the use of centromeric probes by FISH revealed the presence of HM-MSC polyploidy in CLL and ALL patients, as previously reported for rat BM-MSC (50). This was observed especially in patients with altered karyotype, as compared to those with a normal one. Although polyploidy has been previously reported in malignant tissues, in clinical conditions such as stress, hypertension and in aged subjects, we cannot exclude “*a priori*” that some microenvironment conditions, such as those occurring in course of CLL or other diseases, could be responsible for the HM-MSC altered ploidy (61, 62). These data are in agreement with Flores-Figueroa’ results (56), reporting the presence of MSC hypodiploid in myeloid malignant cells even though a normal diploid karyotype has been detected by other authors. Moreover, the presence of binucleated HM-MSC maintaining a normal nuclear diploid numeric pattern was also documented (41) in MSC from B-CLL as otherwise reported for rabbit MSC (63). The functional implications (defective or abnormal production of cytokines as suggested by the literature findings) of HM-MSC aneuploidy and binuclearity especially in patients with altered karyotype in lymphoproliferative disorders is a question that needs to be further addressed since a possible pathogenetic/pathophysiologic role of the BM-MSC have been suggested especially in chronic lymphoproliferative disorders.

5. MSC AND IMMUNOREGULATION

Several studies have demonstrated the immunosuppressive effect of MSC in allogeneic or mitogenic interactions. Cell-cell contact inhibition and secretion of immunomodulatory soluble factors have been suggested as potential mechanisms of action. The inhibitory functions of MSC involve several soluble molecules such as transforming growth factor-beta (TGF-beta), interleukin-10 (IL-10), tumour necrosis factor-alfa (TNF-alfa), prostaglandin E2 (PGE2), TRAIL (58), but a large consensus has been obtained on the immunomodulatory

role of indoleamine 2,3-dioxygenase (IDO) and soluble HLA-G antigens (38, 64). HLA-G antigen is a non-classical HLA class I molecule with potent immunomodulatory activity. Both soluble and membrane-bound HLA-G isoforms are able to inhibit several immune functions, such as the lytic and cytotoxic activity of NK cells and CD8 lymphocytes, maturation of dendritic cells, alloproliferation of CD4+ T lymphocytes and to induce the formation of regulatory T cells. Interestingly, high levels of HLA-G have been associated with a negative prognosis in tumors and viral infections where the tolerogenic function of HLA-G molecules allows the mutated/infected cells to escape the immune system. This intriguing role of HLA-G produced by MSC have been shown to have important implication in CLL bone marrow niche, as detailed below.

5.1. MSC immunophenotype and immunoregulation

Some authors have focused on the MSC immunophenotype in relation to their immunomodulatory properties. MSC share surface markers with thymic epithelial cells and express adhesion molecules for T lymphocyte interaction, such as vascular adhesion molecule 1 and intracellular adhesion molecule 2. It is well known that the low antigenicity of MSC is mainly caused by the low expression of classical HLA (human leukocyte antigen) class I molecules and the complete absence of HLA class II antigens and co-stimulatory molecules such as CD80 (B7-1), CD86 (B7-2) and CD40 (65). Nevertheless, the expression of class II HLA-DR molecule on BM derived MSC from patients with lymphoproliferative disorders has been described (66). Moreover, other studies reported up-regulation of MHC class II molecules only after pre-treatment with IFN-gamma and TNF-alfa or FGF. Of note, BM-derived MSC obtained from hematological malignancies isolated and cultured under *in vitro* angiogenic conditions exhibit a depressed expression of CD90 antigen that resulted associated with high *in vitro* proliferative rate but with a diminished immunosuppressive cell activity on T cell proliferation (40). These data suggest the Thy-1 molecule may be considered a marker implicated in the MSC inhibitory ability and might cooperate with HLA-G molecule in regulating suppressive *versus* stimulatory properties of MSC (67, 68). Thus, MSC may have an intriguing immunological function in the BM microenvironment in particular due to the production of HLA-G and IDO molecules that may allow leukemic cells to escape the immune system.

6. MSC AS SUPPORTIVE BM MICROENVIRONMENT FOR LEUKEMIC CELLS

It is thought that BM hemopoietic stem cells (HSC) reside in special microenvironment areas termed niches. The nature and functions of these niches, however, should be further elucidated. For example, HSC are thought to be in contact with self-renewing osteoprogenitors MCAM^{pos} or cadherin^{pos} and CD45^{neg}, but recent studies have suggested that only a small population of HSC reside in these endosteal niches (69-71). By contrast, many HSC are associated with sinusoidal endothelium, which is referred to as vascular niche in which for example, the presence of CD146^{pos} pericytes has been described (72, 73).

Recent data have also reported that MSC progenitor cells, including LNGFR^{pos} cells, adventitial reticular cells (ARC) (74), CXC chemokine ligand 12-abundant reticular cells (CAR cells) and nestin^{pos} expressing cells (33) can act as HSC niches suggesting that distinct and identifiable non-hemopoietic progenitor cells create distinct microenvironment niches in the BM, producing growth factors and cytokines necessary for stem cells survival. Based on these evidences, the integrity of BM microenvironment is the condition “*sine qua non*” that provides support for normal hematopoiesis and B-cell development. Nevertheless, in spite of the reported absence of stromal cytogenetic alterations, even not without controversies, a possible pathogenetic/pathophysiologic role for BM-MSC has been suggested in HM diseases.

Although the genetic events are considered primarily responsible for the first step of neoplastic transformation, the development and progression for example of the CLL clone are thought to be affected by various microenvironment signals that regulate proliferation and survival of malignant B cells (75, 76). Emerging data on composition of BM microenvironment, especially in CLL, constitute the best *in vitro* and *in vivo* model studied. These studies seem to confirm an active pathogenetic role of MSC as guilty bystander stromal component affecting homing, survival, drug-resistance and proliferation of long-term surviving leukemic cells (77, 78). A very important morphological aspect of stromal cell function in regulating the survival of B-lineage leukemic cells is the occurrence of “pseudoemperipolesis” firstly described by Burger et al. (79), and more recently by our group (41), suggesting that pathologic lymphocytes may be invaginated in the stromal cytoplasm and can actively interact with MSC.

Thus, pathologic lymphocytes interact with accessory stromal cells that protect CLL cells from spontaneous or drug induced apoptosis thus playing a crucial role in disease progression or resistance to therapy (80-83). Further investigations suggest that CLL B cells need the contact with MSC in BM, but with T-cells in lymph nodes and nurse like-cells in lymphatic tissues (84, 85). In particular, gene expression profile analysis indicated HGF and CXCL-12 potentially involved in leukemic clone maintenance in CLL (86). The CX3CR1/CX3CL1 system may also contribute to the interactions between CLL cells and tumor microenvironment by increasing CXCL-12-mediated attraction of leukemic cells to nurse-like cells (NLC) and promoting directly adhesion of CLL to NLC (80, 87, 88). For example, MSC have been previously described to support the survival of CLL cells in *in vitro* co-cultures and to reduce leukemic cell apoptosis when exposed to Ara-C or VP-16 (79-84). Spontaneous and drug-induced apoptosis of CLL cells was significantly inhibited by human and murine MSC. In particular, Balakrishnan et al. described the spontaneous and drug-induced apoptosis of CLL as a consequence of drug-promoted dGuo triphosphate (dGTP) accumulation and GTP/ATP depletion that was inhibited by MSC, providing a mechanism for resistance (89).

In addition, other literature findings reported that vincristine but not imatinib could suppress MSC niche's

support by suppressing L-asparagine secretion (90) and that MSC protect CLL cells from fludarabine and dexamethasone-induced apoptosis. Moreover, the evidence that drugs acting on the mevalonate biosynthetic pathway can regulate MSC-induced T-cell suppression and B-lymphocyte survival (91) and that lenalinomide can modulate the expression of cell markers and chemokine secretion of MSC *in vitro* (92) can have a clinical effect *in vivo* in patients. Since diverse MSC protect CLL from spontaneous and drug-induced apoptosis, a reliable *in vitro* method to assess stromal cell resistance has also been presented (93). B-CLL chemo-resistance involves innate and acquired leukemic side population (94) and the related mechanisms should be better clarified. Indeed, it has been described that MSC seem to increase B-cell viability, while inhibiting proliferation, arresting B-lymphocytes in G0/G1 (76). Moreover, it has been observed that stromal cell-mediated notch-1, notch-2 and notch-4 signaling has a role in CLL survival and resistance to chemotherapy. The block of this pathway could be an additional tool to overcome drug resistance and improve the therapeutic strategies for CLL (95).

Even though BM stroma in haematological malignancies and in CLL could be considered a guilty bystander, on the other hand, other mechanisms are responsible for pathogenesis of B-leukemic proliferation and survival. For example, an upregulation of CD38, CD71, CD25, CD69 and CD70 is described on *in vitro* cultured B-CLL pathologic lymphocytes and in this case the activation by MSC seem to be mediated by soluble factors (96) such as cytokines and pro-angiogenic factors, that can help tumor cells to escape from immune system (97). In addition, the intriguing role of HLA-G tolerogenic molecule produced by MSC and monocytes has been shown to have important implication in B-CLL bone marrow niches. Thus, HLA-G seems to contribute to the impaired immune response in B-CLL supporting disease progression (98) even though more recent studies sustain an irrelevant prognostic value of HLA-G production in CLL (99). Moreover, it has also been reported that CD38+CD49d CLL clones are able to attract CD68+ monocytes at site of infiltration by CCL3 and CCL4 synthesis. Other evidences demonstrated that CD73+/CD39+ CLL B- leukemic cells generate ADO from ADP and activate type 1 A2A purinergic receptors inhibiting chemotaxis and drug induced apoptosis of CLL cells (100). Macrophages release TNF-alfa that induces the up-regulation of VCAM-1 on MSC. VCAM-1/CD49d binding significantly increases neoplastic proliferation and survival (101). The presence of bystander immune cells and HLA-G production by MSC, as above reported, and upregulation of Treg can contribute to immune escape. Moreover, neo angiogenesis processes sustained by hemopoietic and other stromal cells such as endothelial cells and pericytes maintain also the neoplastic survival. All together these evidences could provide useful informations relative to a biochemical mechanism for an MSC-derived resistance to some drugs and emphasizes

the need to move toward combinations with agents that interfere with the microenvironment's protective role for improving current therapeutic efforts.

7. SUMMARY AND PERSPECTIVE

By our knowledge, bone marrow microenvironment plays an important role in the pathogenesis of some myeloid and lymphoid hematological malignancies. In this regard, the cytogenetic, immunological and functional integrity of MSC appear to play a key role. In particular: i) different markers may be prospectively used to identify the most primitive MSC progenitors with embryonic stem cell like-features before *in vitro* expansion, that can be identified by *in situ* immunophenotype of non-expanded MSC; ii) several data suggest that the tissue source of MSC is likely to be biologically relevant. Analysis of MSC transcriptoma has led to the identification of subpopulations of MSC expressing a variety of regulatory proteins that play a role in angiogenesis, hematopoiesis, neural activities and immunity, suggesting that the MSC biochemical heterogeneity may have an impact on the therapeutic applications of these cells; iii) most of the available studies reported a normal cytogenetic pattern of BM derived MSC, some non-specific alterations, not associated to leukemia abnormalities, have also been demonstrated probably as consequence of the *ex vivo* expansion and because of an intrinsic susceptibility; iv) several evidences suggest a "pathological" *in vivo* supportive function of MSC by producing soluble factors (such as cytokines, pro-angiogenic factors, and tolerogenic molecules such as HLA-G) that can help tumor cells to escape from immune system. MSC also provide important drug resistance signals to leukemic cells especially in lymphoproliferative disorders; v) even though BM stroma could be considered only a guilty bystander in haematological malignancies and CLL, other mechanisms are responsible for abnormal B-leukemic proliferation and survival in the bone marrow niche. All together these evidences could provide useful information about a possible mechanism to explain some MSC-derived drug resistances and emphasize the need of new therapeutic combinations that may interfere with the protective role of the stroma microenvironment on tumor cell survival.

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