ELISANGELA RIBEIRO

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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Fisiopatologia Médica, área de concentração em Medicina Experimental.

ORIENTADORA: *Profa. Dra. Irene Lorand-Metze*

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SUMÁRIO

AcMo	Anticorpo monoclonal
AMF	Amifostina
APC	Allophycocyanin
AR	Anemia refratária
AREB	Anemia refratária com excesso de blastos
AREB-t	Anemia refratária com excesso de blastos em transformação
ARSA	Anemia refratária com sideroblastos em anel
ATG	Globulina anti-timócito
BD	Becton Dickinson
BFU-E	Burst Forming Unit-Erytroid
CRDM	Citopenia Refratária com Displasia Multilinear
CD	Cluster Differentiation
CFU-GM	Granulocytic - Monocytic Colony Forming Unit
CF	Citometria de Fluxo
Del	Deleção
EPO	Eritropoetina
FAB	French-American-British
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
G-CSF	Granulocitic-Colony Stimulator Factor
GM-CSF	Granulocytic - Monocytic Colony Forming Unit
Gly A	Glicoforina A

Hb	Hemoglobina
HPN	Hemoglobinúria Paroxística Noturna
IFN	Interferon
IMF	Intensidade Média de Fluorescência
IPSS	International Prognostic System of Score
LMA	Leucemia Mielóide Aguda
LMC	Leucemia Mielóide Crônica
LMMC	Leucemia Mielomonocítica Crônica
MO	Medula Óssea
n	Número Absoluto
NK	Natural Killer
OMS	Organização Mundial de Saúde
PBS	Phosphate Buffer Solution
PE	Phycoerythrin
PERCP	Peridin Chlorophyll Protein
PL	Plaquetas
QT	Quimioterapia
SFB	Soro Fetal Bovino
SMD	Síndrome Mielodisplásica
SP	Sangue Periférico
SSC	Side Scatter
t	Translocação
TGF	Tumoral Growth Factor
TNF	Tumoral Necrosis Factor
WHO	World Health Organization

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RESUMO

Síndromes Mielodisplásicas (SMDs) são desordens clonais da célula precursora hematopoética pluripotencial caracterizadas por alterações na proliferação e maturação celulares e presença de apoptose medular excessiva. Alterações cariotípicas na mielodisplasia são comuns. Apesar dos critérios de diagnóstico estarem bem estabelecidos, o uso da citometia de fluxo (CF) no estudo desta doença tem sido muito explorado, pois desordens não-clonais (DNCs), que também apresentam citopenias periféricas, podem dificultar o diagnóstico. Populações eritroblástica, granulocítica, monocítica, linfóide e blástica em medula óssea (MO) de pacientes com SMD foram estudadas e comparadas à MO normal (doadores para transplante de MO) e às DNCs. Foram incluídos no estudo 32 casos de SMD (20 AR, 05 ARSA, 07 AREB), 11 controles normais e 10 pacientes com DNCs confirmadas. Selecionamos as populações celulares pelo dot plot CD45/side scatter (SSC). A análise quantitativa dos marcadores eritroblásticos CD71 e Glicoforina A (GlyA); granulocíticos e monocíticos CD11b, CD13, CD16, CD10 e CD64; linfóide CD19, CD3, CD4, CD8 e CD16; além do precursor CD34 e o pan leucocitário CD45, foi obtida através da intensidade média de fluorescência (IMF). A granularidade citoplasmática das células granulocíticas foi observada pela baixa IMF do SSC. Mielopoiese, presença de aberrações fenotípicas e o padrão maturacional também foram avaliados nestas células. Todos os pacientes com SMD tiveram algum tipo de alteração nas populações avaliadas pela CF. Os eritroblastos apresentaram perfil maturacional alterado em 20 casos (64,4%) e os monócitos em 22 (68,7%). Os granulócitos em 7 casos tiveram 1 anormalidade imunofenotípica, em 9 pacientes houve 2, em 9 casos 3 e em 2 pacientes 4 expressões anormais foram encontradas. O SSC esteve alterado em 7 casos (23%), os perfis do CD16/CD11b e CD16/CD13 em 18 (56,2%) e 20 casos (66%), respectivamente, , o CD13 em 22 (73%), o CD11b em 17 (56%), , o CD16 em 11 (36%), o CD45 em 17 (56%), e por fim a deficiência do CD10 encontrada em 3 casos (10,3%).

Assincronismo maturacional na população granulocítica foi encontrado enquanto nas DNCs a maturação granulocítica foi mantida. Os monócitos tiveram número aumentado tanto nos pacientes com SMD quanto nos com DNCs. A expressão do SSC, CD13 e CD11b foram similares nos 3 grupos. CD64 esteve aumentado nos 2 grupos de pacientes enquanto o CD16 apenas na SMD. O CD45 esteve diminuído somente no grupo das DNCs. Os linfócitos B nos pacientes AREB tiveram uma diminuição intensa em relação aos outros

subtipos FAB. A maturação B está comprometida na SMD tanto numericamente quanto na expressão antigênica dos grupos de baixo e alto risco. Uma aberração fenotípica $CD19^{+}/cCD79a^{-}$ foi encontrada nas células B na SMD mas não nos controles. As células T não tiveram alterações consideráveis. Os mieloblastos foram analisados nos dot plots CD16/CD11b e CD16/CD13 e CD34. Houve uma correlação entre a porcentagem de blastos visualizados pela morfologia (mielograma) com os do dot plot CD16/CD13 e número de células CD34 positivas, mas não com aqueles quantificados pelo dot plot CD16/CD11b. A técnica da CF pôde demonstrar presença de assincronismo maturacional, aberrações fenotípicas, granulopoiese alterada e utilidade na detecção de anormalidades imunofenotípicas na SMD. Além disso, possibilita monitorar a evolução da doença, observada pelo aumento de blastos na MO, e é capaz de diferenciar a SMD das citopenias periféricas não-clonais.

ABSTRACT

Myelodysplastic syndromes (MDSs) are characterized by abnormalities in proliferation, apoptosis and maturation of the hemopoietic cell lines. MDS is known by bone marrow (BM) cells abnormalities identified by morphology. Although MDS diagnostic criteria are well established, flow cytometry (FCM) technique has been explored. Even so, non-clonal disorders that present peripheral cytopenias such as MDS can difficult the diagnostic, mainly in MDS cases with normal karyotype. Initially, we analyzed the erythroid, granulocytic and monocytic lineages maturation patterns, and the blastic population in BM from patients with MDS and compared them with normal BM (BM transplantation donors) and patients with pancytopenia due to non-clonal diseases (NCD). MDS are considered as hematopoietic myeloid stem cells clonal disorders. However, accumulating evidence support that more immature stem cell, which has ability to differentiate into both myeloid and lymphoid cells, could origin the disease. BM cell populations were separated in CD45/side scatter (SSC) plot. Staining intensity quantitative patterns assessment for CD45, CD16, CD13, CD11b, CD10, CD34 and CD64 were performed in 32 MDS BM cases and compared with 10 non-clonal diseases and 11 controls. Granulocytic and monocytic abnormalities were identified per case and with 2 or more detected alterations. Antigenic expression patterns were determined during maturation. CD16/CD13, CD16/CD11b and CD10/CD64 plots were used for monitoring of granulocyte maturation and monocytic cells, CD71/GlyA for erythroblastic population and CD34+/SSC for blasts cells. Mostly MDS patients presented some kind of expression abnormalities: 7 (23%) low SSC, 18 (56.2%) CD16/CD11b and 20 (66%) CD16/CD13 abnormal patterns, 11 (36%) CD16, 22 (73%) CD13, 17 (56%) CD11b, 17 (56%) CD45, 3 (10.3%) CD10 deficiency. Monocytes were increased in both MDS and NCD, but increase in expression of CD16 occurred exclusively in MDS. The monocytes had similar expression in SSC, CD13 and CD11b among 3 groups. CD64 had increased in both patients groups but the CD45 was decreased more pronounciated in NCD. Asynchronous maturation was found in MDS patients, but was maintained in NCD. Several abnormalities in CD71/Gly A expression were found in erythroblasts in MDS, but they were normal in NCD. Neither GlyA nor CD71 deficiency were observed in NCD. Myeloblastic cells were analyzed in the CD16/CD11b and CD16/CD13 dot plots and CD34 expression. There was positive correlation between blasts percentage with CD16/CD13 dot plot and absolut number of CD34 positive cells, but not

with those quantified by CD16/CD11b dot plot. Ongoing the study, specific enumeration and characterization of CD34+/CD19+ and CD34-/CD19+ B-cell precursors and mature B-lymphocytes was performed in all MDS cases by multiparameter FCM. Both quantitative and qualitative immunophenotypic abnormalities involving BM B-cells were shown in B population. CD19+/cCD79a- phenotype asynchronous antigen expression was detected on CD34+ B-cell precursors but only when detectable levels of those precursos were shown in the MDS BM. There were not any considerable abnormalities for T lymphocytes. Abnormal B-cell maturation in MDS patients were shown by imunophenotypic evidences, and further investigations shall be used to undertand its nature. Concluding, FCM is an useful tool for differential diagnosis between clonal and non-clonal disorders.

INTRODUÇÃO

Síndrome Mielodisplásica

As síndromes mielodisplásicas (SMDs) são desordens da célula precursora hematopoética pluripotencial (stem cell) caracterizada pela hiperplasia da medula óssea (MO) com anormalidades de maturação e proliferação celulares e consequente citopenias periféricas. É principalmente uma doença acometida em sua maioria nos pacientes com mais de 50 anos, sendo rara na infância. O prognóstico é menos favorável nos casos que apresentam alta porcentagem de blastos na MO, citopenias periféricas acentuadas e alterações citogenéticas (GREENGERG et al., 1998; RIGOLIN et al., 1998). O agravamento da SMD pode ocorrer em um terço dos pacientes com a evolução para leucemia mielóide aguda (LMA) (LICHTMAN e BRENNAN, 1990; JENSEN e HOKLAND, 1994; BOUSCARY et al., 1997, RIBIZZI et al., 2001). A transformação para leucemia linfoblástica aguda é extremamente rara. No entanto, a maioria dos casos são de origem B (SATO et al., 2004).

Pacientes com SMD apresentam aumento significativo de risco a infecções sendo um dos motivos de morte em 40% dos casos. Entretanto, anormalidades funcionais dos neutrófilos, monócitos, e linfócitos T e B podem ser alguns dos fatores que contribuem para este quadro, sugerindo a presença de um defeito intrínseco do sistema imunológico (HAMBLIN, 1996). Não existe um mecanismo preciso destas anormalidades nem uma etiologia bem definida, embora a SMD possa ocorrer após exposição a agentes mielotóxicos, quimioterapia antineoplásica e transplante de MO, caracterizando uma SMD secundária.

Citopenias, displasias e assincronismo maturacional nas células da MO podem ocorrer em grau variável nas células do sangue periférico (SP), em uma ou mais linhagens. As anormalidades qualitativas mais comuns são assincronia maturacional núcleo-citoplasma, fragmentação nuclear dos eritroblastos, presença de sideroblastos em anel, bastonetes gigantes, granulócitos hipersegmentados e anomalia de pseudo Pelger-Huet, ou dos micromegacariócitos.

Várias doenças não-clonais que apresentam citopenias periféricas e displasias celulares simulam uma SMD, como hipotireoidismo, hiperesplenismo, doenças infecciosas, auto imunes e neoplásicas, citopenias transitórias devidos à ingestão de álcool ou

medicamentos, contato com produtos químicos, anemias carenciais e alterações na função renal. Portanto, os dados clínicos e testes laboratoriais, histologia da MO (biópsia), análise morfológica para detectar displasia multilinear na MO e no SP, anormalidades cromossômicas e mais recentemente dados imunofenotípicos são utilizados para estabelecer o diagnóstico de SMD (KRISTENSEN e HOKLAND, 1990; JENSEN e HOKLAND,1994; YOSHIDA, 1996; STETLER-STEVENSON et al., 2001).

O grupo French-American-British (FAB) estabeleceu uma classificação (1982) baseada na morfologia e na porcentagem de blastos no SP e na MO, propondo 5 subgrupos: anemia refratária (AR), anemia refratária com sideroblastos em anel (ARSA), anemia refratária com excesso de blastos (AREB), anemia refratária com excesso de blastos em transformação (AREB-t) e leucemia mielomonocítica crônica (LMMC) (Tabela 1) (BENNETT et al., 1982; SCHMITT-GRAEFF et al., 2000). Entretanto, a modificação desta classificação tem sido proposta pela Organização Mundial de Saúde (OMS) com a intenção de melhorar a definição de entidades clínicas da SMD. Entre as mudanças, uma leucemia já seria definida quando apresentasse blastos > 20% no mielograma, lembrando que pelo grupo FAB é considerada pela presença maior que 30%, o que acaba por eliminar o subtipo AREBt, ou pela presença de bastonetes de Auer ou t(8;21) independente do número de blastos. A inclusão da categoria citopenia refratária com displasia multilinear (CRDM) é outra proposta da OMS , já que o grupo AR compreende um espectro amplo de pancitopenia de gravidade variável (SCHIMITT-GRAEFF et al., 2000; VARDIMAN, HARRIS, BRUNING, 2002). Um estudo brasileiro comparou a classificação FAB com a da OMS em 150 pacientes diagnosticados com SMD. Metade dos pacientes que tiveram diagnóstico de AR foram incluídas na nova categoria de CRDM. Além disso, a classificação da OMS foi melhor para predizer sobrevida (LORAND-METZE et al., 2004b).

Para avaliação prognóstica destes pacientes usa-se o IPSS (GREENBERG et al., 1997), que considera a porcentagem de blastos, as anormalidades cariotípicas e o grau de citopenias periféricas (Tabela 2) (fig. 1).

Tabela 1 - Aspectos hematológicos dos subtipos da SMD de acordo com a classificação FAB

	BLASTOS		Corpúsculo de Auer	Monócitos SP ($1x10^9$/L)	Sideroblastos em anel >15% MO
	MO (%)	SP (%)			
AR	< 5	<1	--	--	--
ARSA	<5	<1	--	--	+
AREB	5-20	<5	--	--	+ ou -
AREB-t	20-30	>5	+	+ ou -	+ ou -
LMMC	<20	<5	-	+	+ ou -

AR: anemia refratária; **ARSA:** anemia refratária com sideroblastos em anel; **AREB:** anemia refratária com excesso de blastos; **AREB-t:** anemia refratária com excesso de blastos em transformação; **LMMC**: leucemia mielomonocítica crônica, **MO**: medula óssea; **SP**: sangue periférico

Tabela 2 - Escore IPSS utilizado em pacientes com SMD

Parâmetros Prognósticos	Valores				
	0	**0.5**	**1.0**	**1.5**	**2.0**
Blastos na MO(%)	<5	5-10	--	11-20	21-30
Cariótipo	Bom	Inter	Desf	--	--
Número citopenias	0/1	2/3	--	--	--

Inter: intermediário; **Desf:** desfavorável

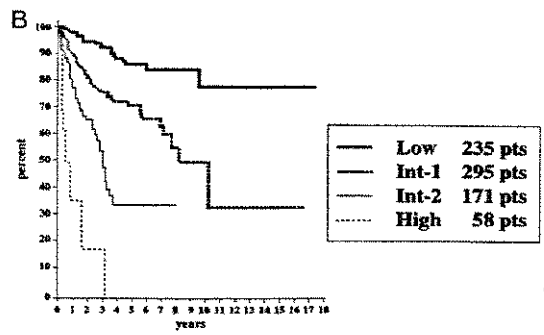
O valor para a determinação do grupo de risco é a somatória dos valores obtidos. Assim, quatro grupos foram identificados:

Baixo risco (Low): somatória dos valores igual a zero

Risco intermediário 1 (Int-1): somatória dos valores 0.5 - 1.0

Risco intermediário 2 (Int-2): somatória dos valores: 1.5 – 2.0

Alto risco (High): somatória dos valores maior ou igual a 2.5



Greenberg et al., 1997

Fig 1. - Gráfico representativo da sobrevida de pacientes com SMD pelo IPSS

Alterações cariotípicas na mielodisplasia são comuns sendo que as anormalidades mais complexas são associadas a um pior prognóstico da doença (HOY et al., 1989). As anormalidades citogenéticas mais frequentes na mielodisplasia com bom prognóstico compreendem deleção do cromossomo Y (45,X,-Y), del(5q), del(20q) e cariótipo normal, e com prognóstico de risco intermediário ocorrem a trissomia do cromossomo 8 (+8) e outras anomalias simples. Já as SMDs que apresentam alterações complexas e anomalias do cromossomo 7, apresentam curso agressivo da doença.

Fisiopatologia

A fisiopatologia tem sido muito discutida. O conjunto de características próprias das SMDs (citopenias, tecido hematopoético hipocelular com atipias) é resultante final de um conjunto heterogêneo de alterações funcionais da hematopoese. Numerosos estudos têm analisado a célula de origem, a clonalidade e as anormalidades de proliferação, maturação e apoptose na SMD (GOASGUEN e BENNETT, 1992; DAR et al., 1999).

Estudos recentes têm demonstrado que os progenitores hematopoéticos sofrem inibição por parte de linfócitos T supressores ($CD8^+$) em uma parte dos casos de mielodisplasia, especialmente naqueles com medula hipocelular (SHETTY et al.,1996). Este seria um outro mecanismo fisiopatológico que causa citopenias periféricas. Têm-se questionado também se a hiperexpressão dos Fas-L (Fas ligante) tornaria os precursores mais susceptíveis à ação inibitória dos linfócitos $CD8^+$ (GOASGUEN, 1992; BENNETT, 1992; DAR et al., 1999). A apoptose excessiva nas células blásticas da MO pode ser um dos principais mecanismos causadores das citopenias periféricas nessa doença. A apoptose pode ser desencadeada por diversos fatores, incluindo danos irreversíveis ao DNA das células precursoras hematopoéticas causadas por agentes quimioterápicos, pela radiação, imunossupressão medular mediada pelo aumento de antígenos indutores da apoptose, Fas/FasL, e dos linfócitos T citotóxicos circulantes (EPPERSON et al., 2001). Essas células que, além de secretarem citocinas inibitórias como TNF-α e TGF-β, que estão aumentadas no estroma da MO e no soro desses pacientes, liberam perforina e granzima B no citosol das células levando-as posteriormente à morte cellular programada. Além disso, a relativa deficiência de sinais como fatores de crescimento, interleucinas, que garantem a sobrevida celular e a alteração do balanço entre proteínas ou genes indutores e/ou supressores da apoptose, são agentes responsáveis pela hematopoese ineficaz e consequente citopenias periféricas na mielodisplasia (SHETTY et al., 1996; GREENBERG, 1998; PARKER e MUFTY, 2000; RIBEIRO et al., 2004).

Em um trabalho anterior foi demonstrado o aumento da expressão dos antígenos Fas/FasL nas células $CD34^+$ em SMD de baixo risco (< 5% de blastos na MO), comparada à MO normal, diminuindo a medida que aumentam o número de blastos (RIBEIRO et al., 2004).

Estudos recentes demonstraram que o sistema imune exerce um papel na fisiopatologia da SMD. Há evidências de que, nesta doença, uma expansão clonal ou oligoclonal de linfócitos T CD8 ativados seria produzida por linfócitos T, como observado na estimulação antigênica crônica (SUGAWARA et al., 1992; IWASE et al., 1995; MOLLDREM et al., 1998; KOOK et al., 2001; EPPERSON et al., 2001; BAUMANN et al., 2002; MELENHORST et al., 2002). Envolvimento das subpopulações linfóides foi demonstrado na SMD (HILBE et al., 1994; MACIEJEWSKI et al., 1994).

Transformação para leucemia linfoblástica aguda é extremamente rara mas a maioria ocorre para linhagem B como se verificou em casos anteriores (SATO et al., 2004). SATO et al (2004) relataram um caso em que o número de blastos aumentou na MO de um paciente com SMD, apresentando subsequentemente leucemia linfóide aguda B do tipo LLA-3.

Estudos controversos têm mostrado a possível presença de clonalidade em casos de SMD. As anormalidades cromossomais têm sido verificadas por técnicas de hibridização *in situ* fluorescente (FISH) e pela reação de cadeia polimerase (PCR) (BOULTWOOD e WAINSCOAT, 2001). A síndrome da deleção do braço curto do cromossomo 5 (5q-) e trissomia do 8 (+8) foram demonstrados. No entanto, +8, que ocorre em células tronco hematopoéticas, parece ocorrer secundariamente à 5q- (NILSSON et al., 2002). SAITOH et al (1998) demonstraram que a +8 está presente apenas a nível de unidades formadoras de colônias de granulócito-eritrócito-macrófago-megacariocítico (CFU-GEMM). A deleção do cromossomo 20 (20q-), observado pelo PCR, deve surgir na célula progenitora capaz de se diferenciar para as linhagens mielóide e linfóide B mas não para T (WHITE et al., 1994). Em outro estudo, 10 casos foram analisados com 3 ocorrências de 5q- em células B e apenas 1 caso em células T (NILSSON et al., 2000).

A apoptose, em precursores hematopoéticos, está aumentada em MO de pacientes com SMD, principalmente em grupos de baixo risco (AR e ARSA- tipo FAB) (GREENBERG, 1998; RIBEIRO et al., 2004). Entretanto, AMIN et al (2003) demonstraram que tal fenômeno também ocorre em linfócitos B medulares mas não em células T.

Em estudo anterior, encontramos correlação inversa entre o número de linfócitos medulares e a porcentagem de blastos e de células $CD34^+$ na MO (RIBEIRO et al., 2003a). Observamos ainda, que pacientes que responderam ao tratamento com amifostina apresentaram menor proporção de linfócitos na MO antes do tratamento. O aumento do número dos neutrófilos após este tratamento foi inversamente relacionado ao número de linfócitos medulares antes do tratamento, sugerindo que os linfócitos têm um efeito inibitório sobre a hematopoese (RIBEIRO et al., 2004a). Em um outro estudo

encontrou-se decréscimo de linfócitos B em pacientes com SMD (LORAND-METZE et al., 2004b). Diante destes resultados, no presente estudo nós analisamos a proporção de linfócitos B na MO de pacientes com SMD bem como suas características imunofenotípicas durante a maturação.

Citometria de Fluxo

A SMD é uma doença que apresenta critérios de diagnóstico bem estabelecidos (LORAND-METZE et al., 2004). Há um número significativo de casos em que a análise do SP e da MO não permitem um diagnóstico seguro de mielodisplasia. Todavia, entidades não clonais podem apresentar citopenias e atipias celulares em uma ou mais séries da MO (STETLER-STEVENSON et al., 2001).

As características fenotípicas necessárias para se caracterizar um diagnóstico de SMD têm sido atualmente muito estudadas e têm-se observado alterações na expressão individual dos antígenos tanto em SP quanto em amostras de MO em pacientes com esta doença (ORFAO et al., 2001, 2003, 2004). As anormalidades fenotípicas podem ser detectadas pela citometria de fluxo (CF) com a utilização de anticorpos monoclonais (AcMo) podendo auxiliar nos casos suspeitos de SMD, mas são de difícil diagnóstico pela citologia convencional, ou quando o cariótipo apresenta-se normal (KRISTENSEN e HOKLAND, 1990; FELZMANN et al., 1992; JENSEN e HOKLAND,1994; KUIPER-KRAMER et al., 1997; ELGHETANY, 1998; STETLER-STEVENSON et al., 2001).

A vantagem da CF é o alto grau de eficiência e sensibilidade, com melhor reprodutibilidade em relação ao microscópio e possibilita o acesso simultâneo a vários parâmetros (NGUYEN et al., 2003). Segundo STETLER-STEVENSON et al. (2001) a CF tem uma correlação de 78% em relação à morfologia. Entretanto, a morfologia convencional avalia melhor as linhagens eritroblásticas e megacariocíticas, enquanto que para a série granulocítica a imunofenotipagem se mostrou mais sensível, talvez pela maior quantidade de marcadores disponíveis. No entanto, esses autores observaram que 100% dos casos de SMD que tiveram anormalidades citogenéticas clonais apresentaram alterações bi ou trilineares pela CF.

A caracterização imunofenotípica pode avaliar nas linhagens celulares (linfocitária, granulocítica, blástica, eritroblástica e megacariocítica) valores quantitativos, estágios maturacionais e anormalidades de expressão antigênica, como ausência, expressão anômala ou quantidades excessivas de antígenos comparadas a pacientes sadios (BASSO et al., 2001; WEIR e BOROWITZ, 2001).

Especificamente, a CF pode ser utilizada para identificar diferenças maturacionais da linhagem eritroblástica pelas características físicas e expressão de marcadores, como glicoforina A (GlyA), o receptor de transferrina (CD71) e o CD45 (pan leucocitário). É importante ressaltar que a população eritroblástica pode se expressar diferentemente de um estudo para outro devido ao tipo de reagente de lise utilizado na preparação do material. O lisante pode eliminar os eritroblastos mais maduros, os policromáticos e ortocromáticos, predominando os mais imaturos na análise (NGUYEN et al., 2003). Outra dificuldade na análise da população eritroblástica é a região onde está situada quando observada tanto em dot plot forward scatter-FSC/side scatter-SSC quanto no CD45 x SSC. A contaminação por células mortas, eritrócitos ou restos celulares (debris) com os eritroblastos restantes após a lise pode dificultar a análise. Por isso, FORNAS et al (2002) aplicaram um novo método de análise baseado na marcação das células viáveis com RNA/DNA (SYTO-13), separando assim as células eritróides nucleadas dos debris.

Entre os marcadores eritróides já citados podemos correlacionar a GlyA, que está normalmente presente em estágios mais maduros a partir do eritroblasto basófilo, ao CD71 que se expressa a partir das células BFU-E até o reticulócito (fig. 2) (LOKEN et al., 1987). Este último marcador, apesar de se expressar intensamente nas células eritróides, não é um marcador específico para esta linhagem. Pode estar positivo, mas com menor intensidade de expressão nos mieloblastos, monoblastos e nos precursores megacariocíticos, além de ser um marcador de proliferação.

A maturação das células eritróides é acompanhada pela queda de expressão do CD45 com ganho gradativo de Gly A. O CD71, como já foi dito, se expressa antes mesmo da GlyA é altamente expresso nestas células. (STETLER-STEVENSON et al., 2001; NGUYEN et al., 2003, ORFAO et al., 2003). KUIPER-KRAMER et al. (1997) observaram um decréscimo na expressão do CD71 pelos eritroblastos na SMD. Entretanto, essa

deficiência pode ser encontrada também em casos de anemia aplástica com alteração morfológica eritróide evidente como em hiperplasias eritróides e diseritropoeses (deficiência de folato/B12). Contudo, nestas 2 últimas situações o CD71 apresenta uma distribuição heterogênea com intensidade de expressão variável. Outras anormalidades são observadas como assincronismo da dupla marcação CD45 e CD71 ou GlyA e no CD71 com GlyA (NGUYEN et al., 2003).

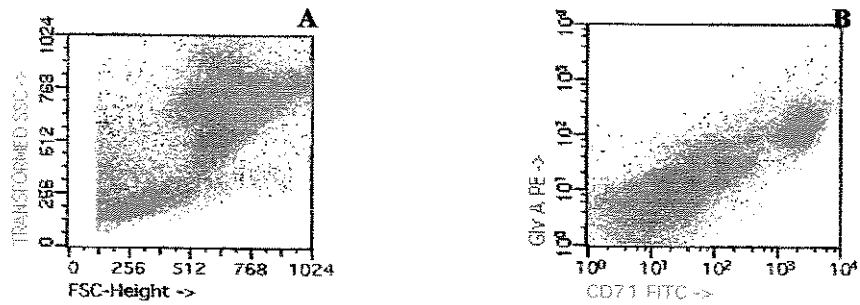
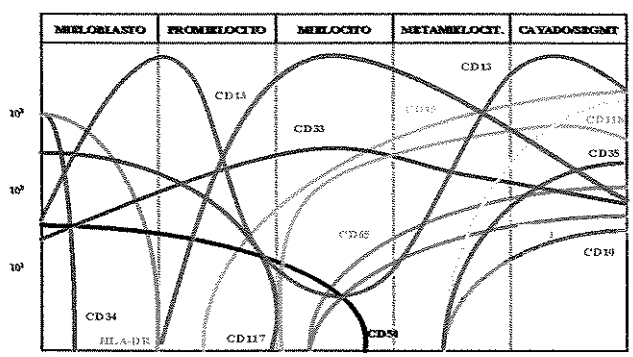


Fig 2. - População eritroblástica em MO normal. **A:** Dot plot FSC/SSC. **B:** Dot plot CD71/GlyA. Expressão dupla positiva.

Em relação à população granulocítica muitos marcadores podem ser utilizados para identificar assincronismo maturacional, diferenciação mielóide, presença de desvio à esquerda, além de detectar deficiências e/ou aumento de expressão antigênica. Há ainda marcadores que podem indicar prognóstico e progressão da SMD para leucose aguda. O entendimento da maturação celular normal é imprescindível para que se possa identificar estas anormalidades antigênicas na SMD (fig. 3).



ORFAO et al., 2001

Fig 3. - Diferenciação mielóide em MO normal utilizando anticorpos monoclonais (AcMo).

A maturação granulocítica pode ser monitorada pela combinação antigênica do CD11b x CD16 e do CD13 x CD16 (STETLER-STEVENSON et al., 2001; WELLS et al., 2003). A alteração do perfil de maturação na SMD, quando comparado à MO normal, pode demonstrar um assincronismo maturacional granulocítico. Na maturação mielóide normal, o CD13, que é um marcador que se expressa mais imaturamente, a partir da célula comissionada CFU-GM (TERSTAPPEN et al., 1990), é perdido nos mielócitos mas é expresso novamente nos metamielócitos e progressivamente até a maturação final, fase em que o CD16 inicia sua expressão sendo mais forte nos granulócitos maduros (fig. 4). O aumento de expressão do CD13 pode significar um aumento de células imaturas na MO.

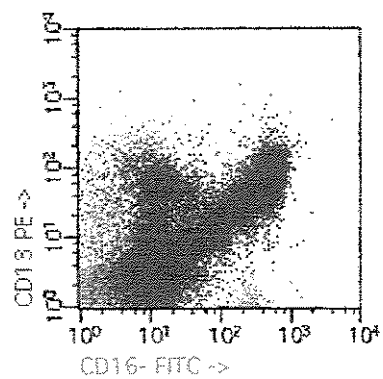


Fig 4. - Dot plot CD16/CD13. Perfil maturacional da população granulocítica em MO normal.

O CD11b é um marcador que aparece a partir do mielócito mais jovem, aumentando sua intensidade de expressão gradativamente à medida que as células vão se diferenciando. A expressão máxima ocorre na fase final da maturação granulocítica, nos neutrófilos maduros (fig. 5) (TERSTAPPEN et al., 1990).

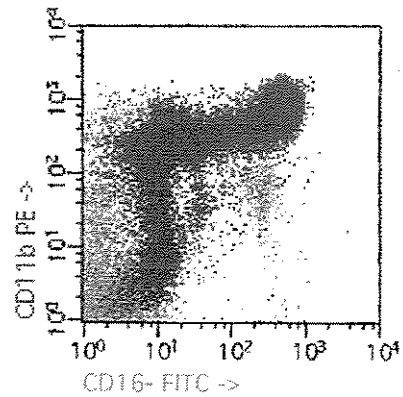


Fig 5. - Dot plot CD16/CD11b. Perfil maturacional da população granulocítica em MO normal.

O CD16 é um marcador negativo para os monócitos que são $CD14^+/HLADR^+$. A população minoritária de células dendríticas (células iniciadoras da resposta imune, apresentadores de antígeno e potente ativador do sistema imunológico), apresenta imuunofenótipo $CD16^+/HLADR^+/CD14^-$. Diante disso, a análise deve ser feita com cuidado para não contaminar a primeira população com a segunda, respectivamente, e considerar erroneamente os monócitos CD16+, como comentado por WELLS et al. (2003) e ALMEIDA et al. (2001). As células dendríticas se situam entre a população monocítica e linfóide (fig. 6), mas mais próxima da primeira (ALMEIDA et al., 2001).

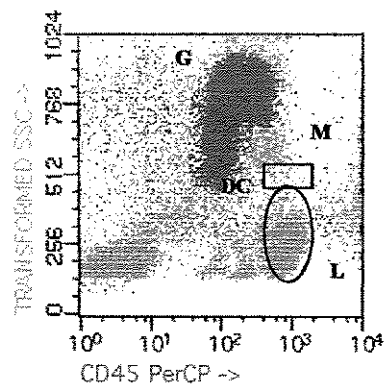


Fig 6. - Dot plot CD45/SSC. Azul: População granulocítica (G); Cianina: População monocítica (M), Populaçnao de células dendríticas (dendritic cells-DC) e População linfóide (L)em MO normal.

O CD64 é um marcador mielo-monocítico e representa uma molécula efetora presente nos grânulos das células granulocíticas. Apresenta positividade a partir dos mieloblastos (+/-), aumenta nos prómielócitos e mielócitos (++) e diminui progressivamente com a maturação (+/-). No entanto, o CD64 aparece antes que o CD15, um marcador mielóide mais maduro. Nas SMDs de alto risco o CD64 parece ter um aumento na intensidade de expressão enquanto o CD16 está diminuído nas células granulocíticas (OHSAKA et al, 1997). Na população monocítica, o CD64 é o único marcador expresso já nas células $CD34^+$ apresenta-se com maior intensidade que na população granulocítica (OLWEUS, JOHANSEN, TERSTAPEN, 1995).

Bowen e Davis (1997) estudaram o CD11b e CD16 nas SMDs e detectaram um baixo número de células granulocíticas que expressavam um e/ou outro marcador quando comparados a grupos controle. Elghetany (1998) observou baixa intensidade de fluorescência do CD16 nas células granulocíticas da MO mielodisplásica em 64% dos casos. Estes resultados sugerem um assincronismo de maturação da série mielóide em pacientes mielodisplásicos (SAWADA et al., 1995). A expressão anormal dos pares de AcMo CD16 x CD11b e CD16 x CD13 nas células granulocíticas foi detectada primeiramente na SMD. É necessário ressaltar, porém, que outras doenças como as mieloproliferativas, especialmente a leucemia mielóide crônica (LMC), doenças não neoplásicas (induzidas por drogas; agranulocitose) ou MO com intenso efeito de G-CSF podem apresentar alterações nas curvas de expressão antigênica destes AcMo (NGUYEN et al., 2003).

Citocinas como o IFN-γ, TNF-α e GM-CSF, presentes em processos inflamatórios causados por infecções bacterianas e processos autoimunes, alteram a expressão dos antígenos de superfície das células mielomonocíticas. O CD64 e CD11b são marcadores mielo-monocíticos. Ambos têm expressão mais forte na série monocítica do que nos granulócitos. Estes AcMo podem ser correlacionados com a classificação FAB nas duas populações celulares e no SP de pacientes com mielodisplasia. Na SMD de baixo risco tanto o CD11b quanto o CD64 apresentam expressão aumentada nos monócitos mas não nos granulócitos. Já nas SMDs de alto risco o CD11b está hiperexpresso nas duas populações celulares. O CD11b está envolvido na fagocitose e processos de adesão celular (FELZMANN et al., 1993).

Chang e Cleveland (2000) observaram a diminuição da expressão do CD10 (marcador característico das células granulocíticas maduras) nos granulócitos das SMDs. Essa deficiência pode provocar uma maior susceptibilidade a infecções nos pacientes porque esse marcador possui uma sequência idêntica à enzima presente na membrana dos granulócitos, a qual é responsável pelo controle da quimiotaxia e da resposta inflamatória promovidas por essas células. NGUYEN et al (2003) observaram que a perda ou alteração do CD10 é mais frequente em SMD de baixo risco que de alto risco. No entanto, quando há alteração em casos de baixo risco há uma correlação com alterações citogenéticas e consequentemente uma propensão maior à progressão da SMD para LMA.

As populações linfóides medulares têm sido estudadas na SMD desde há muito tempo. Inicialmente tentou-se verificar se os linfócitos participariam do clone geneticamente anormal. Isso foi comprovado em raros casos. (HAMBLIN et al., 1996; BARRETT et al., 2000).

Têm crescido as evidências de que, nas SMDs, ocorre uma expansão oligoclonal de linfócitos T CD8 ativados, compatíveis com uma estimulação antigênica crônica (HAMBLIN, 1996; MOLLDREM et al., 1998; BARRET et al., 2000; EPPERSON et al., 2001). Estes achados têm sido a base para o tratamento dos pacientes com SMD, especialmente nos casos AR e naqueles com medula óssea hipocelular que respondem ao tratamento imunossupressor (MOLLDREM et al., 1998; ASANO et al., 2001).

Apesar disso, há uma série de controvérsias referentes às alterações que ocorrem tanto nos linfócitos T $CD4^+$ e linfócitos B como na interação dos linfócitos com o clone anormal e na proliferação dos blastos e sobrevida dos pacientes (HAMBLIN, 1996; BARRET et al., 2000; NILSSON et al., 2000; EPPERSON et al., 2001; KOOK et al., 2001; BAUMANN et al., 2002). Em estudo anterior mostramos que há uma correlação inversa entre o número de linfócitos medulares e a porcentagem de blastos e o número de células $CD34^+$ (RIBEIRO et al., 2003a). Verificamos também que os pacientes que respondem ao tratamento com amifostina têm, antes do tratamento, uma proporção menor de linfócitos na medula (mediana 14,5% e 27,4% para os não-respondedores). Encontrou-se uma correlação inversa entre o aumento de neutrófilos após o tratamento e a proporção de linfócitos na medula antes do tratamento (RIBEIRO et al., 2003b).

Na população dos blastos em MO a presença do CD34 pode indicar o número de células precursoras (KUIPER-KRAMER et al., 1997, STETLER-STEVENSON et al., 2001), podendo correlacionar com os grupos FAB. Nas SMDs AR há um valor absoluto de $CD34^+$ anormalmente baixo enquanto nas AREB e AREB-t este marcador é extremamente alto (FUCHIGAMI et al., 1999). Por outro lado, RIBEIRO et al. (2004) não encontraram correlação entre as células $CD34^+$ e o número de blastos.

As alterações quantitativas dos marcadores da séries eritróides, mielo-monocítica, linfocitária e blástica podem ser determinadas pela CF, assim como as qualitativas podem ser visualizadas em citologia de MO. Entretanto, como as SMDs são diagnosticadas e classificadas pela FAB, é importante que se obtenha as alterações antigênicas aberrantes nesse tipo de material em casos de difícil análise morfológica. Além do mais, as anormalidades imunofenotípicas também podem ser detectadas através do SP. Orfao et al. (2003) observaram uma frequência variável de expressão dos AcMo nos neutrófilos do SP em pacientes mielodisplásicos e detectaram decréscimo de CD11b (50% dos casos), CD15 (26%), CD16 (80% das SMDs hipocelulares), entre outros. Além do mais, houve aumento de expressão nestas mesmas células de CD33 (18-54%), CD14 (40%) e CD64.

Em resumo, a CF pode contribuir para o diagnóstico da SMD revelando assincronia de maturação, deficiência de marcadores e/ou alterações na expressão antigênica. Estas alterações podem ainda ser correlacionadas com prognóstico e as classificações FAB e OMS. A CF pode auxiliar nos casos em que a morfologia convencional não consegue detectar atipias morfológicas características e o cariótipo é normal. Além disso, esta técnica pode contribuir para o entendimento da fisiopatologia da SMD como o envolvimento de várias linhagens celulares nesta doença.

A CF, como visto, é uma técnica complementar à citologia convencional que pode ser utilizada como uma ferramenta a mais para se chegar à conclusão diagnóstica de um quadro de mielodisplasia.

OBJETIVOS

Ao considerar a importância de se estudar casos de SMD de difícil diagnóstico pelos métodos usuais, foi proposto:

1) Descrever as alterações de maturação nas séries eritroblástica, granulocítica, monocítica e blástica utilizando a citometria de fluxo, além de analisar as atipias celulares pelo mielograma.

2) Avaliar a importância da citometria de fluxo no diagnóstico das SMDs em relação aos critérios convencionais.

3) Observar o valor da citometria de fluxo no diagnóstico diferencial entre SMD-AR com cariótipo normal e das desordens não-clonais que podem simular um quadro de mielodisplasia.

4) Determinar as subpopulações e possíveis alterações da população linfóide na medula óssea de pacientes com SMD.

MATERIAIS E MÉTODOS

Pacientes

Amostras de aspirado de MO, em EDTA, foram examinadas em 3 grupos de pacientes da Universidade Estadual de Campinas (UNICAMP) no período de agosto/2002 a maio/2004. **1)** grupo controle (MO normal), foi obtido de doadores para transplante alogênico sem uso de G-CSF ou outros fatores de crescimento, **2)** pacientes com SMD (casos novos e com diagnóstico confirmado), **3)** pacientes que apresentavam doenças não-clonais com citopenias periféricas ou pacientes suspeitos de SMD ou nos quais não foi possível confirmar este diagnóstico. Foi utilizado um protocolo (ver em Anexos) para confirmar o diagnóstico de mielodisplasia com a exclusão de outras doenças que continham citopenias no sangue periférico.

Critérios mínimos para o diagnóstico de SMD (LORAND-METZE et al., 2004)

- Presença de citopenias no SP;
- Citologia de MO com atipias em pelo menos 2 linhagens hemopoéticas ou presença de alteração citogenética clonal.

O estudo cariotípico foi colhido em todos os casos. A presença de alterações clonais confirmou o diagnóstico de SMD

Critérios de Inclusão dos Pacientes com Doenças Não-Clonais (Grupo 3)

1. Casos com citopenias periféricas, mielograma com atipias e cariótipo normal (quando realizado). Confirmação de: hipotireoidismo, doenças infecciosas, autoimune, neoplásicas, transitórias devido ao consumo de álcool, medicamentos e contato com produtos químicos; anemias carenciais e alteração da função renal.
2. Casos sem confirmação para SMD, segundo o protocolo de exclusão das desordens não clonais (Anexo 1).

Exames realizados nos grupos 2 e 3

- Hemograma completo com o número de reticulócitos;
- Mielograma corado pelo May-Grünewald Giemsa e para ferro (azul da Prússia);
- Biópsia de medula óssea (MO);
- Determinação do cariótipo pelo aspirado de MO;
- Imunofenotipagem de amostras de MO para determinação antigênica;

Avaliação Laboratorial

A análise do SP (hemograma) e da citologia de MO (mielograma) foram realizados em cada paciente e nos controles no laboratório de hematologia do Hemocentro-UNICAMP. A dosagem da hemoglobina, contagem de leucócitos, neutrófilos e de plaquetas foram obtidos pelo contador hematológico (Cell Dyn 3000, USA). Os mielogramas foram analisados em esfregaços corados pelo May-Grünewald Giemsa e pela coloração do azul da Prússia para detectar acúmulo de ferro e/ou presença de sideroblastos em anel. Este exame foi realizado ao diagnóstico e quando houve suspeita de transformação leucêmica. O diagnóstico da SMD e a identificação dos seus subtipos obedeceram os critérios citomorfológicos das classificações FAB e OMS.

A técnica da CF foi utilizada para quantificar, em número absoluto e porcentagem, e detectar a intensidade média de fluorescência (IMF) dos AcMo conjugados com fluorocromos. Estes parâmetros foram obtidos nos 3 grupos de pacientes nas populações celulares: eritroblástica, granulocítica, monocítica, linfocítica e blástica. Os 2 grupos de pacientes foram comparados ao grupo controle e entre si.

Realizou-se o cariótipo pelo método convencional, bandeamento G.

Reagentes

1- Anticorpos monoclonais

2- Meio RPMI 1640;

3- Solução lisante Becton Dickinson (BD) 10x concentrada;

4- Tampão fosfato (PBS) com 2% de soro fetal bovino e 0,5% de azida sódica pH = 7,4;

5- Tubos de polietileno BD;

6- Tubos Falcon de 15 ml.

Anticorpos Monoclonais Anti-Humanos

Os anticorpos foram adquiridos comercialmente sendo todos conjugados diretamente com fluorocromos:

1. CD45/PerCP (Proteína Piridina Clorofila-clone 2D1, BD): marcador pan leucocitário;
2. CD34-anti HPCL2/PE (Ficoeritrina- clone 8G12, BD): marcador de células precursoras hematopoéticas;
3. CD16/FITC (Isotiocianato de Fluoresceína- clone DJ130c, DAKO): expresso em linfócitos NK e granulócitos maduros;
4. CD11b/PE (clone 2LPM19c, DAKO): presente nas séries mielóide, apartir do mielócito jovem, e monocítica;
5. CD13/PE (clone WM17, DAKO): células mielóides mais imaturas, fase intermediária (exceto nos mielócitos) e maduras e, nos monócítos.
6. CD64/FITC (clone 10.1, DAKO): presente em células mielóides e monocíticas;
7. CD71/FITC (clone Ber-T9, DAKO): receptor transferrina – expressão desde o BFU-E à reticulócitos em linhagens eritróides; e fraca positividade nos mieloblastos, monoblastos e precursor megacariocítico;

8. Glicoforina A/PE, CD235a (clone JC159, DAKO) : expresso desde o eritroblasto basófilo ao eritrócito maduro;
9. CD10/FITC (clone W8E7, BD): células granulocíticas maduras, linfócitos B mais imaturos;
10. CD19/FITC (HIB19, BD): linfócitos B;
11. CD3/FITC (clone VCHTI, DAKO): linfócitos T;
12. CD4/FITC (clone MT310, DAKO): linfócitos T indutor/helper e monócitos;
13. CD8/PE (clone DK25, DAKO): linfócitos T citotóxicos e, em linfócitos NK;
14. IgG1/FITC: Controle negativo
15. IgG1/PE: Controle negativo
16. IgG1/PERCP: Controle negativo.

Técnica da Citometria de Fluxo

Para marcação de superfície, as células foram utilizadas na concentração de 2,0-3,0 $x10^6$ células/ml. Volume de 100 µl foram utilizadas para posterior incubação com 10µl de cada AcMo em protocolo de 3 cores usando as seguintes combinações:

1. Controle/Controle/CD45-PERCP;
2. CD16-FITC/CD11b-PE/CD45-PERCP;
3. CD16-FITC/CD13-PE/CD45-PERCP;
4. CD10-FITC/CD64-PE/CD45-PERCP;
5. CD71-FITC/GlyA-PE/CD45-PERCP;
6. CD4-FITC/CD8-PE/CD3-PERCP;
7. CD3-FITC/CD19-PE/CD45-PERCP;
8. CD19-FITC/CD34-PE/CD45-PERCP;

Os AcMos foram incubados por 15-20 minutos à temperatura ambiente e no escuro. Os eritrócitos foram lisados com 2 ml de FACS lysing solution (BD) em cada tubo com incubação adicional de 10 minutos. Os leucócitos foram centrifugados a 1500 rotações/ minuto (rpm) durante 5 minutos, lavados com PBS e levados ao citômetro de fluxo FACScalibur da BD para aquisição de 30.000 células (eventos). Foi utilizado o programa Cell Quest, para aquisição e o Paint-A Gate, para análise dos dados, considerando o número de eventos, porcentagem e o IMF dos AcMo para cada população celular.

A população linfóide e seus subtipos na SMD foi analisada primeiramente na UNICAMP para analisar a possibilidade de alterações em número. Devido aos resultados obtidos, demos seguimento a este estudo na Universidade de Salamanca (Espanha), analisando anormalidades quantitativas, maturação e presença de aberração fenotípica dos linfócitos B. Nos casos de Salamanca foi utilizado um protocolo de 4 cores.

Os AcMo conjugados diretamente com os fluorocromos FITC, PE, PERCP e aloficocianina (APC) foram:

1. CD19 FITC BD; (clone: HIB19): linfócitos B
2. cCD79a PE Dako (clone HM57): marcador intracitoplasmástico expresso antes mesmo do CD19.
3. nTdT FITC Dako; (clone HT-6): marcador nuclear. Positivo nas células linfóides imaturas e em torno de 25% nas imaturas mielóides.
4. HLA-DR FITC BD (clone L243): marcador de imaturidade mielóide, linfócitos B, linhagem monocítica, células dendríticas e linfócitos T ativados.
5. CD34 APC BD (clone 8G12): marcador de células precursoras ou imaturas.

Painel de 4 cores seguiu as seguintes combinações:

1. CD19/cCD79a/CD45/CD34
2. TdT/cCD79a/CD45/CD34
3. HLA-DR/cCD79a/CD45/CD34

Análise das Populações Celulares pela Citometria de Fluxo

As populações celulares foram definidas através do quadrante (Dot Plot) CD45/SSC (side scatter- complexidade interna) (fig. 7), e FSC (forward scatter- tamanho celular)/SSC (fig. 8). Posteriormente, foram analisados os marcadores específicos para cada linhagem.

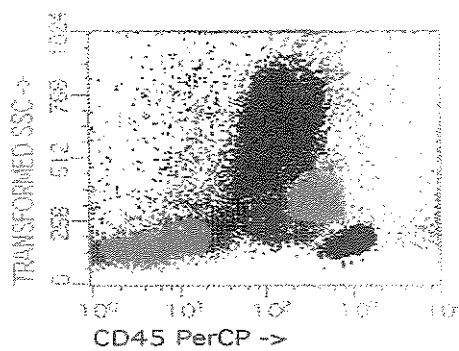


Fig 7. - Dot Plot CD45/SSC. As populações foram definidas de acordo com a intensidade do CD45 (pan-leucocitário) e a granularidade celular (SSC). AZUL: população granulocítica; VERDE: população eritroblástica; CIANINA: população monocítica; VIOLETA: população linfóide; VERMELHO: população blástica

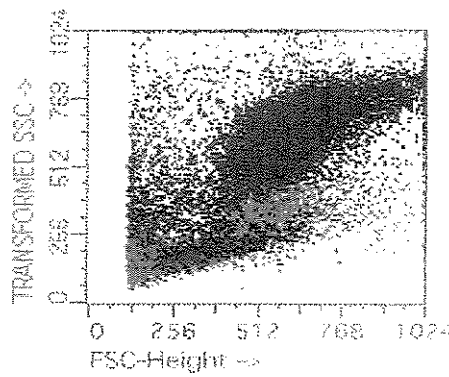


Fig 8. - Dot Plot FSC/SSC. As populações de acordo com tamanho celular (FSC) e granularidade (SSC), com as populações em cores correspondentes à figura anterior.

Análise da População Eritroblástica

A análise da população eritroblástica (fig. 7-VERDE) foi realizada pela expressão do CD71 e da Gly A já que ambos são co-expressos em MO normal (fig. 2B).

Análise das Populações Granulocítica e Monocítica

As populações granulocítica (fig. 9A-AZUL) e monocítica (fig. 9A-CIANINA) foram analisadas no grupo controle e pacientes com SMD e doenças não-clonais. As combinações CD16 x CD11b (fig. 9B), CD16 x CD13 (fig. 9C) e CD10 x CD64 (fig. 9D) foram analisadas em ambas as populações a nível quantitativo considerando o número absoluto, porcentagem e a intensidade média de fluorescência (IMF) em cada marcador. Além disso, foi observado o perfil maturacional apenas para a população granulocítica, enquanto que para os monócitos o CD16 e CD10 não foram considerados.

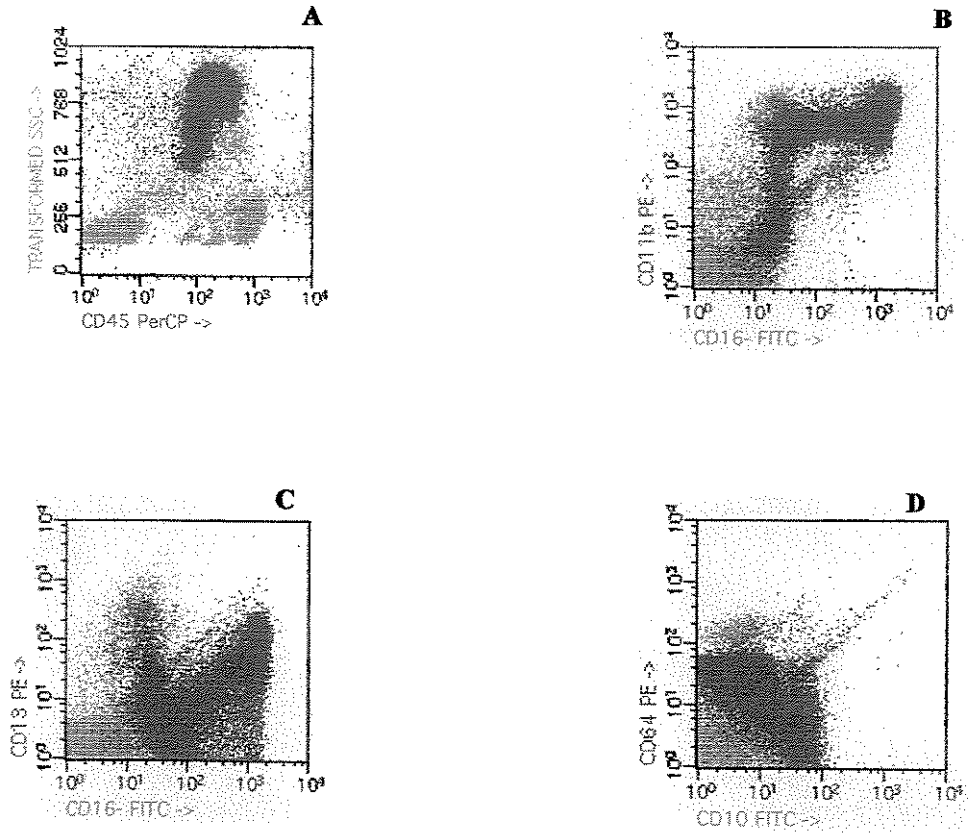


Fig 9. - Populações granulocítica e monocítica em MO normal. A: Dot plot CD45 x SSC. Região da população granulocítica (Azul) e monocítica (Cianina). B: Dot plot CD16 x CD11b. Perfil maturacional dos granulócitos (AZUL) e forte expressão do CD11b nos monócitos (CIANINA). C: Dot plot CD16 x CD13. Perfil maturacional dos granulócitos e forte expressão do CD13 nos monócitos (CIANINA). D: Dot plot CD10 x CD64. Forte expressão do CD64 nos monócitos (CIANINA).

A mielopoiese foi definida e avaliada individualmente de acordo com a maturação granulocítica: mieloblastos, prómielócitos, mielócitos, meta/bastão e neutrófilos maduros (figs. 10 e 11).

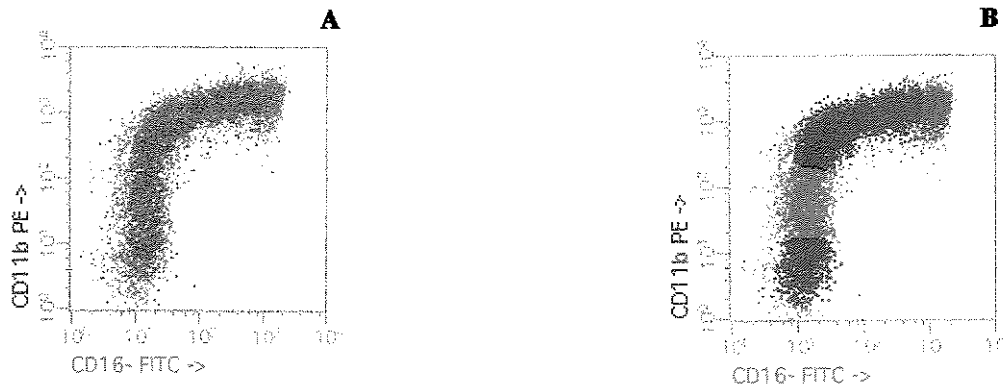


Fig 10. - Dot plot CD16/CD11b. Maturação normal da população granulocítica. **A:** Perfil maturacional da população total. **B:** Mielopoiese por citometria de fluxo determinada em 5 populações. VERMELHO: Mieloblastos; VERDE: Prómielócitos; AZUL: Mielócitos; VIOLETA: Meta/bastão; CIANINA: Neutrófilos maduros.

Fig 11. - Dot plot CD16/CD13. Maturação normal da população granulocítica. **A:** Perfil maturacional da população total. **B:** Mielopoiese por citometria de fluxo determinada em 5 populações; VERMELHO: Mieloblastos; VERDE: Prómielócitos; AZUL: Mielócitos; VIOLETA: Meta/bastão; CIANINA: Neutrófilos maduros.

Análise da População Linfóide

A população linfóide foi analisada considerando número absoluto e porcentagem para as subpopulações T (CD3/CD4, CD3/CD8) (figs.12 A-C) e NK (CD16). As células B foram analisadas pelos marcadores CD19, cCD79a, TdT, e HLA-DR. Todas as amostras foram identificadas como sendo $CD19^{+}$ (fig.13-A) e considerou-se o SSC baixo. As células B foram observadas em 3 diferentes subgrupos identificadas em dot plot CD45/SSC (fig.13-B) e FSC/SSC (fig.13-C), nos quais foram determinadas de acordo com o grau de maturação: 1) células B precursoras $CD34^{+}/CD45^{fraco}$, 2) intermediário $CD34^{-}/CD45^{int}$ e 3) linfócitos B maduros $CD34^{-}/CD45^{forte}$ (fig. 13 D). Para a análise fenotípica de cada uma destas populações B considerou-se tanto a intensidade do SSC como a IMF obtida para cada marcador individualmente. Baseado na marcação dos controles, as células foram consideradas positivas uma vez que os valores da IMF eram superiores a 5.

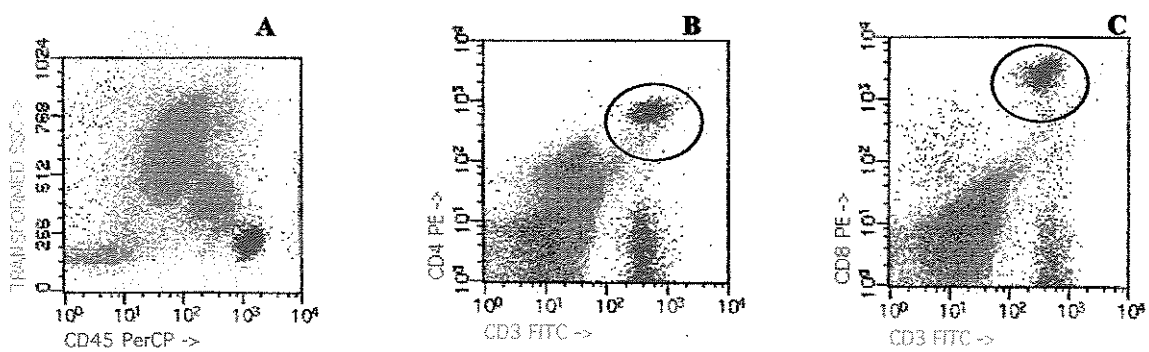


Fig 12. - População de células T em MO normal. **A:** Dot plot CD45/SSC. População de linfócitos maduros com forte IMF para o CD45 e baixa IMF do SSC. **B:** Dot plot CD3/CD4. Identificação das células T helper com a dupla positividade para CD3 e CD4 (círculo). **C:** Dot plot CD3/CD8. Linfócitos T citotóxicos positivos para CD3 e CD8 (círculo).

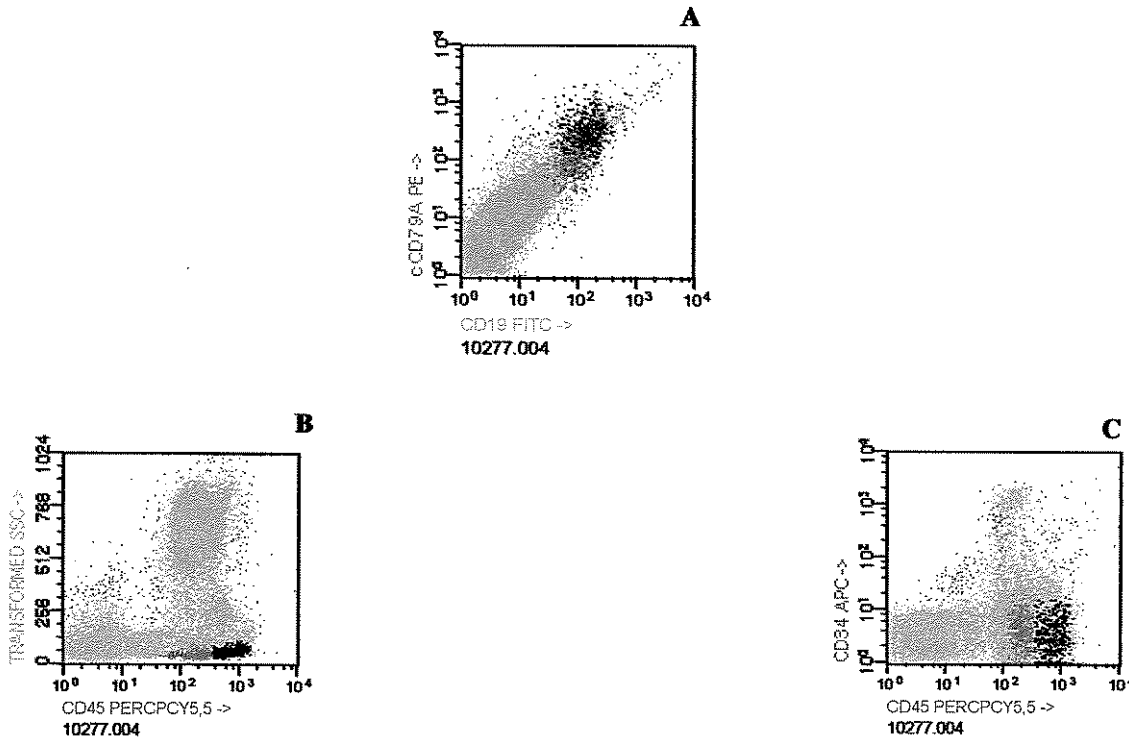


Fig 13. - Dot plots representando as 3 subpopulações de células B em MO normal. População 1 (VERMELHO): precursores de células B, CD34+/CD45^{fraco}; População 2 (AZUL): precursores de células B CD34-, CD34-/CD45^{int}; População 3 (PRETO): linfócitos B maduros, CD34-/CD45^{forte}. **A:** Células B CD19+/cCD79a+; **B**: Dot plot CD45/SSC constando as 3 populações de células B de acordo com a intensidade do CD45 (maturação B) e SSC baixo; **C:** Vizualização das 3 subpopulações B pela intensidade de fluorescência do CD45 e CD34.

Análise da População Blástica

A população dos blastos foi avaliada pelo dot plot CD34 x SSC considerando-se as células fortemente positivas para este marcador de imaturidade e baixo SSC. Número absoluto, porcentagem e IMF das células CD34$^+$ foram analisados. A porcentagem de blastos na MO, quantificada pelo mielograma, também foi considerada e posteriormente correlacionada com o número absoluto de células CD34$^+$.

Análise Estatística

Foi utilizada a análise descritiva para obtenção das médias, medianas, mínimo, máximo e desvio padrão. O teste de Mann-Whitney foi utilizado para comparações entre 2 grupos e o de Kruskal-Wallis entre 3 ou mais. Para correlações usou-se o teste de Spearman. Foram considerados valores significativos quando $p<0,05$. Para todas as análises foram utilizados os programas estatísticos Winstat versão 3.1 e SPSS versão 10.0.

TRABALHOS PUBLICADOS

CYTOMETRY , SUBMETIDO JULHO 2005

DETECTION OF HEMATOPOIETIC MATURATION ABNORMALITIES BY FLOW CYTOMETRY IN MYELODYSPLASTIC SYNDROMES AND ITS UTILITY FOR THE DIFFERENTIAL DIAGNOSIS WITH NON-CLONAL DISORDERS

Elisangela Ribeiro[1], Lilian Suarez [2], Carmen S. P. Lima[3], Konradin Metze[4], Irene Lorand-Metze[1]

[1]Department of Internal Medicine, State University of Campinas, Campinas, São Paulo, Brazil

[2]Centro de Investigación del Cáncer Servicio General de Citometría and Departamento de Medicina, Universidad de Salamanca, Salamanca, Spain

[3]Hematology-Hemotherapy Center, State University of Campinas, Campinas, São Paulo, Brazil

[4]Department of Pathology, State University of Campinas, Campinas, São Paulo, Brazil

Key words: myelodysplastic syndromes, diagnosis, bone marrow, cell maturation, phenotypic aberrations, flow cytometry

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Address for correspondence

Prof Dr Irene Lorand-Metze

Hemocentro – State University of Campinas

P.O. Box 6198

BR - 13081-970 Campinas – SP Brasil

Fax: + 55-19-3788 86 00

Phone number: + 55 19- 3788 87 40

E.mail: ilmetze@unicamp.br

Background: The diagnosis of myelodysplastic syndromes (MDS) is based on peripheral cytopenias, bone marrow (BM) morphology and karyotyping. However, in cases with few dysplastic elements in BM and a normal karyotype, the diagnosis may be difficult.

Methods: We examined the utility of flow cytometric analysis for this differential diagnosis. Quantitative assessment of CD45, CD16, CD13, CD11b, CD10 and CD64 in granulocytes and monocytes and CD71 and glycophorin A in erythroblasts was performed in 32 MDS BM cases and compared with 10 non-clonal disorders (NCD) and healthy controls.

Results and conclusions: At least one abnormality in granulocytic precursors was found in 93% of the patients with MDS. In non-clonal disorders the normal variation of antigen expression along maturation was always maintained. The most important parameter for discrimination between both groups was SSC in CD34+ cells and CD45 in mature granulocytes. Monocytes were increased in both MDS and NCD, but increase in expression of CD16 occurred exclusively in MDS. Several abnormalities in CD71/glycophorin A expression were found in MDS. In NCD this was always normal. In the discriminant analysis, expression of CD16 in monocytes, SSC in CD34+ cells and number of erythroblasts were able to discriminate 90% of the cases. These results should be validated in a larger number of patients.

Key words: myelodysplastic syndromes, diagnosis, bone marrow, cell maturation, phenotypic aberrations, flow cytometry

Myelodysplastic syndromes (MDS) are clonal disorders of the hematopoietic stem cell. Diagnostic criteria are well established, and based on peripheral blood (PB) cytopenias, a cellular bone marrow (BM) with atypias in hemopoietic precursors in and cytogenetics (1-3). However, in cases with only few dysplastic elements in BM and a normal karyotype, the diagnosis may be difficult.

Multiparameter flow cytometry (FCM) has been extensively used to analyze hemopoietic precursors both in normal BM as well as in several diseases (4). Recently, several approaches have been made to examine several features of BM in MDS (5-15).

Abnormal expression of multiple antigens in granulocytic precursors, monocytes and erythroblasts have been reported (7-10) as well as asynchronous expression of antigens related to immature myeloid cells (5,11-14).

Besides analysis of single antigens, an increasing emphasis has been put on the qualitative analysis of the maturation sequence of co-expressions in granulopoiesis, especially in the CD16/CD13 and CD16/CD11b combinations (6-10), and the analysis of individual BM sub-populations gated in the CD45/SSC plot. In most studies, the majority of the patients with MDS present two or more abnormalities. Progression of the disease has been associated with increase of CD34+ cells as well as the number of phenotypic alterations (9,15).

Although in some studies regenerative bone marrow (BM) after chemotherapy (12) and patients with aplastic anemia (6) have been examined, there are few data concerning which features could better discriminate MDS with a normal karyotype from non-clonal disorders with peripheral blood cytopenias (10,15). Therefore, we have investigated the maturation pattern of granulocytes, erythroblasts and monocytes in MDS and non-clonal diseases, in order to search for parameters that could be useful for differential diagnosis between these disorders.

MATERIALS AND METHODS

Patients

We examined prospectively whole BM specimens of newly diagnosed MDS patients that entered our Institution. The diagnosis was made by FAB criteria (1) based on peripheral blood (PB) counts and BM smears stained with May-Grunewald Giemsa and Perls' stain (for iron), considering the cellular atypias in at least two hemopoietic cell lines (2-3) and karyotype. Cases were reclassified according to the WHO proposal (3). Deficiency anemias, renal or hepatic failure, thyroid dysfunction, hepatitis, cytomegalovirus and HIV infections, as well as inflammatory and autoimmune diseases were excluded.

We also collected BM from patients with known non-clonal disorders, including deficiency anemias, hyperesplenism, infection, mielotoxicity and neoplasic disease who had indication of BM aspiration for elucidation of peripheral cytopenias.

In addition, BM from adult donors for allogeneic BM transplantation were used as normal controls. All patients gave informed consent according to the rules of the local's Ethics Comitee.

Flow Cytometric analysis

Immunophenotypic studies were performed using three color combinations of monoclonal antibodies (MoAb). Immunofluorescence staining was made using a standardized direct stain-and-then-lyse-and-wash technique (7). The identification of the different BM granulocytic and monocytic cell compartments was performed on the CD45 vs SSC plot (4). In all samples granulocytic and monocytic cells were identified as being $CD45^{high}$ / SSC^{high} and $CD45^{int}$ / SSC^{int}, respectively (Fig. 1). Immediately after staining, samples were acquired in a FACSCalibur flow cytometer (Becton-Dickinson Biosciences – BDB-San Jose, CA) using the CellQuest software program (BDB). Information on a 30.000 cells was acquired and stored. For data analysis the PAINT-GATE software (BDB) was used.

Monoclonal Antibodies. Direct immunofluorescence staining was performed with isothiocyanate fluorescein (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP). PERCP-CD45 (clone 2D1), PE-CD34 (clone 8G12) and FITC-CD10 (clone W8E7) were purchased from BDB. CD16 FITC (clone DJ130c), CD11b PE (clone 2LPM19c), CD13 PE (clone WM17) and CD64 PE (clone 10.1), CD71 FITC (clone Ber-T9) and CD235a, glycophorin A (GlyA), (clone JC159) were purchased from Dako Corporation (Carpinteria, CA).

Data collection and analysis. CD16/CD11b and CD16/CD13 were used in the present study for the phenotypic characterization of the granulocytic maturation. These combinations permit to distinguish five differentiation stages: myeloblasts, promyelocytes, myelocytes, metamyelocytes/band and mature cells (Fig. 2). Granulocytes were also analyzed in the CD10/CD64 combination (Fig 3). Deficiency of CD10 was examined in mature granulocytes (Fig.3B). The mean fluorescence intensity (MFI) of CD64 observed in the CD64/CD10 combination of granulocytic population was used to quantify the shift to the left (increase of immature forms). Monocytes were gated as shown in Fig 1 and analyzed for expression of CD45, CD11b, CD64, CD13 and CD16. Erythroblasts were gated as a $CD45^{low}$ -/SSC^{low} population. MFI of CD71 and GlyA were analyzed in the CD71/GlyA combination.

The MFI for each individual antigen was obtained in relative linear arbitrary channel units scaled from 0 to 1024 (13). Cells were considered positive for a given antigen when MFI value was higher than 5. MFI was considered altered when values were below or above the mean ± standard deviation of the MFI of each antigen in normal BM.

Statistical analysis

For all variables under study a descriptive analysis was performed. Differences between groups were tested by ANOVA. An univariate linear discriminant analysis was performed to find out which data obtained by flow cytometry could be helpful for the differential diagnosis between MDS and non-clonal disorders. The two diagnostic

categories, MDS or non-clonal disorder as defined by clinical and laboratory examinations represented the dependent variable and the FCM data were considered as independent variables. Since for this method normally distributed data are necessary, logarithmic transformations of the variables were made if necessary, in order to obtain a good approximation to a Gaussian distribution. The goodness-of-fit was evaluated according to the Kolmogorov - Smirnov test. In order to evaluate quality, stability and robustness of the analysis and to obtain a more realistic classification matrix, the leave-one out method ('jack-knife' procedure) was applied. This method was regarded as the method of choice considering our relatively small sample sizes (16-19). The WINSTAT 3.1 and SPSS 8.0 softwares were used for all calculations.

RESULTS

Thirty-two MDS cases were included in the study. There were 16 males and 16 females; median age: 61.5 years (18-93). According to the FAB criteria 20 cases had refractory anemia (RA), 5 RA with sideroblasts (RARS) and 7 RA with excess of blasts (RAEB). According to the WHO classification, 6 had RA, 11 refractory cytopenia with multilineage dysplasia (RCMD), 5 refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS), 2 refractory anemia with excess of blasts (RAEB-1), 5 refractory anemia with excess of blasts (RAEB-2) and 3 were unclassified. In 25/32 (78%) cases a normal karyotype was found; 2 patients presented 45,X,-Y, 1 had a complex alteration and 1 presented 5q- abnormality.

Ten cases of non-clonal disorders were analyzed: 3 patients with megaloblastic anemia, 2 with combined deficiency anemia (iron and folate), 1 non-Hodgkin lymphoma (BM not involved), 1 had hyperesplenism, 1 hepatic failure and 2 patients had a drug-induced mielotoxicity. Median age was 54 years (28-70). In 3 of them, the karyotype was performed and was normal.

In addition, 11 BM donors for allogeneic transplantation, corresponding to healthy BM were included as controls.

Flow cytometric analysis

Granulocytic population

The MFI of all analyzed parameters, separated by cell type of the granulocytic cell line in normal BM, MDS patients as well as those with non-clonal disorders are shown on Table 1. Comparing these parameters as whole groups (normal, MDS and non-clonal disorders) the only significant difference was found in SSC MFI of myeloblasts (p=0,003). Among MDS patients, only 2 cases with RA presented a normal expression for all antigens examined in the whole granulocytic population (Table 2). In 7 one abnormality was found, in 9 patients there were 2 abnormalities, in 9 there were 3 and in 2 patients 4 abnormal expressions were found. In addition, deficiency of CD10 expression in mature granulocytes was found in 3 patients. These abnormalities were equally found in all FAB or WHO categories. The maturation pattern observed in the CD16/CD11b in plot was altered in 18/32 patients and that visualized in the CD16/CD13 plot was abnormal in 20/30 patients (Fig.4).

Myeloblasts were analyzed in BM smears, in the CD16/CD11b and CD16/CD13 plots (Fig.2), and CD34 positive cells were quantified (Table 3). In MDS patients, there was a significant correlation between percentage of blasts counted on BM smears and those found in the CD16/CD13 combination ($r = 0.38$; $p = 0.002$) as well as the number of CD34 positive cells ($r = 0.35$; $p = 0.03$), but not with those quantified in the CD16/CD11b plot.

Among the patients with non-clonal disorders, the sequence of the variation in the expression of each of the antigens examined was always similar to that found in normal controls (Table1). However, when the MFI of the whole granulocytic population was examined, expression of CD45 was decresed in 5 cases. Expression of CD11b was increased in 5 cases. MFI of SSC was always in the same range as in normal controls.

Monocytes

An increase in number of monocytes, when compared to normal BM, was found both in MDS patients and in those with non-clonal disorders (Table 4). SSC and expression of CD11b and CD13 was similar in all three groups. However, that of CD64 was increased in MDS as well as in non-clonal disorders. That of CD16 was only increased in MDS. MFI of CD45 was decreased, more pronouncedly in non-clonal disorders.

The number of patients presenting abnormal antigen expression is presented in Table 5. Immunophenotypic alterations were observed in 22 cases (68.7%) of patients with MDS. Partial loss of expression of CD45 was found in 4 cases, of CD11b in 7 cases, of CD13 in 15 and CD64 in 17 cases.

In patients with non-clonal disorders, a decreased expression of CD45 was observed in 7 cases, that of CD11b in 4, and that of CD13 in only one case. None of these patients presented deficiency of CD64. Comparing both groups, MDS patients had a significantly higher frequency of these alterations only for CD13 ($p=0.04$) and CD64 ($p=0.02$).

Erythroblasts

The erythroid cell line was analyzed in the CD71/GlyA combination (Fig. 5). A double positive expression was found in all controls and also in all cases with non-clonal disorders. In MDS, 20 patients presented an abnormal pattern (Tables 6 and 7). A partial loss of CD71 expression was found in 5 cases, a deficiency in expression of both antigens in 5 cases. In 10 patients, 2 populations with different antigen expression were found: CD71+-/GlyA+- and CD71+/GlyA+- in 3 cases; CD71+-/GlyA+- and normal: 3 cases; CD71neg/GlyA+ and normal: 2 cases; CD71neg/GlyA neg and normal: 1 case; CD71+-/GlyA+- e CD71 heterogeneous/GlyA+: 1 case. Underexpression of GlyA was not found in any case examined.

In the discriminant analysis, the parameter which best separated both diagnostic groups was MFI of CD16 in monocytes. This parameter permitted a correct diagnosis in 82,9 % of all cases, followed by MFI CD45 of mature granulocytes with 77.5%. Both results showed a high internal stability, since the percentages were equal after the jackknife procedure.

The number of erythroblasts was able to separate 77,5 % of the cases and SSC of CD34+ cells separated 76.5 %. But there was less stability for these variables, since the percentages fell to 75%, and 67.5 % respectively after the leave-one–out procedure.

Combining MFI CD16 of monocytes and number of erythroblasts, the algorithm obtained 90.0% (90.0%) of correctly classified cases. When adding MFI of SSC of CD34+ cells as a third variable the value rose to 92.5 %, but fell again to 90.0% after the leave-one out procedure. Therefore, in terms of parsimony and stability the combination of MFI CD16 of monocytes and number of erythroblasts separated best both diagnostic groups.

DISCUSSION

In the present study, we performed immunophenotyping in the diagnostic work-up of patients with MDS using a relatively small panel of antibodies in a 3-color combinations, analyzing separately the granulocytic cell line, blasts, monocytes and erythroblasts gated in the CD45/SSC plot. A quantitative analysis of expression of each antigen in the several cell types along the myeloid maturation was also performed. This technique showed to be reproducible in normal BM and permitted to make a quantitative description of the maturation pattern, as has been found in previous works (6-8,12). The findings of patients with MDS were compared with those of normal BM and disclosed several changes in the maturation pattern as well as abnormal expression of individual antigens in different cell types. Previous studies have called attention to the fact that number of abnormalities increase with progression of disease (9,14,15) or correlate with IPSS (10). In our patients, these abnormalities were evenly distributed in all FAB or WHO

types. This may be due to the small proportion of patients with RAEB and those with abnormal karyotype in our study. Blasts were examined in BM smears and in the CD16/CD13 and CD16/CD11b plots as well as by quantification of CD34+ cells. A good correlation was found between these and blstas analysed in the CD16/CD13 combination but not in the CD16/CD11b one. In this combination, increase in CD11b along maturation is very gradual making it difficult to separate consistently the blast population. Although some over- and underexpressions of single antigens, mainly those of CD45 and CD11b could be observed in non-clonal disorders, the normal sequence of variation of each antigen along maturation within the granulocytic cell line was always maintained. Therefore, this parameter was useful to distinguish clonal and non-clonal disorders. The most important parameters distinguishing both groups were low SSC of CD34+ cells and decreased expression of CD45 in non-clonal disorders. Underexpression of CD10 in mature granulocytes was infrequent among our patients as has also pointed out by others (9,15,20,21).

The analysis of monocytes was the most important discriminant factor between MDS and non-clonal disorders. They were increased in both groups when compared to normal BM, more pronouncedly in MDS. This is probably an unspecific finding observed in inflammatory processes and increased phagocytosis that occurs in BM both in MDS and non-clonal disorders. Several phenotypic abnormalities have been described in monocytes in MDS and chronic myelomonocytic leukemia (5-9,22), mainly partial or total loss of antigen expression. We found abnormalities of expression in CD64, CD13 and CD11b. Underexpression of CD13 is characteristic of aberrant phenotype or maturational arrest within the monocyte compartment in MDS (22,23). However, among the antigens examined, the expression of CD16 was exclusively increased in this group. Inflammatory and infectious diseases seem to promote phenotypic alterations in myelomonocytic cells (23,24). Surface molecules, such as CD64 and CD11b, can be modulated in vitro by a variety of agents, e.g. cytokines (interferon-γ) or granulocyte-macrophage colony stimulating factor (GM-CSF), complement split products as well as by stimulation of Fcγ receptors (FcγR) and receptors for microbial wall components such as lipopolysaccharides (23-25). Therefore, these factors may stimulate the alterations found of CD64 and CD11b

on granulocytes and monocytes of patients with non-clonal disorders. CD16 was highly increased in the cells gated in the monocytic region in MDS but not on the non-clonal disorders and controls. CD16 is one of the markers that also identify dendritic cells (DCs). Therefore, this increase in CD16 expression may indicate a shift to production of DCs in MDS.

The erythroid cell line was altered in 64% of the patients with MDS. The CD71/GlyA combination was useful to detect several types of abnormalities. However, in the non-clonal disorders no qualitative abnormalities could be detected. The number of erythoblasts was an important hallmark to discriminate between MDS and non-clonal disorders.

Taking into account all the features above described, we can conclude that flow cytometry, used for analysis of maturation of hematopoietic cell lines in BM is useful for the diagnosis of MDS and permits to discriminate between these clonal disorders and non-clonal diseases presenting peripheral blood cytopenias. These results should be validated in a larger patient sample.

Enclosing, these phenotypic characteristics, observed by FCM technique can contribute to make a differential diagnosis between MDS clonal disease with normal karyotype and pancytopenias diseases. Although FCM technique have been very explored and seems to be a useful tool, an association with bone marrow and peripheral blood analysis, and exclusion protocol is important to conclude MDS diagnosis.

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Table 1

Granulocytic Maturation in Patients with MDS According to FAB Criteria Compared to Controls and Non-clonal disorders

	Myeloblasts	Promyelocytes	Myelocytes	Metamyelocyte/Band	Mature Granulocytes
Controls					
MFI SSC	761,6	761,4	657,2	721,6	779,5
MFI CD45	137,5	146,1	180,6	237,4	452,8
MFI CD16	15,9	14,8	18,1	142,1	757,5
MFI CD11b	9,4	63,9	349,0	481,7	913,9
MFI CD13	156,9	24,3	10,9	20,7	78,4
RA					
MFI SSC	713,8	664,9	638,4	706,8	744,6
MFI CD45	126,6	118,2	168,6	227,7	480,3
MFI CD16	48,3	21,4	108,8	281,6	964,9
MFI CD11b	114,7	80,7	387,8	603,2	875,3
MFI CD13	236,1	63,9	21,4	49,4	228,4
RARS					
MFI SSC	662,9	635,5	586,4	658,5	694,1
MFI CD45	106,0	126,3	248,9	308,9	402,6
MFI CD16	12,3	9,9	11,6	109,2	760,8
MFI CD11b	9,3	66,4	432,1	594,7	680,7
MFI CD13	471,1	73,1	10,6	45,9	232,5
RAEB					
MFI SSC	666,7	574,8	653,1	688,5	698,4
MFI CD45	173,2	117,1	281,3	335,3	445,1
MFI CD16	41,4	11,4	16,2	151,8	922,1
MFI CD11b	299,9	69,7	739,5	1207,1	1340,7
MFI CD13	267,9	63,4	68,2	89,3	149,8
Non-clonal disorders					
MFI SSC	721,3	735,4	673,9	722,5	765,8
MFI CD45	99,7	122,8	165,9	177,6	281,1
MFI CD16	14,0	14,2	19,7	158,0	902,4
MFI CD11b	10,4	67,8	413,9	565,9	939,6
MFI CD13	247,1	45,9	8,1	25,3	122,5

Table 2

Antigen Expression in the Granulocytic Population in Normal Bone Marrow, MDS, and Non-Clonal Disorders

	RA (n=20)			RARS (n=5)			RAEB (n=7)			Non-Clonal Disorders (n=10)		
	N	↓	↑	N	↓	↑	N	↓	↑	N	↓	↑
MFI SSC	16	4	0	2	2	0	5	1	0	8	0	2
MFI CD45	9	7	4	2	1	1	3	0	4	3	5	2
MFI CD16	10	1	8	4	0	0	5	0	1	8	0	2
MFI CD11b	11	2	7	0	2	2	0	3	3	3	2	5
MFI CD13	7	2	9	0	0	5	1	1	5	7	0	3
MFI CD10	13	3	3	3	0	1	5	0	1	9	1	0
MFI CD64	8	0	11	2	0	2	1	1	5	6	1	3

N MFI within normal range; ↑ overexpression; ↓ underexpression

*These parameters could not be analyzed in one case of RARS and one of RAEB. ** Not performed in two cases with RA. *** not performed in one case each type of MDS; **** Not performed in one case with RA and one of RARS.

Table 3

Analysis of myeloblasts by different antigen combinations

	Controls	Non-Clonal Disorders	MDS	P value*
Nr Mb CD16/CD11b	1297 ± 270	1005 ± 392	968 ± 551	0.17
SSC Mb CD16/CD11b	761.6 ± 34.8	721.3 ± 28.2	697.6 ± 10.7	0.08
Nr Mb CD16/CD13	600 ± 446	303 ± 125	323 ± 316	**0.05**
SSC Mb CD16/CD13	783.2 ± 35.7	726.8 ± 42.4	285.8 ± 199	0.03
nr CD34	227 ± 59	250 ± 127	431 ± 645	0.41
SSC CD34	251.8± 11.8	247 ± 8.15	814.5 ± 570	**0.006**
MFI CD34	541.6 ± 46.3	652.4 ± 59.1	17.7 ± 8	0.57

nr Mb: number of myeloblasts.

* in ANOVA

Table 4

Antigen Expression in Monocytes of MDS and Non-Clonal Disorders Compared to Controls

	Controls	**Non-Clonal Disorders**	**MDS**	**P value***
Absolute number	469.2 ± 195	949.4 ± 495	1189.7 ± 860	**0.001**
MFI CD45	698.0 ± 227	415.7 ± 288	596.3 ± 398	**0.02**
MFI CD13	142.7 ± 86	275.4 ± 422	285.8 ± 199	0.07
MFI CD64	134.5 ± 61	207.8 ± 113	253.4 ± 154	**0.04**
MFI CD11b	771.8 ± 170	625.9 ± 401	814.5 ± 570	0.36
MFI CD16	10.2 ± 5	10.9 ± 9	17.7 ± 8	**0.001**

* in ANOVA

Table 5

Abnormalities in Antigen Expression in Monocytes in MDS and Non-Clonal Disorders

	RA (n=20)			**RARS (n=5)**			**RAEB (n=7)**			**Non-Clonal Disorders (n=10)**		
	N	↑	↓	N	↑	↓	N	↑	↓	N	↓	↑
MFI CD45	8	9	2	3	0	1	0	5	1	2	7	1
MFI CD11b	9	6	5	1	2	1	1	4	1	5	4	1
MFI CD13	11	1	8	3	0	2	2	0	5	7	1	2
MFI CD64	7	0	11	1	0	3	3	0	3	4	0	6

N MFI within normal range; ↑ overexpression; ↓ underexpression

*These parameters could not be performed in one case each type of MDS; Not performed in one case of RARS and one of RAEB. *** Not performed in two cases with RA and one case with RARS and RAEB.
MFI: Mean Fluorescence Intensity

Table 6

Parameters of the erythroblastic population in patients with MDS and non-clonal disorders compared to controls

	Control	MDS	Non-Clonal Disorders	P value*
Nr EB	2417±664	3124±2461	4909±2629	**0.04**
MFI SSC	176.2	205.4	211.1	0.21
MFI FSC	314.2	297.5	303.0	0.42
MFICD71	1730.1	1950.2	3179.2	0.38
MFI GlyA	144.1	331.9	578.2	**0.04**

Nr EB: total erythroblasts **SSC:** side-scatter; **FSC:** forward-scatter; * in ANOVA

Table 7

Overwiew of all Immunophenotypic Abnormalities found in MDS and Non-Clonal Disorders

	MDS (%)	Non-Clonal Disorders (%)
Granulocytic Population		
low SSC	7/30 (23)	0/10 (0)
Abnormal CD11b expression	17/30 (56)	7/10 (70)
Abnormal CD13 expression	22/30 (73)	3/10 (30)
Abnormal CD16 expression	11/30 (36)	2/10 (20)
Abnormal CD45 expression	17/30 (56)	7/10 (70)
CD16/CD11b pattern abnormal	18/32 (56.2)	0/10 (0)
CD16/CD13 pattern abnormal	20/30 (66)	0/10 (0)
Deficiency CD10 on mature granulocytes	3/29 (10.3)	1/10 (10)
Monocytic Population		
Underexpression of CD11b on monocytes	12/30 (40)	4/10 (40)
Underexpression of CD13 on monocytes	1/30 (3.3)	1/10 (10)
Overexpression of CD16 on monocytes	14/32 (43.7)	1/10 (10)
Erytroblastic Population**		
Abnormal CD71/GlyA pattern	20/31 (64.4)	0/10 (0)
Partial loss of CD71	5/31 (16.1)	0/10 (0)
Presence of 2 populations	10/31(32.2)	0/10 (0)
Loss of both antigens	5/31 (16.1)	0/10 (0)

* CD64+/CD10- combination

**When GlyA was normal only one case had abnormal expression in CD71

FIGURE 1

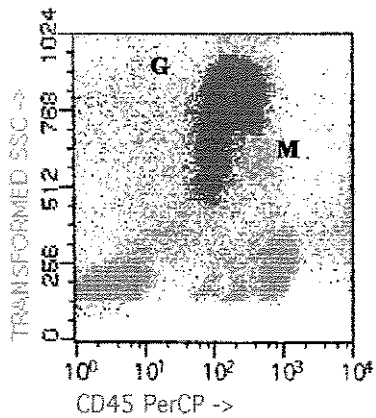


FIGURE 2

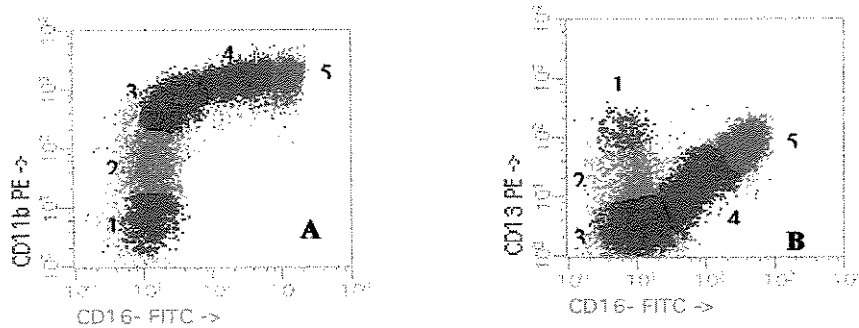


FIGURE 3

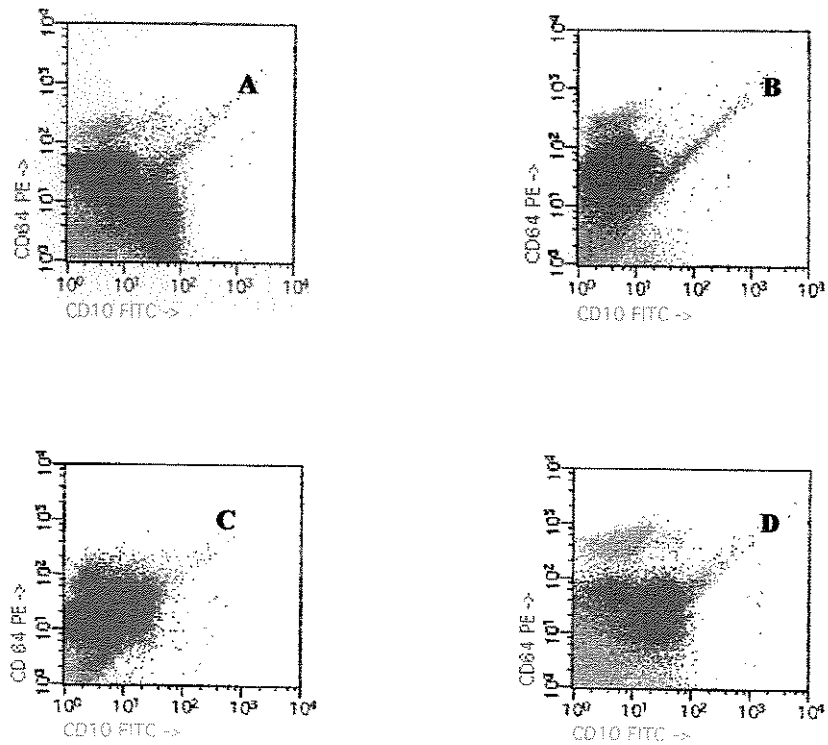


FIGURE 4

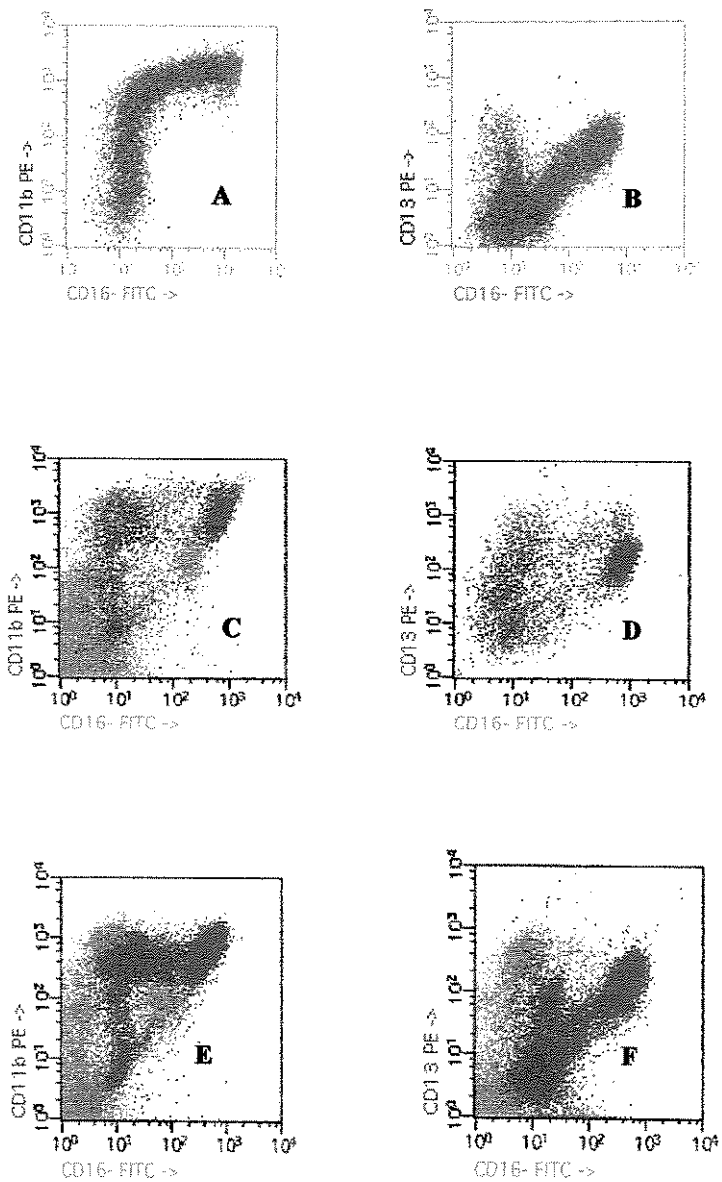
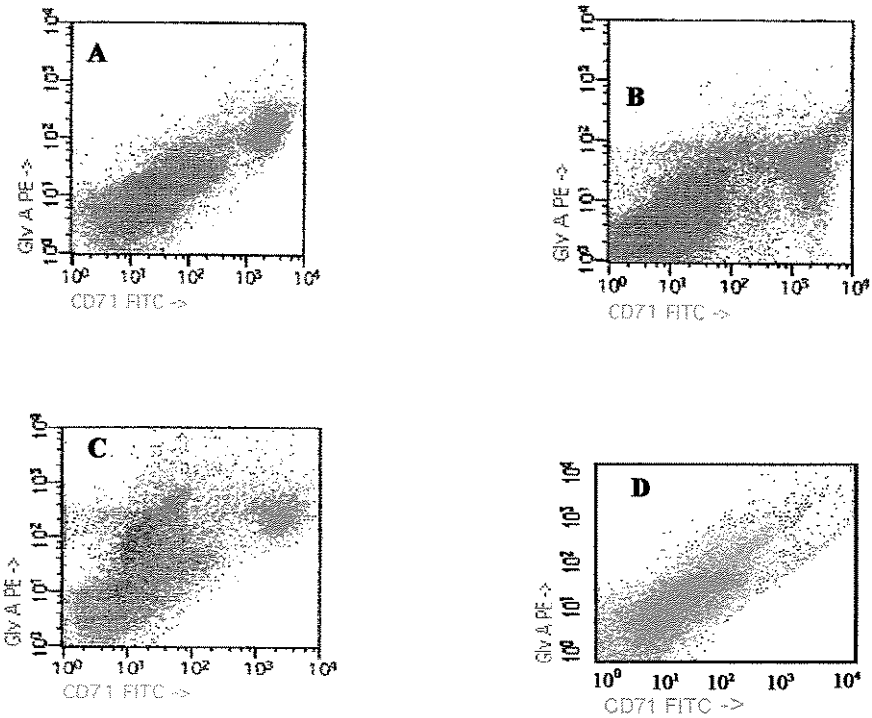

CD11b PE ->
CD16- FITC ->
A
CD13 PE ->
CD16- FITC ->
B
CD11b PE ->
CD16- FITC ->
C
CD13 PE ->
CD16- FITC ->
D
CD11b PE ->
CD16- FITC ->
E
CD13 PE ->
CD16- FITC ->
F

FIGURE 5



LEGENDS TO FIGURES

FIGURE 1. G: Granulocytic population. High light scatter (SSC^{high}) in combination with $CD45^{int}$ expression. M: Monocytic population. CD45 $^{low\text{-}high}$ combined with a SSC ^{low}.

FIGURE 2. Pattern of antigen expression in normal BM. **A:** CD16/CD11b/CD45. **B:** CD16/CD13/CD45. These combinations permit to distinguish five differentiation stages of granulocytic maturation. **1**: Myeloblasts, **2:** Promyelocytes, **3:** Myelocytes, **4:** metamyelocytes/band forms, **5:** mature neutrophils.

FIGURE 3. CD10 / CD64 plot of the granulocytic population. **A:** normal pattern of expression (normal BM). **B:** Deficient expression of CD10 on mature neutrophils in a patient with RA. **C:** increase in immature granulocytes in a patient with RA. **D:** Predominance of mature neutrophils RCMD.

FIGURE 4. Abnormalities in antigen expression of granulocytes in MDS and non-clonal disorders detected by the analysis of CD16/CD11b and CD16/CD13 plots. **A:** Normal pattern of CD16/CD11b. **B:** Normal pattern of CD16/CD13. **C and D**: Abnormal pattern in a patient with RCMD-RS. **E and F:** normal pattern in a patient with a combined deficiency anemia.

FIGURE 5. Patterns of expression found in the CD71/GlyA plot. **A:** normal pattern. **B** decreased expression in Gly A and variable expression of CD71 in a case of RAEB-1. **C**. two populations in a case with RCMD. **D**. double deficiency in a patient with RAEB-2.

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Maturation-associated immunophenotypic abnormalities in bone marrow B-lymphocytes in myelodysplastic syndromes

Elisangela Ribeiro[a,1], Sergio Matarraz Sudón[b,1], María de Santiago[b], Carmen S.P. Lima[a], Konradin Metze[c], Manuel Giralt[d], Sara T.O. Saad[e], Alberto Orfao de Matos[b], Irene Lorand-Metze[e,*]

[a] *Hematology and Hemotherapy Center, State University of Campinas, Brazil*
[b] *Centro de Investigación del Cáncer Servicio General de Citometría and Departamento de Medicina, Universidad de Salamanca, Salamanca, Spain*
[c] *Department of Pathology, Faculty of Medicine, State University of Campinas, Brazil*
[d] *Department of Hematology, Hospital Miguel Servet, Zaragoza, Spain*
[e] *Department of Internal Medicine, Faculty of Medicine, State University of Campinas, Rua Carlos Chagas 480, P.O. Box 6198, BR 13081 970 Campinas, Sao Paulo, Brazil*

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Abstract

Recent studies concerning the pathophysiology of myelodysplastic syndromes (MDS) have shown evidences for the existence of complex interactions between hematopoietic stem cells and the bone marrow (BM) microenvironment. We analyzed the B-lymphocyte maturation in BM of patients with MDS. For this purpose, 41 newly-diagnosed patients were analyzed. Enumeration and characterization of CD34+ and CD34− B-cell precursors and mature B-lymphocytes was performed using multiparameter flow cytometry. BM from eight transplant donors and six orthopedic surgery patients were used as controls. CD34+/CD45^{lo} B-cells were found in 17/22 patients with RA/RARS and in 5/13 with RAEB. In patients with RAEB-t and CMML no CD34+ B-cell precursors could be detected. A positive correlation was found between CD34+ and CD34− B-cell precursors ($r = 0.52$). CD34+ B-cell precursors presented an inverse correlation with BM percentage of blasts and peripheral leukocytes and a positive one with hemoglobin. Asynchronous antigen expression (CD19+/CD79a− cells) was found in 7/11 cases of RA/RARS and 6/18 cases of RAEB in which this phenotype was examined. Abnormal patterns of expression for at least one antigen was found in 91% of RA/RARS cases and in 74% of RAEB. Underexpression of TdT and CD79a were the most frequent abnormalities. Our results present evidences of an abnormal B-cell maturation in MDS. This may be an evidence that B-lymphocytes are derived of the abnormal clone. But it may also be the consequence of influences of abnormalities of BM microenvironment leading to an impaired commitment and maturation of the B-cell line in MDS. Studies performed with purified well-characterized B-cells may further elucidate these abnormalities.

Keywords: Myelodysplastic syndromes; Bone marrow; B-cell maturation; Flow cytometry

1. Introduction

Myelodysplastic syndromes (MDS) are a group of clonal disorders originating from myeloid hematopoietic stem cells [1]. Despite this, accumulating evidence indicates that the neoplastic event in MDS could target a more immature stem cell, which still retains the ability to differentiate into both the myeloid and lymphoid lineages [2–7]. On the other hand, progression of MDS into acute lymphoblastic leukemia (ALL) although rare, has been documented [3,8–11]. ALL has been diagnosed mainly by phenotypic features, more frequently by the presence of TdT, HLA-DR but also by the expression of CD19 and CD10. In some cases, IgH rearrangement has also been documented [3,9]. In others, the same cytogentetic abnormalities found in the

* Corresponding author. Tel.: +55 19 37888740; fax: +55 19 37888600.
E-mail address: ilmetze@unicamp.br (I. Lorand-Metze).
[1] Both authors have equally contributed to this work and should be considered as first authors.

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original MDS clone, were also observed in blasts of ALL [10,11].

More recently, several groups have attempted to demonstrate the clonal nature of lymphoid cells in MDS through the identification of common genetic abnormalities in myeloid precursors, multipotent stem cells and lymphoid cells. Presence of circulating clonal B-lymphocytes was observed in 5/68 cases of MDS analyzing the hypervariable region CDR3 of the immunoglobulin heavy chains [2]. White et al. [4] demonstrated the presence of 20q deletions in a multipotent precursor of both myeloid cells and B-cells. In keeping with these results, Nilsson et al. [5] studied 10 patients with MDS with del(5q) and showed that this cytogenetic abnormality was also present in mature B-lymphocytes (one of 10 cases studied) as well as CD34+/CD19+ B-cell precursors (three cases). In contrast, Saitoh et al. [6] could not demonstrate the presence of trisomy 8 neither in lymphoid cells nor in CD34+/CD90+ hematopoietic stem cells in a group of seven MDS patients carrying this cytogenetic abnormality in the neutrophil, monocytic and erythroid compartments. More recently, Nilsson et al. [7], suggested that these apparently discrepant results could be related to the fact that trisomy 8 is a secondary cytogenetic change which is usually restricted to the more mature myeloid committed stem cell progenitors, while other primary cytogenetic abnormalities such as 5q− would involve more immature CD34+/CD38−/CD90+ stem cells with the capacity to differentiate into both the myeloid and lymphoid lineages. Additional indirect evidence of the involvement of lymphoid cells in MDS has been found by Amin et al. [12] who have shown that BM B-cells, but not T-lymphocytes, have increased apoptosis. Increased susceptibility to undergo apoptosis of BM myeloid precursor cells from MDS patients, particularly among the low risk cases [13–16] has been described by several authors. Recent reports have shown the existence of a high number of phenotypic aberrations on different myeloid compartments of BM cells in MDS [16–19].

In a preliminary work, we have found a decreased number of B-lymphocytes in MDS patients [20] especially in RAEB. However, to the best of our knowledge, no study has been reported so far in which the distribution and immunophenotype of the different compartments of maturing B-cells has been analyzed in detail in a large series of MDS patients. Our aim was to analyze the distribution and immunophenotypic characteristics of the different maturation-associated compartments of BM B-cells in patients with MDS, in order to identify the presence of numerical and phenotypic abnormalities that would support the neoplastic nature of B-cells.

2. Materials and methods

2.1. Patients and samples

A total of 41 patients: 23 males, 18 females with mean age of 60 years (20–93) with newly diagnosed MDS classified according to the FAB classification [21] were included. From these patients, 12 were diagnosed at the Hematology Center of the State University of Campinas (Brazil) and 29 at the Cytometry Service (University of Salamanca, Spain). According to the FAB classification, 20 cases had refractory anemia (RA), 2 RA with sideroblasts (RARS) (22 low risk cases), 13 RA with excess of blasts (RAEB), 2 RAEB in transformation (RAEB-t) and 4 chronic myelomonocytic leukemia (CMML) (19 high risk cases). IPSS was not performed as karyotype was not available is all cases and was frequently normal. Instead, in order not to split the 41 cases in too many categories and lower too much the statistic power of the tests performed, the correlations between the bone marrow (BM) B-cell populations and the peripheral blood (PB) counts and BM blasts were analyzed as continuous variables.

In each patient, a BM aspirate was obtained for diagnostic purposes and used for flow cytometry immunophenotypic studies described below, according to the guidelines of the Local Ethics Committees. In addition, 14 BM samples from normal individuals (donors of allogeneic BM transplantation—eight cases or patients of orthopedic surgery—six cases) were analyzed as normal BM controls. All subjects gave their informed consent to participate in this study.

2.2. Flow cytometry immunophenotypic studies

In all cases immunophenotypic studies were performed using three- and four-color combinations of monoclonal antibodies (MAb). Immunofluorescence staining was performed using a standardized direct immunofluorescence stain-and-then-lyse-and-wash technique [22,23] combined with fixation and permeabilization for the detection of intracellular antigens. For the identification of the different BM B-cell compartments, CD45 (clone HLE-1); Becton-Dickinson Biosciences (BDB) San Jose, CA together with CD19 (clone HIB19, BDB) were used (Fig. 1). Other markers combined with CD19 and CD45 used in the present study for the phenotypic characterization of BM B-cells included: CD34 (clone 8G12), CD16 (clone NKP15) and HLA-DR (clone L243) purchased from BDB; CD3 (clone VCHT1), CD4 (clone MT310), CD8 (clone DK25), CD79a (clone HM57) and TdT (clone HT-6) purchased from DakoCytomation (Glostrup, Denmark). Immediately after staining, samples were acquired in a FACSCalibur flow cytometer (BDB) using the CellQuest software program (BDB). The following combinations of antibodies were used: CD19/cCD79a/CD45/CD34, TdT/cCD79a/CD45/CD34 and CD/cCD79a/CD45/CD34.

Information on a minimum of 300,000 cells (corresponding to all nucleated BM cells) was acquired and stored. At the two participating centers, identical instrument set-up and calibration procedures were used. In addition, comparison of the light scatter and immunophenotypic characteristics of the different B-cell subsets in normal bone mar-

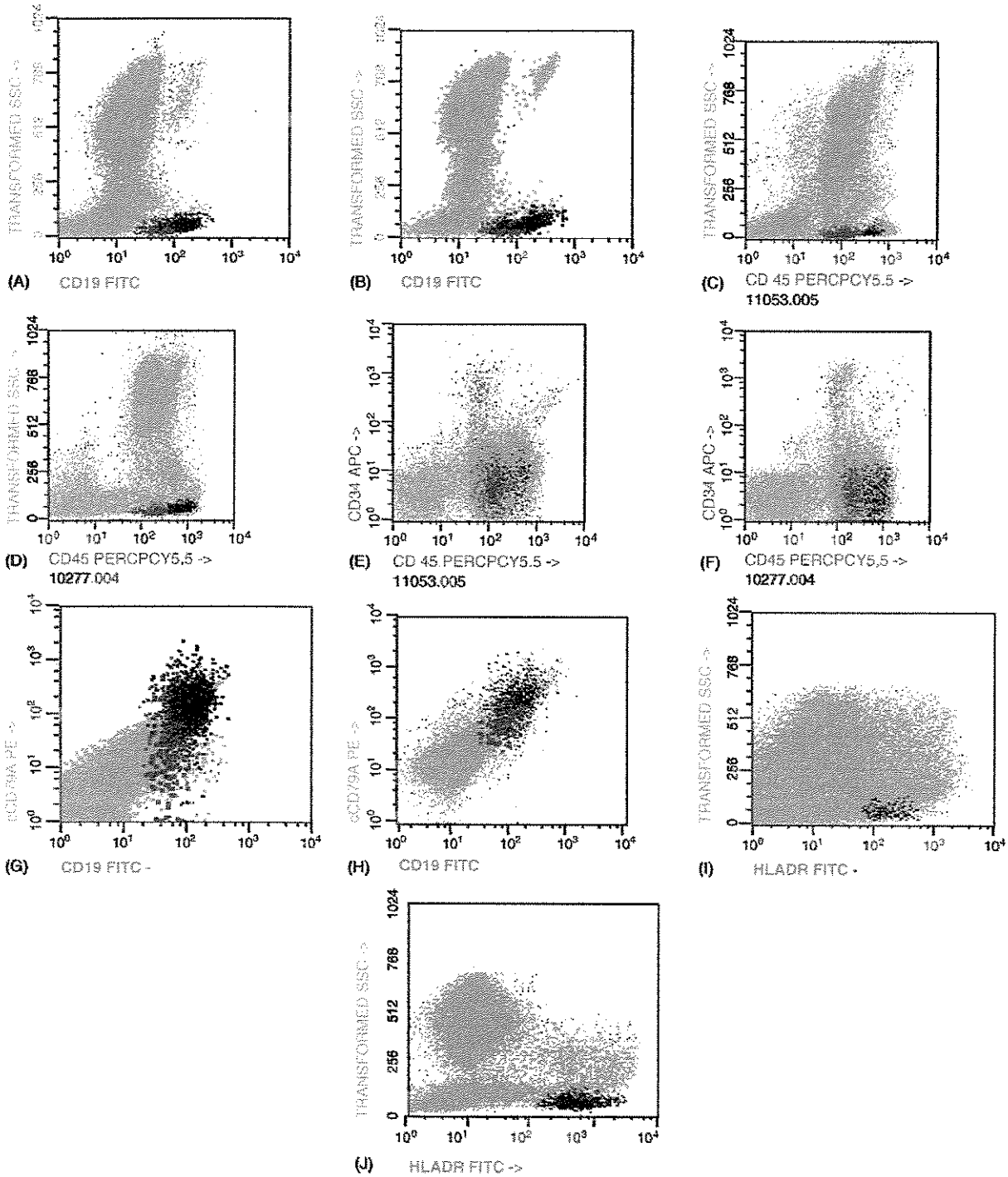

Fig. 1. Dot plots illustrating the different B-cell sub-populations (CD19+ gate) which were identified in the bone marrow (BM) of a normal control (panels B, D, F, H and J) and a patient with MDS (panels A, C, E, G, and I). In all dot plots, CD19+/CD45^{lo}/CD34+ and CD19+/CD45^{int}/CD34− B-cell precursors are painted red and blue, respectively. Mature B-lymphocytes are painted black. Grey events in all dot plots correspond to all other BM cells. Dot plot G: decreased MFI of cCD79a in immature and intermediary B-cells of a patient compared to control (H). Dot plot I: decreased MFI of HLA-DR in mature-B-cells of a MDS patient compared to control (J).

row samples was performed showing comparable results for the cases analyzed at each of the two centers ($p > 0.05$). The PAINT-A-GATE software (BDB) was used for data analysis.

In all samples, B-cells were identified as being either CD19+ or cCD79a+ and having low sideward light scatter (SSC) as shown in Fig. 1. Among B-cells, three different subsets were identified in a CD45/SSC and CD45/CD34 bivariate dot plots which included, according to their degree of maturation: (1) early CD34+/CD45^{lo} B-cell precursors; (2) CD34−/CD45 intermediate (int) B-cell precursors; and (3) CD34−/CD45^{hi} mature B-lymphocytes. For the phenotypic characterization of each of these B-cell populations their mean light scatter intensity as well as the mean fluorescence intensity (MFI) obtained for each individual antigen, both expressed in relative linear arbitrary channel units scaled from 0 to 1024 were used. Based on the staining observed for isotype-matched negative controls, cells were considered to be positive for a given antigen once MFI values higher than 5 were found.

2.3. Statistical analysis

For all variables under study, their median and range values were calculated. The differences observed between groups was assessed using X^2 or the Kruskal–Wallis tests when suitable. p-values < 0.05 were considered to be associated with statistical significance. Correlations were examined by the Spearman's test. We calculated partial correlation coefficients to examine interdependency of variables. This method allows to eliminate the influence of one variable on the correlation between two other variables, keeping the first variable constant [24]. The Winstat and SPSS softwares (SPSS 10.0, Chicago, IL) were used.

3. Results

3.1. Distribution of BM B-cell maturation compartments

The distribution of the different B-cell maturation compartments in BM of low risk (LR) and high risk (HR) MDS patients as compared to normal BM are shown on Table 1. For each individual, the sum of the percentages of all three compartments was 100%. Concerning normal BM, early CD34+/CD45^{lo} cells were found in 11/14 cases. However, in RA/RARS they were found in 17/22 cases. In RAEB, only 5/13 cases presented these cells. None of the RAEB-t and CMML cases presented CD34+ B-precursor cells. This frequency was significantly different (in X^2 $p < 0.0001$).

All normal BMs presented cells in the intermediate and mature compartments. CD34− intermediate cells were found in all cases of RA/RARS but only in 10/13 patients with RAEB and 3/6 patients with RAEB-t/CMML cases.

Considering only cases who presented CD34+ B-cell precursors (population 1), their percentage was similar in RA/RARS but were increased in the five cases of RAEB in which these cells were detected (Table 1). The CD34− B-cell precursors (population 2) were also similar in normal BM and RA/RARS but was decreased in RAEB. A positive correlation was found between populations 1 and 2 ($r = 0.52$). A positive correlation was found between percentage and MFI of FSC in CD34+ B-cell precursors ($r = 0.79$) as well as in CD34− B-cells ($r = 0.43$).

CD34+ B-cell precursors showed an inverse correlation with BM percentage of blasts ($r = -0.28$) and with PB leukocytes ($r = -0.43$). A positive correlation was found with hemoglobin ($r = 0.38$). In the partial correlation, maintaining MFI of FSC constant, all these correlations lost their significance.

Table 1
Distribution of subsets of B-cell maturation compartments in BM of low risk (LR) and high risk (HR) MDS patients as compared to normal BM as median among cases that presented cells in each compartment

B-cell subset	NBM ($n = 14$)	Low risk MDS ($n = 22$)	High risk MDS ($n = 19$)	p-value
CD34$^+$ B-cell precursors[a]				
Percent of B-cells	4.5 (1.8–15.9)	4.5 (1.2–19.2)	11.9 (5.3–46.1)	0.01
Percent of all BM cells	0.11 (<0.01–0.23)	0.15 (<0.01–0.73)	0.25 (0.13–0.6)	0.01
CD34− B-cell precursors[b]				
Percent of B-cells	29 (7.35–80.1)	19.5 (4.5–83.5)	10.4 (5.3–66.4)	<0.001
Percent of all BM cells	0.62 (0.15–1.07)	0.74 (<0.01–3.7)	<0.01 (<0.01- 2.5)	<0.001
Mature B-lymphocytes[c]				
Percent of B-cells	72.1 (50.4–98.2)	77.4 (25.7–100)	92.0 (4.8–100)	0.01
Percent of all BM cells	1.47 (0.3–3.3)	1.5 (0.1–6.3)	0.8 (<0.01–6.1)	0.10
CD19+/cCD79a− aberrant cells				
Percent of B-cells	0	9.0 (4–36)	8.8 (1.0–20)	0.56
Percent of all BM cells	0	0.33 (<0.01–1.9)	0.16 (<0.01–0.6)	0.25

NBM: normal bone marrow; BM: bone marrow; MDS: myelodysplastic syndrome. p-values obtained by the Kruskal–Wallis test.
[a] 11/14 NBM, 17/22 in RA/RARS, 5/13 RAEB and none of the cases of RAEB-t and CMML.
[b] All NBM and low risk patients showed cells in this compartments. In high risk, only 13/19 patients showed these cells.
[c] All patients in this group showed cells in this compartment.

Concerning CD34− B-cells these correlations were: $r = -0.38, -0.36$, and $+0.26$, respectively. In the partial correlation however, these correlations were accentuated for blast percentage ($r = -0.75$) but remained unaltered for leukocytes ($r = -0.33$) and hemoglobin ($r = +0.22$).

3.2. Immunophenotypic characteristics of BM B-cells

Concerning the two pan-B-cell markers used (CD19 and cCD79a) only CD19 was constantly expressed by all B-cells from all individuals. However, aberrant CD19+/cCD79a− B-cell precursors were observed in both LR-MDS and HR-MDS patients but not in normal BM (Table 1). CD19+/CD79a− cells were found in 7/11 cases of LR- and 6/18 cases of HR-MDS (Fig. 1G). They were associated with a statistically significant low expression of cCD79a in early and intermediate B-cells but not in mature ones (Fig. 2). In addition to cCD79a, other B-cell associated markers were also abnormally expressed in different BM B-cell compartments. Thus, reactivity for CD45 was abnormally low on immature as well as on mature lymphocytes (Fig. 3). Expression of TdT was also decreased (Fig. 4A) on CD34+ B-cell precursors. A lower amount of HLA-DR was found on CD34− B-cells (Fig. 4B). For all markers analyzed except TdT, abnormal antigen expression on CD34+ B-cell precursors was more frequently observed in LR-MDS patients whereas abnormal antigen expression on the two CD34− B-cell compartments predominated among HR-MDS cases. Overall, 91% of all LR-MDS and 74% of HR-MDS cases showed aberrant expression of at least one marker in one or more subpopulations of B-cells. From all antigens studied, aberrant expression of CD3, CD4, CD8 and CD16 on BM B-cells was never observed. Mean forward light scatter (FSC) values (Fig. 4C) on B-lymphocytes were variable but overall not decreased. However, a significant positive correlation was found between percentage of CD34+ B-cells and their FSC MFI ($r = 0.61$) and those CD34− ones ($r = 0.51$).

4. Discussion

Myelodysplastic syndromes (MDS) are clonal disorders originated in the myeloid hematopoietic stem cell[1]. However, recently, several studies using FISH and immunological markers as well as several functional essays have brought evidences indicating that, in at least a part of the cases,

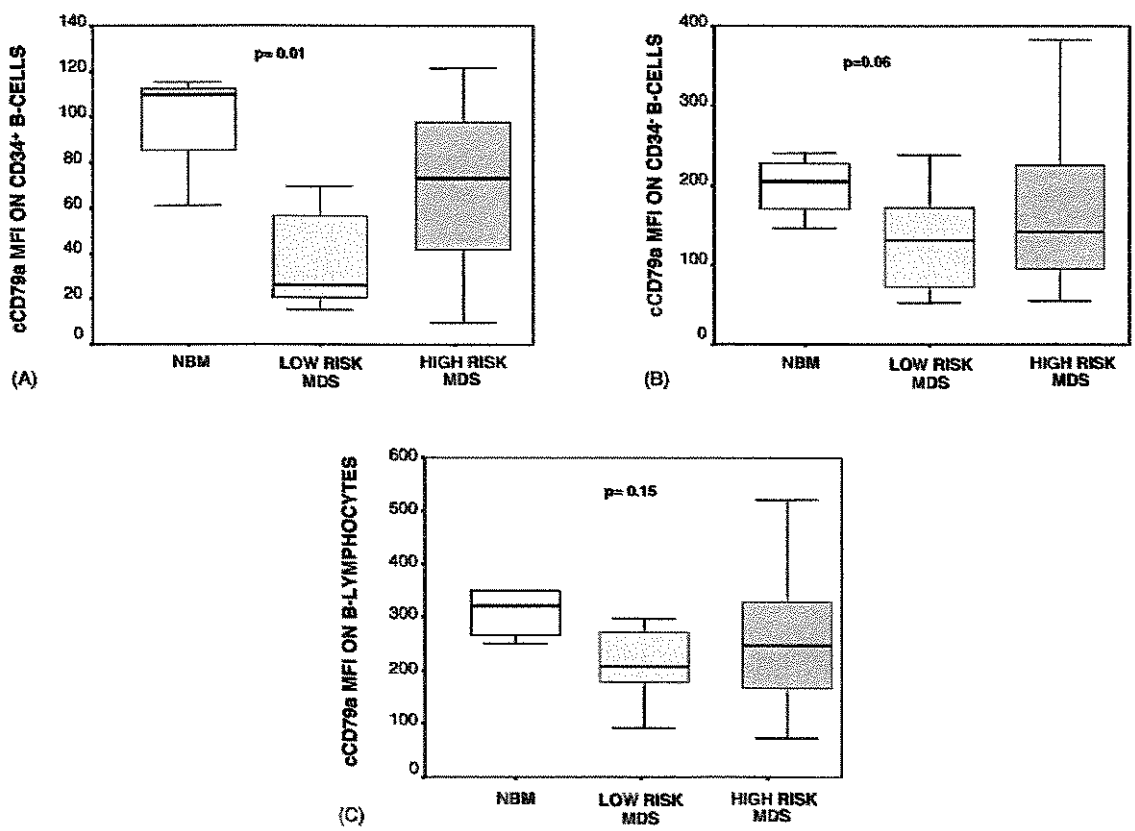

Fig. 2. Intensity of CD79a expression (measured by MFI) observed in the B-cell sub-populations in normal controls versus low risk and high risk MDS. (A) CD34+ (immature) B-cells (B) intermediately mature cells (CD34−) (C) mature lymphocytes.

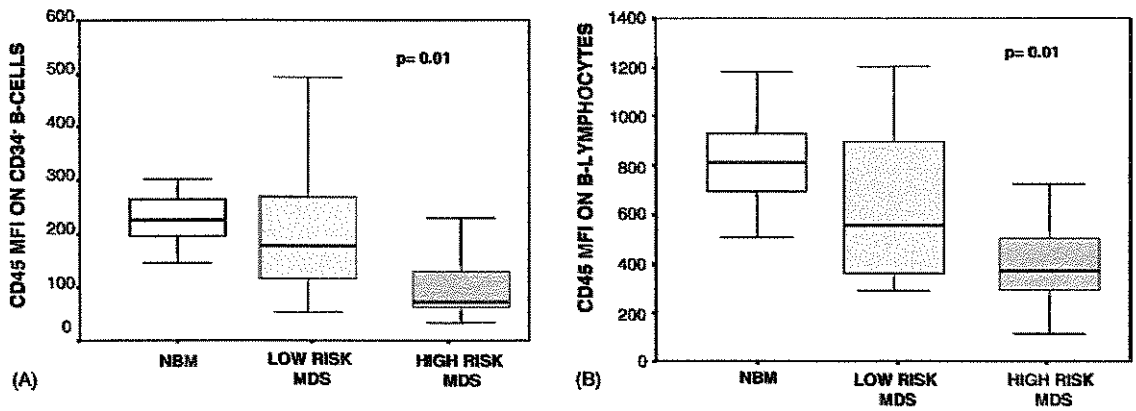

Fig. 3. Expression of CD45 (measured by MFI) on the intermediately mature (A) and mature (B) B-lymphocytes in normal controls versus low risk and high risk MDS.

the neoplastic event could occur in a more immature stem cell, with ability to differentiate into both the myeloid and lymphoid lineages [4,5,7]. In the present study, we describe both quantitative and qualitative immunophenotypic abnormalities involving BM B-cells in MDS patients. Numerical abnormalities were more pronounced in the CD34+/CD45^{lo} and CD34−/CD45^{int} compartments of B-cell precursors and were more frequent in RAEB/RAEB-t. In a previous study we have found decreased numbers of B-lymphocytes in BM [20] of patients with MDS, more pronouncedly in RAEB. However, to the best of our knowledge this is the first report in which the number and distribution of B-lymphocytes as well as their precursors are analyzed in BM of a relatively large series of patients.

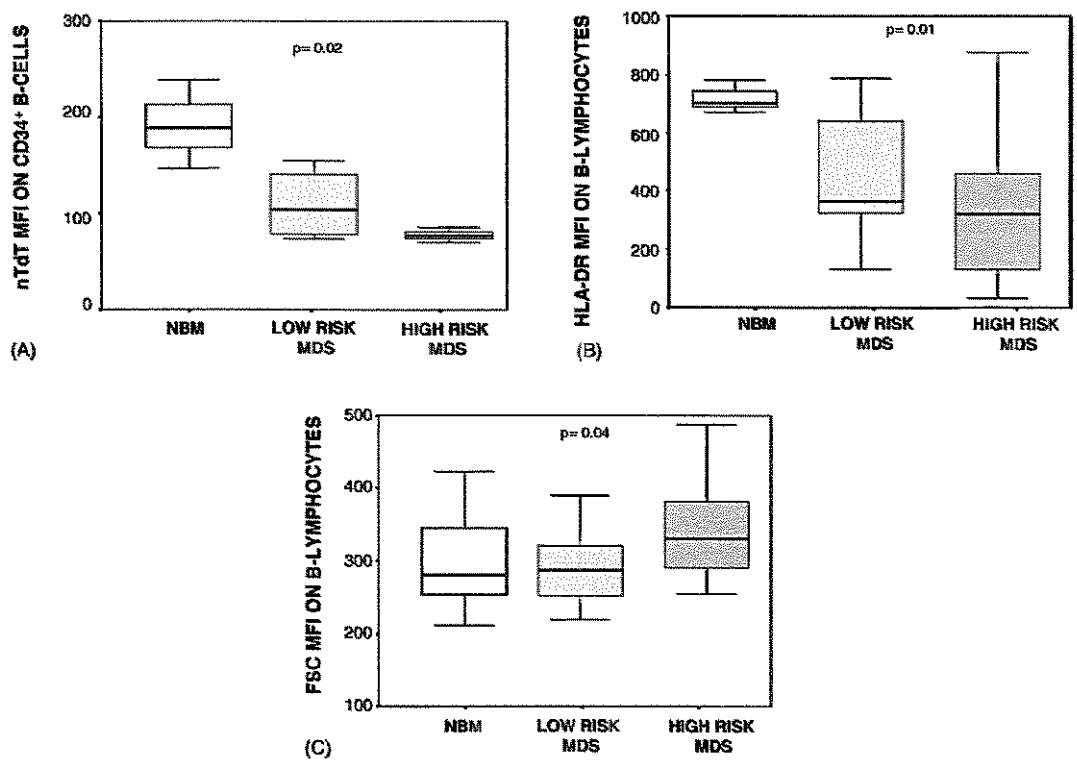

Fig. 4. Immunophenotypic differences observed in sub-populations of bone marrow B-cells in normal controls and MDS. (A) Differences in expression of nTdT in immature (CD34+) B-lymphocytes. (B) Differences in expression of HLA-DR on mature lymphocytes. (C) Features of FSC (forward side scatter) of mature lymphocytes in the three groups.

The numerical changes found are in keeping with the hypothesis that the abnormal stem cell has a decreased ability to produce B-lymphocytes, as has also been postulated by others [25]. The apparent high proportion of mature B-lymphocytes presenting less phenotypic alterations could suggest that in this sub-population there is a mixture of abnormal cells derived from the MDS clone and normal "pre-clonal" B-lymphocytes as has already been pointed out [25].

This decrease of CD34+ and less pronounced of CD34− B-cells, was more evident with increasing numbers of BM blasts. We also observed a relation between these cell populations and PB leukocyte count and hemoglobin values. This could suggest that, impaired B-cell production is associated with progression of the MDS clone. Proportion of BM blasts as well as PB counts are well recognized prognostic factors in MDS [1,13,14,26]. Therefore, one may hypothesize that a more abnormal stem cell looses its capacity to differentiate to B-lymphocytes.

However, it has been recently shown that the early phase of commitment of the multipotent hematopoietic stem cells to the B-cell line in BM is driven by their attachment to stromal cells expressing CXCL12 [27]. As several stromal abnormalities have been described in MDS, these may be associated with the impairment of B-cell commitment and maturation [28]. This would also be in keeping with the increased apoptosis recently described in BM B-lymphocytes in MDS [12].

Further supporting the hypothesis on the occurrence of an abnormal B-cell maturation, most of our cases presented abnormal patterns of expression for at least one of the markers analyzed. Such phenotypic abnormalities were more frequently observed in the early CD34+/CD45^{lo} B-cell precursors and were more evident in advanced cases. Regarding the type and frequency of the different phenotypic abnormalities found in the present study, it should be noted that they affected most of the B-cell-associated antigens analyzed and they highly varied both between different populations of B-cells and between different individuals.

Asynchronous antigen expression was restricted to the CD19+/cCD79a− phenotype on CD34+ B-cell precursors. During B-cell maturation in normal and regenerating BM, expression of cCD79a always precedes that of CD19 [29,30]. No normal B-cell precursors showing a cCD79a−/CD19+ phenotype has been reported in the literature. This phenotypic abnormality is not frequent in B-cell precursor ALL where most blast cells display a CD19+/CD79a+ phenotype [29–31]. Therefore, this abnormality may be specific for MDS. Recently, it has been shown that expression of lineage antigens are frequently lost during apoptosis of T lymphocytes and B-lymphocytes (CD19 positive) of chronic lymphocytic leukemia as well as in cell lineages in culture [32,33]. One may speculate if absence of CD79a expression is due to a downregulation of its gene in a neoplastic cell, specifically occurring in MDS but rarely seen in ALL or if this expression is lost during apoptosis of B-cell precursors influenced by abnormalities of BM microenvironment. These are multiple and remain poorly characterized [28]. Studies analyzing apoptosis specifically on these cells with abnormal phenotype could clarify this point.

Clonal subpopulations of several lineages have been described in MDS, more frequently in more advanced cases [2,16,25,34]. Clonality is well recognized in granulocytes, erythroblasts and megakaryocytes and can be observed in most patients, while detection of clonal B-lymphocytes has been controversial, but has been demonstrated in at least few cases [4–7,34]. In order to elucidate this point, further studies examining clonality of BM B-cell precursors using FISH and lineage-specific markers should be performed. But also the influence of the stromal abnormalities on B-cell production in MDS should be further elucidated.

In summary, the present study shows phenotypic evidence of an abnormal B-cell maturation in MDS patients, more pronounced in advanced disease. This could be due to a participation of the B-lymphocytes in the MDS clone, but could also be a result of an influence of the altered microenvironment on these cells. Further research, using purified well characterized cells would better describe the role of each cell type in the complex pathophysiology of MDS.

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CONCLUSÕES

População Eritroblástica

1. Uso da combinação CD71/GlyA apresentou padrão alterado na SMD mas não nas doenças não-clonais (DNC).

População Granulocítica

1. IMF SSC baixo (hipogranulação citoplasmática) encontrado nos mieloblastos e prómielócitos na SMD. DNC foi semelhante aos controles
2. Deficiência do CD10: pouco frequente na SMD
3. Expressão do CD64 e CD45: sem alterações significativas na SMD
4. CD16 e CD11b: hiperexpressão nos mieloblastos
5. CD16: hiperexpresso em toda a maturação granulocítica no grupo AR
6. CD13: hiperexpresso em toda maturação granulocítica nos 3 subtipos FAB da SMD.
7. DNCs: sequência maturacional da expressão dos antígenos esteve conservada
8. DNCs: não apresentaram aberrações fenotípicas, como encontradas na SMD

População Monocítica

1. MO de pacientes com DNCs teve número aumentado de monócitos, como a SMD. No entanto, este aumento é inespecífico
2. Expressão mais intensa na SMD e DNCs do marcadores CD13 e CD64. O CD11b não se diferenciou em ambos os grupos
3. O CD16 esteve aumentado nos monócitos da SMD. Este resultado pode ser por contaminação das células dendríticas na gate dos monócitos ou evidência da diferenciação dos monócitos em céluals dendríticas

População Blástica

1. De todos os parâmetros usados para avaliar os blastos, o melhor foi a quantificação do CD34, que teve melhor resultado com a combinação do CD16/CD11b

População Linfóide

1. Diminuição na produção de linfócitos B mais intensa nos tipos FAB mais avançados da SMD
2. Em relação à maturação B, a população mais imatura $CD34^{+}/CD45^{fraca}$ apresentou maior diminuição nos casos AREB. Na LMMC e AREB-t esta população não foi detectada
3. Presença de expressão anômala CD19+/cCD79a- nos precursores B da SMD

Os parâmetros que melhor discriminam a SMD das DNCs foram:

Granulócitos

1. SSC nos mieloblastos e prómielócitos
2. Padrões de expressão alterados na mielopoiese da SMD pelos dot plots CD16/CD11b e CD16/CD13

Eritroblastos

1. Perfil maturacional alterado nos eritroblastos da SMD pela combinação CD71/GlyA

Monócitos

1. Hiperexpressão do CD16 na SMD

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ANEXO

***Confirmar* ou *não* se os exames abaixo foram realizados para excluir as doenças que simulam a SMD**

Positivo	Negativo	
( )	( )	Avaliação das carências vitamínicas: dosagens do ferro sérico, transferrina e ferritina, ácido fólico e vitamina B12;
( )	( )	Avaliação da função tireóide: dosagens T4L e TSH;
( )	( )	Avaliação da função renal: uréia, creatinina e clearance da creatinina;
( )	( )	Avaliação da função hepática: transaminases, bilirrubinas, eletroforese de proteínas séricas e do tempo de protrombina;
( )	( )	Avaliação das doenças auto-imunes: células LE, fator anti-núcleo, fator reumatóide, fator anti-DNA, Coombs direto e indireto;
( )	( )	Avaliação das doenças infecciosas: sorologias para toxoplasmose, mononucleose infecciosa, hepatites B e C, HIV e citomegalovírus;
( )	( )	Investigação de doenças neoplásicas: raio-X tórax e ultrasonografia abdominal;
( )	( )	Investigação da hemoglobinúria paroxística noturna: testes de Ham e da sacarose.