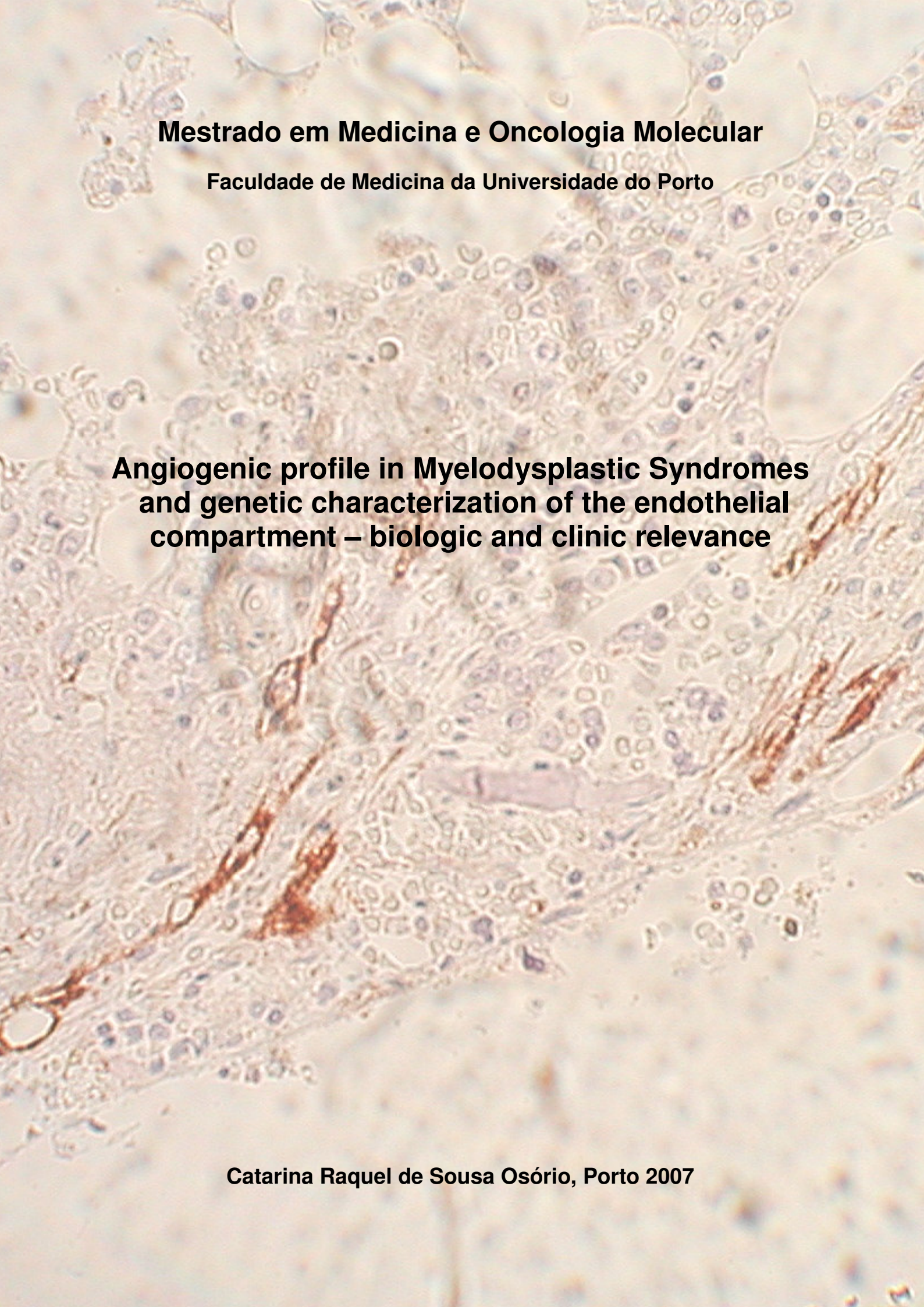

Mestrado em Medicina e Oncologia Molecular

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Angiogenic profile in Myelodysplastic Syndromes and genetic characterization of the endothelial compartment – biologic and clinic relevance

Catarina Raquel de Sousa Osório, Porto 2007

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Abstract

Myelodysplastic syndromes (MDS) are a group of clonal bone marrow (BM) disorders characterized by hematopoietic insufficiency and potential leukemic transformation. Recent studies suggest a substantial increase in BM vascularity of MDS and acute myeloid leukemia (AML). However, the mechanisms that regulate angiogenesis and angiogenic factors role in MDS progression are poorly understood. In this study, we hypothesized that changes in MDS BM microenvironment can induce BM angiogenesis leading to AML onset and progression.

Bone marrow biopsies were colleted from patients; we analysed 19 MDS patients that were not under treatment at the time of analysis and 9 patients under MDS treatment (like Thalidomide, Danazol and Vidaza).

Flow cytometry analysis of BM biopsies revealed an increase in $CD34^+$, $CD117^+$ and $CD133^+$ progenitor cells, $CD133^+KDR^+$ EPC and KDR^+ endothelial cells (EC) in intermediate risk patients (accordingly with the International Prognostic Scoring System (IPSS) classification). Patients under MDS therapeutics had lower progenitor cells levels when compared with patients without treatment. Secondary MDS caused by radiotherapy had augmented $CD133^+$ progenitor cells, $CD133^+KDR^+$ EPC and KDR^+ EC. The incidence of apoptosis of $AC133^+$ progenitor cells and KDR^+ EC in intermediate risk patients was higher than in low risk patients. These data revealed an increase in progenitor cell pool (or increased turnover of this lineage), in particular EPCs, with disease progression, and with radiotherapy MDS related, which seems to be reduced with specific therapeutics for MDS. BM microvessel density quantifications were made, indicating that intermediate risk patients had higher levels of the angiogenic markers: VEGF, CD31 and vWF (that is, increased BM MVD and increased VEGF levels).

To understand how the MDS BM microenvironment might contribute to leukemic transformation, we measured the expression of factors like TNF-α, TFG-β and PlGF by Real-Time PCR as well as the different VEGF isoforms. We also determined VEGF levels by ELISA in BM plasma samples.

TNF-α and TFG-β expression was higher in intermediate-risk MDS patients and VEGF levels were similar among patients. We also observed that the proportion between VEGF isoforms changed. In MDS patients the most frequent isoform was $VEGF_{121}$ and there was an increase in $VEGF_{189}$ with MDS progression. Together, these results confirmed an abnormal BM environment in MDS patients may be due to a particular regulation of angiogenic factors and augmented angiogenesis which might contribute to leukemic progression.

To determine whether $AC133^+$ progenitor cells are already transformed in MDS early stages (ie. are part of the malignant process), we isolated $AC133^+$ cells from a low-risk MDS patient with a chromosomal deletion del(20q). Using FISH analysis, we detected this cytogenetic alteration in some of those cells, which is strongly suggestive of an early progenitor cell transformation. We also performed immnohistochemistry analysis for the EPC marker homeobox HoxA9 in the same cells and we conclude that subset of EPC are transformed (ie. malignant) cells in MDS.

Besides these Human studies, we tried to validate the importance and putative involvement of EPC/vascular cells in MDS-leukemia progression in a murine model of irradiation-induced BM malignant transformation. In this model, mice that develop BM disease (MDS-leukemia) earlier had lowering EPC levels, again suggesting that EPC turnover is a crucial feature of the pathophysiology of these BM diseases.

Taken together, these findings suggest that an increase in the BM progenitor cell pool is directly related with MDS clinical stage risk and may contribute to the angiogenic response in MDS. The altered microenvironment and malignant cell transformation can also lead to disease progression and leukemia onset. The presence of an EPC malignant clone in MDS BM may indicate a possible role of these cells in BM abnormal vascularization, but also in the disruption of normal hematopoiesis and, finally, acute leukemia progression.

Resumo

Os síndromes Mielodisplásicos (SMD) são um grupo de doenças clonais da medula óssea (MO), caracterizadas por uma desregulação na hematopoiese e susceptibilidade em desenvolver leucemia mieloide aguda (LMA). A maioria dos doentes têm uma idade média de 65 anos e acaba por falecer, a maior parte das vezes, antes de desenvolver leucemia. A mielodisplasia pode surgir de novo ou ser secundária devido a tratamentos de radioterapia ou quimioterapia.

Os SMD podem resultar de alterações genéticas e epigenéticas que ocorrem nas células progenitoras hematopoieticas e de alterações no micro-ambiente da MO. As alterações genéticas mais frequentes são as cromossomais que surgem em 50% dos casos de SMD primários e 80% nos secundários. O micro-ambiente medular, pode potenciar o desenvolvimento do clone maligno devido sobretudo aos níveis patológicos das citoquinas secretadas.

A angiogénese nos tumores hematológicos não está tão bem caracterizada como nos tumores sólidos. Contudo há evidências de um aumento da vasculatura da MO em SMD e em LMA. Algumas drogas anti-angiogenénicas como a Talidomida já são usadas clinicamente e com sucesso em doentes específicos. Contudo, os mecanismos que regulam a angiogénese e a produção de factores angiogénicos na progressão da mielodisplasia são pouco conhecidos. A caracterização do perfil angiogenico destes doentes é importante para o prognóstico, diagnóstico e avaliação das respostas terapêuticas.

Neste estudo colocamos a hipótese de as alterações no micro-ambiente da MO dos doentes com SMD poderem conduzir a LMA.

Foram recolhidos os aspirados medulares dos doentes dos quais 19 não tinham recebido nenhuma terapêutica e 9 estavam em tratamento para a mielodisplasia (com Talidomida, Danazol e Vidaza).

Análises por citometria de fluxo nas MO mostraram que as células progenitoras para os marcadores $CD34^+$, $CD117^+$ e $CD133^+$, células progenitoras endoteliais (CPE) $CD133^+KDR^+$ e células endoteliais (CE) KDR^+ nos doentes com risco intermédio (de acordo com a classificação do

International Prognostic Scoring System (IPSS)) existem em maior percentagem. Nos doentes em tratamento a percentagem destas células era menor quando comparada à percentagem dos doentes sem tratamento. Os SMD secundários causados por tratamento anterior com radioterapia têm um número aumentado de células progenitoras $CD133^+$, CPE $CD133^+KDR^+$ e CE KDR^+. Também foram medidos os níveis de apoptose nas células da MO destes doentes. Os doentes de risco intermédio apresentam níveis de apoptose mais elevados nas células progenitoras $CD133^+$ e CE KDR^+ que os doentes de baixo risco. Estes resultados sugerem um aumento de células progenitoras, em particular CPE, um aumento dos vasos sanguíneos na MO que acompanha a progressão da doença, nos doentes sem tratamento e expostos previamente à radioterapia. Pelo contrário, estes valores parecem ser reduzidos nos doentes em tratamento específico para a mielodisplasia.

Para perceber de que modo o micro-ambiente medular pode contribuir para a transformação em leucemia, foi determinada a expressão do mRNA, por PCR Quantitativo, nos seguintes factores angiogenicos: TNF-α, TFG-β e PlGF. Também foi medida a expressão das isoformas do VEGF por PCR Quantitativo e os níveis totais de VEGF por ELISA nas células da MO.

A expressão de TNF-α e TFG-β era maior nos doentes com risco intermédio. Verificou-se que o padrão da expressão das isoformas do VEGF estava alterado; a isoforma $VEGF_{121}$ estava aumentada e a isoform $VEGF_{189}$ aumentava com a progressão da doença. Análises de imunohistoquímica com marcadores de vasos sanguíneos nomeadamente VEGF, CD31 e vWF revelaram um aumento de vasos nos doentes de risco intermédio comparativamente aos doentes de baixo risco. Estes resultados sugerem um aumento da angiogenese nos doentes com SMD, bem como uma regulação alterado de factores no micro-ambiente da MO que podem contribuir para a progressão da doença em LMA.

Para perceber se as CEP poderiam também ser células participantes no processo de transformação maligna, isolaram-se células progenitoras $AC133^+$ de um paciente com uma delecção no cromossoma 20 (del(20q)). Usando técnicas de FISH foram detectadas células com a alteração citogenética, revelando assim uma transformação em células progenitoras. Estas células foram posteriormente submetidas a um ensaio de imunohistoquímica para o

marcador de CPE HoxA9, do qual se concluiu que uma parte das CPE estarão provavelmente envolvidas no processo de transformação maligna em SMD.

Além dos estudos em humanos tentou-se validar a importância do envolvimento das CEP na progressão de SMD para leucemia usando para isso um modelo de carcinogénese em ratinho induzido por irradiação. O grupo de ratinhos que ficou doente mais cedo apresentou um menor número de CPE em circulação no sangue periférico que o grupo que não desenvolveu doença. Isto sugere uma contribuição das CPE na génese de algumas doenças hematológicas tais como SMD e leucemia aguda.

Concluindo, estes resultados sugerem que uma maior renovação ("turnover") de progenitores medulares está directamente relacionado com o grupo de risco dos SMD, podendo originar uma resposta angiogenica na MO do SMD. Um micro-ambiente medular alterado e a presença de uma CEP transformada podem originar um aumento dos vasos sanguíneos e a uma alteração da hematopoiese normal podendo culminar no desenvolvimento da LMA.

Índex

Aims

1) To characterize the angiogenic profile in MDS patients according with the International Prognostic Scoring System (IPSS) classification.

2) To contribute towards a better understanding the involvement of bone marrow endothelial progenitor cells and soluble angiogenic factors in the origin of the angiogenic response in MDS bone marrow.

3) To test the “common origin of malignant hematopoietic and vascular cells hypothesis” in MDS.

4) Development and characterization of a murine model of MDS, in order to validate the biological readouts investigated in the previous aims.

1) Introduction

Basic mechanisms of carcinogenesis

Carcinogenesis is a multistep process in which normal cells undergo vascular and functional changes, resulting in their conversion into neoplastic ones. Several genetics alterations are acquired in this process due to mutations in genes with dominant gain of function, oncogenes, and inactivating mutations in tumor suppressor genes leading to genetic instability. During neoplasic transformation cell physiology is altered, resulting in the acquisition of distinct properties responsible and essential for tumor progression: augmented proliferative potential and self-sufficiency in growth signals, apoptosis evading and insensitivity to anti-growth signals, induction of tumor angiogenesis and invasion/metastasis dissemination. It is believed that most human tumors share these characteristics although each tumor may acquire them by various mechanisms.[1,2]

Angiogenesis

Mechanism

Angiogenesis is a process that results in the growth of new blood vessels. During embryonic development, vasculogenesis/angiogenesis establishes the primary vascular tree and provides the nutrients and oxygen needed for organ development. During adulthood, angiogenesis occurs in physiologic processes, such as during the ovarian cycle and placenta development, and in pathological processes like wound healing and cancer.[3]

Tissue metabolic demands in nutrients and oxygen comprise the main stimulus for blood vessel growth. Oxygen can diffuse to a distance of 100-200μm, but above that limit a hypoxic environment is originated; exposure of cells to a hypoxic environment is the best known molecular “trigger” for the onset of angiogenesis.

For instance, hypoxia is a strong inducer of angiogeneic stimulators, including vascular endothelial growth factor (VEGF). VEGF is one of the best characterized angiogenic factors, which has a potent action at several steps of the angiogenic process: vessel permeability, endothelial cell proliferation and migration and vessel chemoattraction fusion pre-existing structures. Alternative splicing generates six VEGF-A isoforms consisting of 121, 165, 145, 189 and 206 amino acids which differ mostly in their bioavailability (ie, some isoforms are freely soluble whereas others bind collagen or heparin). $VEGF_{121}$ is a fully soluble isoform whereas $VEGF_{165}$ can exists either in the soluble isoform or trapped in the membrane, $VEGF_{145}$ and the largest isoforms $VEGF_{189}$ and $VEGF_{206}$ because their affinity for cell-surface proteoglycan are membrane isoforms.[4] While $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ stimulate endothelial cell proliferation and migration in adult angiogenesis, $VEGF_{206}$ appears to be expressed only in embryonic tissue. The largest VEGF isoforms are responsible for prolonged mitogenic signals than the shorter isoforms, which are more diffusible.[5]

VEGF binds to three known tyrosine kinase receptors: Flt-1/VEGFR-1, KDR/Flk-1/VEGFR-2 and Flt-4/VEGFR-3. These receptors differ mainly in their cell pattern of expression, and also in their binding specification (the 3 receptors bind members of the VEGF family with different affiliation).

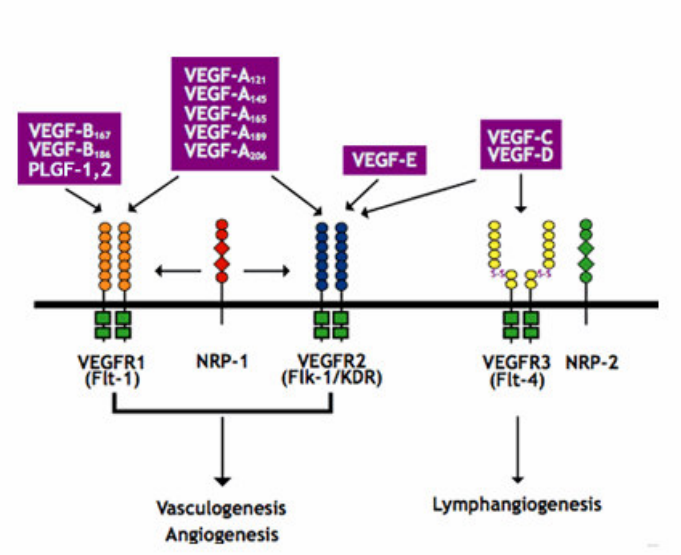


Figure1. Interactions between VEGF ligands and receptors.
From Hicklin DJ, Ellis LM. J Clin Oncol. 2005;23:1011-1027

Adult Vasculogenesis

Vasculogenesis is the *de novo* formation of blood vessels from the differentiation of endothelial precursors. In the embryo, endothelial cells arise from mesodermal precursor cells, the angioblasts, to form the primitive capillarity network. Embryonic vasculogenesis involve the fusion of multiple blood islands. These, are spatially arranged with hematopoietic stem cells that are going to origin hematopoietic (blood) cells at the center of the blood island and endothelial progenitor cells (EPC) at the periphery. These progenitors have the capacity of differentiating into functional mature endothelial cells.[6]

Some studies revealed that HSC and EPC may have a common ancestor, the hemangioblast.[7] HSC and EPC express common surface markers like KDR (VEGFR2), CD34 and Sca-1. EPCs were initially isolated using KDR and CD34 as markers, but more recently, it was shown that EPC expressed also the stem cell marker AC133 (whose function is still undisclosed); interestingly, the expression of AC133 in EPC is lost when cells differentiate into mature endothelial cells.[8] EPC maturation and differentiation is crucial to form functional endothelial cells and consequently blood vessels. The potential to differentiate into EC is regulated by several signalling pathways including Notch/Delta.[9] Notch/Delta signalling regulates embryonic arterial differentiation[10] and in postnatal vasculature regulates arterial EC differentiation.[11] Concerning the activation of an endothelial-specific transcription profile, recent evidence as suggested the Hox family of transcription factors may be involved. In fact, the homeobox transcription factor HoxA9, which is regulated by histone deacetylases, is also a critical regulator of adult neovascularization and directs the expression of other endothelial specific genes.[12]

Indeed, neovascularization in the adult was though to be maintained only by sprouting angiogenesis. However, there is recent evidence that suggested EPC in adult blood and bone marrow are involved in angiogenesis by replacing lost endothelial cells; EPC have been shown to incorporate healing wounds and even tumor vasculature.[14] The pathways and mechanisms that regulate EPC differentiation are important also for post-natal angiogenesis.

Tumor angiogenesis

In the early 70s Judah Folkman proposed that angiogenesis is required for tumor growth after a 1 to 2 mm^3 volume (which formed the basis for the research in hypoxia regulation of neo-angiogenesis). He also postulated that angiogenic inhibition would be a good strategy to prevent tumor development. It was showed that if angiogenesis was prevented tumor growth decreased and tumor cells died.[15]

As mentioned early, avascular tumors grow until inner regions become hypoxic which leads to the angiogenic stimuli. In normal conditions, or even in some tumors, maintenance of a quiescent vasculature results from a balance between angiogenic activators and inhibitors, between proliferation and apoptosis. The loss of equilibrium between such activators and inhibitors such as in response to hypoxia, results in an angiogenic "switch", favouring proliferation and blood vessels growth, eventually contributing towards tumor cells dissemination (metastases). Angiogenic inhibitors identified to date include endostatin (it is a fragment from type XVIII collagen and blocks VEGF signalling, though its binding to VEGF receptor 2); angiostatin (it is a fragment from plasminogen and links to hepatocit growth factor receptor), thrombospondin-1 (binds to p53 and alter its regulation) and tumstatin (derived from type IV collagen degradation, binds integrins which avoid their connection to endothelial cells and their migration).[16]

Mechanistically, tumors use the same molecules used in normal angiogenesis to promote their own blood supply. Globally, tumor vascularization may occur by several mechanisms such as sprouting, recruitment of EPC, cooption, vasculogenic mimicry and mosaic vessels.[17] Blood vessels can growth through sprouting which results in the branching of new capillaries from pre-existing vessels or non-sprouting processes like fusion of pre-existing vessels.[18]

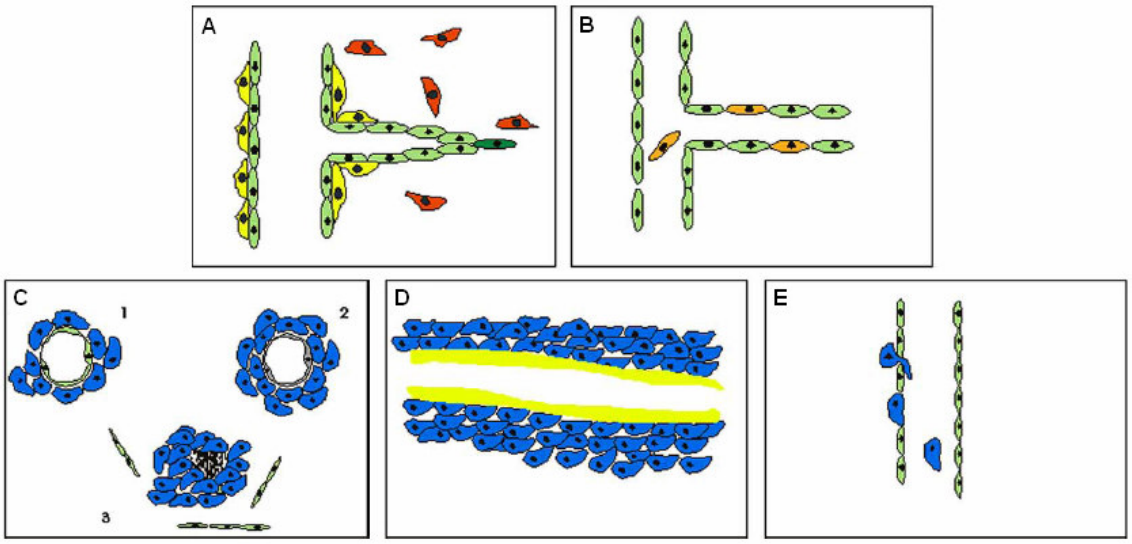


Figure2. Vascular angiogenic mechanisms: sprouting (A), EPC recruitment (B), cooption (C), vasculogenic mimicry (D) and mosaic vessels (E).
From Auguste, P., et al, Crit Rev Oncol Hematol, 2005; 54:53-61

In addition, recent years have shown that molecular therapeutic strategies to block this process have potential in the treatment of murine and human tumors. Most notably, VEGF blockade has shown significant success in the treatment of colo-rectal and renal cancer. [19]

The neovascularization process is crucial for tumor growth and survival.[15,20] Given the recent identified involvement of EPC in the angiogenesis process, it is important to understand the mechanisms that regulate endothelial cells growth from EPC in order to identify new anti-angiogenic therapeutic thargets. In a vascular stress situation, these precursor cells are recruited to the circulation and, mediated by angiogenic inducers and inhibitors; they incorporate the new formed vessels (or may contribute towards vessel formation in an indirect manner).

Hematopoietic organs: The Bone Marrow

Structure

Bone marrow is the major hematopoietic organ in adulthood and exists in the central bone cavities of long and axial bones. Marrow spaces form a trabecular structure with stromal cells, hematopoietic and endothelial progenitors. In long bones, one or two large vessels (arteries and veins) enter the marrow cavity and in flat bones there are several blood vessels with different sizes. Myelinated and non-myelinated nerves constitute BM enervation. The hematopoietic tissue consists in various types of mature blood cells and their immature precursors, organized in a spatially, temporally and molecularly ordered manner. [21]

The bone marrow microenvironment consists of BM stroma cells and factors, growth factors and cytokines, provided from stroma and blood vessels. Stromal cells have been considered the responsible in maintaining the BM microenvironment but the vasculature is also seen as very important since it provides the BM with oxygen and nutrients. Globally, the BM stroma is thought to consist of different types of cells: endothelial cells, macrophages, adypocytes, fibroblasts, osteoblasts and extracellular matrix elements like fibronectin.[22]

For hematopoiesis to take place it is necessary a stable microenvironment where stem cells are located in their appropriate niches, where they are provided with the factors for self-renewal, proliferation, migration, differentiation and lineage commitment. Hematopoietic stem cells self-renewal and proliferation are maintained by low level secretion of stromal cells factors and others. Hematopoiesis is stimulated by up-regulation of specific cytokines in response of hypoxia, anemia, inflammation or bacterial infection.[23]

Hematopoiesis

Hematopoiesis is a continuous process that starts with a pluripotent hematopoietic stem cell. It has been suggested that this cell may originate non-hematopoietic cells including hepatocytes, neurons, osteoblast, adipocytes, condrocytes and fibroblasts. The commitment, proliferation and differentiation of hematopoietic cells are controlled by cytokines, growth factors, hormones and the activation of a global (cell-specific) transcriptional profile. After maturation, cells transverse blood vessels wall and enter the bloodstream, in response to chemoattractant signals.

Pluripotent HSC have the capacity to maintain their number through a self-renewal process and the potential to commit to the myeloid and lymphoid lineages if the BM microenvironment provides the right conditions. HSC may originate a common myeloid precursor – CMP – and a common lymphoid precursor – CLP. CMP can develop into a granulocytic and monocytic precursor – GMP, or a erytroid and megakaryocyte precursor – EMP. GMP can differentiate into monocytes and neutrophils and EMP gives rise to erytrocytes and megakaryocytes. CLP differentiate in B lymphocytes and pro-T lymphocytes.[23] importantly, the process of myeloid/hematopoietic differentiation results also from the balance between survival and differentiation signals and apoptosis control within the BM microenvironment.

As mentioned before, HSC and EPC have been suggested to share a common ancestor. It is believed that, in the adult, the majority of EPC reside in BM in association with HSC and BM stroma. EPCs incorporate nascent vasculature of pathological lesions (tumor angiogenesis) but the precise signals required for EPC recruitment from the BM and differentiation are not completely understood; however, ischemia and vascular trauma stimulate EPC mobilization.[6,14] It is also known that upregulation of $VEGF_{165}$ and Angiopoetin 1 results in mobilization of endothelial and hematopoietic progenitors.[24]

Tumor angiogenesis may be supported by the co-mobilization of HSC and EPC from bone marrow and incorporation of EPC in the tumor vasculature.[25] EPCs have been already detected in circulation of lymphoma patients[8,26] and other haematological diseases like leukemia.[27] Since these

originate in the BM microenvironment it is extremely important understand how EPC contribute for vasculogenesis in haematological malignancies and its role in the genesis of BM diseases.

Myelodysplastic Syndromes

Disease description

The Myelodysplastic syndromes (MDS) are a heterogeneous group of disorders of hematopoietic stem cells that are characterized by ineffective hematopoiesis and susceptibility to development into acute myelogenous leukemias.[28] Most patients with this die before leukemia progression due to BM failure, infections or hemorrhagic complications.

MDS is seldom observed in patients under 50 years old but it is the most frequent hematologic disease in patients between 60 and 70 years old. Above 70 years old disease incidence is about 50 cases per 100.000 per year. Several risk factors are implicated in the MDS genesis: age, alcohol, cigarette smoking, ionizing radiation among others. Myelodysplasia may arise de novo or may be secondary due to previous radiotherapy or chemotherapy treatments (therapy-related MDS).[29]

Classification systems

In 1982, the French-American-British (FAB) group established the first classification of MDS and defined 5 subtypes based on morphology, the number of blasts in bone marrow and peripheral blood and monocyte counts. MDS patients were then classified as having refractory anemia (RA; <5% marrow blasts), RA with ringed sideroblasts (RARS; <5% marrow blasts >15% BM ringed sideroblasts), RA with excess of blasts (RAEB; 5-20% marrow blasts), RAEB in transformation (RAEB-T; 21-30% marrow blasts) and chronic myelomonocytic leukaemia (CMML; <5% marrow blasts, > $10^3/mm^3$ monocytes). Although, the FAB classification provides important diagnostic prognostic information, patients with single or multiple cytopenias, other than

anemia could not be easily classified; also, controversy persisted about the number of BM blasts needed for AML diagnosis.

To overcome some of this limitations, in 2001 the World Health Organization (WHO) classification suggested new subtypes of MDS. Refractory anemia (RA) and RA with ringed sideroblasts (RARS), were kept but new subtypes were introduced such as refractory cytopenia with multiple displasia (RCMD), RCMD with ringed sideroblasts (RCMD-RS). RA with excess of blasts I (RAEB-I; 5-10% blasts in BM) and RA with excess of blasts II (RAEB-II; 10-20% blasts in BM) were defined, and two more particular groups: MDS unclassified (for patients with neutropenia or thrombocytopenia alone) and MDS with 5q- syndrome (since this is a specific group with particular biology and a better prognosis) were introduced. On the other hand, CMML was excluded. [30]

However, the WHO classification did not include relevant information on cytogenetic abnormalities, one of the main prognostic factors in MDS patients. Also the threshold of 20% BM blasts count for defining AML is still debatable. In 1996, the International Prognostic Scoring System (IPSS) based on known significant prognostic factors (% of blasts, number of cytopenias and type of cytogenetic abnormalities) defined four risk groups: Low, Intermediate I, Intermediate II and High (table 1.).[31] Each risk group has different prognosis, as shown by survivals ranging between 0,4 to 5,7 years.

	Score Value				
Prognostic Variable	0	0.5	1.0	1.5	2.0
BM blasts (%)	<5	5-10	—	11-20	21-30
Karyotype*	Good	Intermediate	Poor		
Cytopenias	0/1	2/3			

Scores for risk groups are as follows: Low, 0; INT-1, 0.5-1.0; INT-2, 1.5-2.0; and High, ≥2.5.

* Good, normal, −Y, del(5q), del(20q); Poor, complex (≥3 abnormalities) or chromosome 7 anomalies; Intermediate, other abnormalities.

Table 1. IPSS for MDS: Diagnostic and Prognostic.
From Greenberg, P. et al, Blood, 1997; 89:2079-88

Pathogenesis

MDS is a clonal and probably multi-step disease that results from genetic or epigenetic alterations in the myeloid hematopoietic stem cell and abnormalities in BM microenvironment. The molecular mechanisms underlying the genetic alterations are: chromosomal deletions (frequent) and translocations (rare), genetic mutations and epigenetic alterations. Cytogenetic abnormalities appear in 50% of primary MDS and 80% of the secondary cases and may influence clinical features, define prognosis and suggest therapeutic strategies. It is frequent to find deletions and monossomies in regions that may have tumor suppressor genes. However these tumor suppressor genes are not yet known. The loss of specific genes that affect cell cycle, apoptosis and hematopoiesis control contribute to MDS phenotype. The most common cytogenetic alterations are: –5, 5q-, -7, 7q-, 11q-, 13q-, 17p- and 20q-. The del(5q) and –7/del (7q) are the most frequent. Interestingly del(5q) has a good prognosis due to the specific treatment with lenalidomide. Monosomy 7 and del (7q) have a particularly bad prognosis,[32-35] as well as complex karyotypes (> 3 cytogenetic alterations).

MDS pathogenesis is also caused by abnormal bone marrow microenvironment that favours the development of the malignant clone.[36] This microenvironment is composed not only by the stromal cells and intercellular matrix, that may be altered and induce abnormal adhesion patters, undifferentiation and apoptosis, leading to altered hematopoiesis, but also by pathological levels of locally secreted cytokines. MDS derived adherent cell layers produce high levels of TNF-α and IL-6.[37] MDS therapy should target not only patterns in the transformed cell but also this aberrant environment. The angiogenesis patterns in haematological malignancies are not as well characterized as they are in solid tumors. However, it is known that angiogenesis in leukaemia and in MDS has an important role, since an increased vascular density is found in these diseases. Anti-angiogenic drugs like Thalidomide and Lenalidomide are already used in patients with some encouraging results, although its mechanism action is not fully understood.[38]

Treatment

Treatments available for MDS patients are scarce and consist mainly in supportive care (transfusions), biologic response modifiers and chemo therapy. Transfusions are given to correct cytopenias like anemia and thrombocytopenia. In selected cases biologic response modifiers (like hematopoietic growth factors: EPO and G-CSF; and the synthetic androgen Danazol[39]) may ameliorate anemia. The benefit of low-intensity chemotherapy (5-azacytidine – AzaC) is not clearly demonstrated. High-intensity chemotherapy, is indicated in high risk patients and has an increased risk of morbidity and mortality: it can only be apply to fit patients and consists in standard chemotherapy for acute leukemia. It can be followed by hemopoietic stem cell transplantation (HSCT) in selected patients. Azatidine and decitabine were recently introduced in the clinical armamentarium and are very important to antagonize the hypermetilation of the promoter regions of cell cycle control found genes in MDS patients. Their clinical benefit, however, is still limited.

As previously mentioned, many studies have already demonstrated the importance of angiogenesis in hematologic diseases such as MDS. It is known that MDS bone marrow has an increased vascularization and higher levels of angiogenic soluble factors, like Vascular Endothelial Growth Factor (VEGF), and its specific membrane receptors.[40,41] Some clinical studies suggest that VEGF and VEGF receptors levels might correlate with disease aggressiveness.[42]

Based on this information, some alternative therapies have been considered in these disorders: anti-angiogenic (anti-VEGF antibody; Bevacizumab) and anti-apoptotic drugs (anti-TNF), Thalidomide and Lenalidomide. Thalidomide inhibits angiogenesis by interfering with both bFGF and VEGF action and inhibits the production of TNF-α by mRNA degradation. Thalidomide, and more recently Lenalidomide, are known to be effective in patients with 5q- syndrome and to induce not only clinical but also cytogenetic responses.

In general, the majority of MDS patients are over 60 years old and their therapeutic options remain very limited. The development of new target drugs able to stop disease progression by interfering with specific pathogenic

mechanisms is urgently needed. For that, it is first necessary to clarify the pathogenic mechanisms inherent to different MDS subtypes, and define patients which have higher probabilities to respond to specific treatments.

MDS is an attractive pathogenic model to study disease progression towards leukaemia, especially because it has many different stages with specific features, during which the neoplastic clone grows and acquires different characteristics.

MDS is also a good model to study cell and microenvironment changes in bone marrow that may be involved in progression to acute myeloid leukemia. As MDS includes many disease subtypes, we can follow disease progression and understand the inherent mechanisms under leukemia development.

Because of this, it is important to characterize the angiogenic profile in patients with different subtypes of MDS and relate them with prognosis, disease evolution and therapeutic responses. The cytogenetic study in BM endothelial progenitor cells is extremely relevant to understand the role of vasculogenesis in this malignancy, the influence of endothelial cells upon hematopoiesis, their relationship with the neoplastic clone and the contribution of the endothelial compartment to an abnormal bone marrow phenotype in hematological tumors.

Recently, in non-Hodgkin lymphoma, cytogenetic alterations in endothelial cells similar to the ones found in the neoplasic clone were described.[43] These results suggest the existence of a common precursor between tumor cells and endothelial cells, or even endothelial cell differentiation though tumoral progenitor cell. Further examples of such process have not been identified in other tumors. Therefore, a close interaction between vascular and hematopoietic elements within the BM is crucial for the cell differentiation in normalcy as well as malignancy. A thorough understanding of the contribution/importance of the vascular BM compartment during leukemia progression from MDS is essential for the discovery of novel therapeutic strategies to treat these deadly diseases.

2) Materials and methods

Patient samples

Bone marrow (BM) biopsies were obtained from 28 patients and were collected after informed consent, according to IPOFG-EPE guidelines. Study protocols were approved by the Institutional Review Board and Ethics Committee. Patients with MDS were 29 - 82 (median 67) years old and were diagnosed according to the International Prognostic Scoring System (IPSS) (see table 1). The study group was divided into 6 low-risk patients, 13 intermediate-risk patients and 9 patients that were under MDS specific treatment at the time of analysis (we consider MDS therapeutics those consisting of Thalidomide, Danazol and Vidaza).

Among these, 8 patients were diagnosed as primary MDS while 10 patients were considered secondary MDS (if the disease occurred after chemotherapy and/or radiotherapy, for breast cancer, endometrial cancer, rectum carcinoma and neuroendocrine carcinoma).

Mononuclear cells (BMNC) from the BM biopsies were isolated by Ficoll-Hypaque (Sigma, St Louis, MO) density gradient centrifugation. After subsequently washing BMNC with phosphate-buffered saline/2mM of EDTA, cells were counted with the aid of a hemacytomer and then resuspended in 30% of RPMI (GIBCO, Grand Island, NY) with 10% fetal bovine serum (FBS), 1x antibiotic and antimycotic and 1x Glutamine (Complete Medium). Finally, BMNC were frozen in Complete Medium, 10% of DMSO and 60% of FBS and kept at -80ºC for further analysis.

Flow cytometry analysis

Fluorescence-activated cell-sorting (FACS) analysis was performed using a BD FACSCalibur flow cytometer (Becton, Dickinson), and was used to identify BM Hematopoietic Stem cells (HSC), Endothelial Progenitor Cells and endothelial cells. For FACS staining, approximately $1x10^6$ cells of each BM sample were incubated with 10µl of FcR blocking reagent (Miltenyi Biotec), with

rotation at room temperature. After an incubation period of 15min, 5µl of the following fluorescent conjugate antibodies were added: CD133-PE (Miltenyi Biotec), ckit (CD117, Immunotech), CD34 (BD Pharmigen) and/or KDR-APC (RnD systems). Cells were incubated 45min, with rotation at room temperature.

For determination of the BM apoptosis index, erythrocyte lysis was performed with RCLB reagent (Red cell lyses buffer) and cells were resuspended in 100µl of Annexin reagent buffer. For apoptosis identification it was applied 2µl of Annexin V- FITC antibody (BD Pharmingen) for 15 min at room temperature. For each sample it was acquired 50.000 cells.

RNA extraction and cDNA synthesis

RNA extraction from BMNCs was performed according to the following protocol: BMNCs were washed in serum-free RPMI, counted and homogenized in Trizol reagent (Invitrogene). The next steps were done with the cells placed on ice. Chloroform was mixed to the homogenized solution, incubated for 15min and after that centrifuged for 15min, 14000 rpm in a refrigerated (at 4ºC) centrifuge. The aqueous phase was collected to a new tube and isopropranol was added to precipitate RNA. The mixture was incubated for 15min, centrifuged for 15min, 14000 rpm at 4ºC, and the precipitated RNA obtained was washed in ethanol 70% and solubilized in DEPC water. Total RNA was quantified for each sample in a spectrophotometer (Beckman Du-650 (USA)).

cDNA was synthesized according to the following protocol: a 13,5µl mix was prepared with 2ng/µl of RNA, 1µl of Random Primers and distilled water. After the annealing step of 10min at 70ºC, a mixture 4µl of buffer 10x, 4µl of dNTPs, 2µl of DTT co-factor, 1µl of SuperScriptII reverse transcriptase and 0,5µl of RNase out inhibitor (Invitrogen), was added, making up a final volume of 11,5µl. This mixture was incubated 1h30min/2hours at 37ºC and a final 10min at 65ºC step to stop the enzymatic reaction.

To validate the presence of the new synthesized cDNA, BCR gene was amplified by PCR.

Quantitative RQ-PCR for TNF-α, TGF-β, PlGF and VEGF isoforms

TNF-α, TGF-β, PlGF and VEGF isoforms (VEGF121, VEGF145, VEGF165 and VEGF189), mRNA expression was determined by quantitative real-time PCR. For TNF-α, TGF-β and PlGF mRNA expression 1μl of cDNA was used, 12,5μl of SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA), 0,75μl of each primer (Sigma), 0,5μl of BSA 2% and 9,5μl of distilled water in a final volume of 25μl. The relative expression level of each target mRNA was normalized to BCR expression. VEGF isoforms mRNA expression was determined based on specific primer and probes. PCR reaction contained 1μl of cDNA, 12,5μl of Taqman universal PCR master mix (Applied Biosystems), 0,75μl of each primer, 0,83μl of probe and 9,17μl of distilled water in a final volume of 25μl. A specific primer and probe set was used for the internal control gene, 18S. All reactions consisted of incubations at 50ºC for 2 minutes, 95°C for 10 minutes, 45 cycles at 95°C for 15 seconds and 60°C for 1 minute and were performed in Micro-Amp optical 96-well plates using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Relative values were calculated by using a comparative cycle threshold (Ct) for each duplicate. The primer and probe sequences are listed in Table 2.

Gene	Sense primer	Antisense primer
TNF-α	TCAGCCTCTTCTCCTTCCTG	GCCAGAGGGCTGATTAGAGA
TGF-β	GTACCTGAACCCGTGTTGCT	CACAACTCCGGTGACATCAA
PlGF	GTCATGAGGCTGTTCCCTTG	GGGTACCACTTCCACCTCTG
BCR	GAGCGTGCAGAGTGGAGGGAGAACA	CACAGTATCCTCAGGGTCTGGGA
VEGF121	CCAGCACATAGGAGAGATGAGCTT	CGGCTTGTCACATTTTTCTTGTC
VEGF145	AATGTGAATGCAGACCAAAGAAAG	CACATACGCTCCAGGACTTATACC
VEGF165	CCAGCACATAGGAGAGATGAGCTT	AGGCCCACAGGGATTTTCTT
VEGF189	AATGTGAATGCAGACCAAAGAAAG	AGGGAACGCTCCAGGACTTATA
Probe		
VEGF121	ACAGCACAACAAATGTGAATGCAGACCAAA	
VEGF145	AGAGCAAGACAAGAAAAAAAAATCAGTTCGAGGAA	
VEGF165	ACAGCACAACAAATGTGAATGCAGACCAAA	
VEGF189	AGAGCAAGACAAGAAAAAAAAATCAGTTCGAGGAA	

Table 2. Primer pairs and probes used in real-time PCR experiments

VEGF quantification by ELISA

Plasma samples from all the BM biopsies were obtained and VEGF levels were quantified by Enzyme-linked immunoadsorbent assay (ELISA) (Calbiochem, Dalmstadt, Germany), following the instructions of the manufacturer. Briefly, we added 100 µl of Assay Diluent RD1W to each well of a 96-well plate coated with anti-human VEGF mouse monoclonal antibody. Afterwards, 100µl of each sample was applied to each well, plate was covered with the sealer provided and incubated at room temperature for 2hours. The wells containing sample and calibration samples were washed extensively with 1X Wash Buffer afterwhich 200 µl of the VEGF Conjugate was added to each well. The Plate was incubated at room temperature for 2 hours, washed with 1X Wash Buffer and 200 µl of Substrate Solution were applied to each well. After an incubation period of 25 minutes at room temperature, 50 µl of Stop Solution was added, and finally absorbance was measured in each well using a spectrophotometric plate reader (Anthos Labtec, ELISA-Reader, Wals, Austria) at 450/540 nm.

$AC133^+$ cells isolation

An attempt to identify a putative common origin for the different affected lineages in MDS BM. Hematopoietic and EPC were isolated from MDS patients harbouring different genetic markers. For example, BMNCs from patients with a del(20) (q11q13) cytogenetic alteration were washed in complete RPMI medium, resuspended and plated in a 1% gelatine-coated well, where they were left to recover. $AC133^+$ cells were isolated using the mini-MACS immunomagnetic separation system from Miltenyi Biotec, following these procedures: we added 100 µl of FcR Blocking Reagent to total cells and resuspended them in 300 µl of BSA/0,1% PBS buffer. Cells were labeled by adding 100 µl CD133 MicroBeads, incubated for 45 minutes at 4–8 ℃ and afterwards they were washed with BSA/0,1% PBS buffer . The column used for the magnetic separation was placed in the magnetic field of a MACS Separator. Cell suspension was applied to the column, which was washed with buffer,

removed from separator and finally the fraction with magnetically AC133 labelled cells was flushed out using the plunger supplied with the column.

FISH analysis

AC133$^+$ isolated cells were added in a small spot in glass microscope slide for fluorescence in situ hybridization (FISH). The isolated cell suspension was dried at 37ºC and then fixed with methanol/acetic acid (3:1) at room temperature. The commercial probe (Vysis, Downwers Grove, IL) LSI® D20S108 (20q12) (conjugated with spectrum orange fluorophore) used to detect del(20) (q11q13) in AC133$^+$ cells, was applied at the hybridization area. The next step involved denaturation for 5 min at 80ºC and a hybridization step for 16hours at 37ºC. Afterwards, glass microscope slide was washed in 0,4xSSC/0,3%NP40 2 minutes at 73ºC, 2xSSC/0,1%NP40 for 30 seconds at room temperature and finally DAPI was applied to the glass microscope slide which was visualized in a fluorescent microscope (Zeiss AxioImager.Z1 microscope) (using the 63x objective).

Immunohistochemistry

To determine whether EPC shared malignant cell markers/genetic alterations, immunohistochemistry detection for HoxA9 was performed on isolated AC133$^+$ cells already submitted to FISH analysis (described above). Cells were rehydrated by washing in decreasing concentrations of ethanol for 5 min each (100%, 90%, 70%, 30%). Cells were washed with PBS1x for 5 min, after which they were incubated for 5 min at room temperature with 0,2% Triton reagent. After membrane permeabilization, the cells were blocked with BSA 0,1% for 45 min at room temperature.. Cells were incubated with HoxA9 (Santa Cruz Biotechnology) diluted at 1:50 overnight at 4ºC, washed with PBS the next day and incubated with donkey anti-goat Alexa 594 (Molecular probes) diluted at 1:500 in 0,2% Triton reagent for 1 hour at room temperature. After secondary antibody incubation sample was washed in PBS, mounted in a medium

composed with Vectashild and Dapi and examined under fluorescent microscope (63x objective).

The same cells were studied for the two markers and in each marker glass microscope slide coordinates were registered. In the end we could compare different markers in the same cells based in their position.

Normal and malignant BM from murine carginogenesis model and BM biopsies from MDS patients were fixed in 10% buffered formalin for a minimum of 24 hours and decalcified in a rapid bone decalcifier (Perudo00-008; Eurobio, Les Ulis, France) for 3 hours and paraffin embedded. For the immunostaining 2μm sections, togheter with thymus, spleen and lungs from murine model, were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol.

Slides for normal and malignant BM, thymus, spleen and lungs from mice were stained with hematoxilin-eosin. Antigen retrieval for slides for VEGF, CD31 and vwF (Von Willebrand factor) staining was performed as follows: 15 min in Tris-EDTA at 98ºC for VEGF, 6 minutes in citrate buffer in a pressure cooker for CD31 and 20 min in pepsin at 37ºC for vwF. VEGF (Dako Cytomation) was used diluted at 1:50, CD31 (Dako Cytomation) was used at 1:20 and vwF (Novocastra) at 1:30. Then, were incubated 1 hour at 4ºC and final staining was performed in a Dako Thechmate500 Plus (Dako Cytomation) at room temperature. The number of vwF and CD31 immunostained blood vessels in tissue sections was quantified in four different fields at 400x magnification. The intensity of the VEGF immunoreactive reaction was graded in four different fields at 100x magnification (0, no immunoreactivity, 1, weak intensity, 2, moderate intensity, 3, strong intensity).

Mice

For the establishment of an in vivo MDS model, FVB/N mice (15 males and 15 females) were divided in two groups: 10 control mice and 20 sublethally irradiated (3Gy; 300rad) mice in a three irradiation cycle with one month of

interval between them. Every month after the last irradiation, the levels of circulating EPC (Flk^+Sca^+) in peripheral blood were quantified by FACS. Briefly, blood was centrifuged and plasma was collected for VEGF ELISA analysis. Then, cells were incubated 1hour with Sca-1-FITC and Flk-1-PE (BD Pharmigen) in PBS/2% FBS at 4ºC, with rotation. In the end cells were washed and read in the flow cytometer.

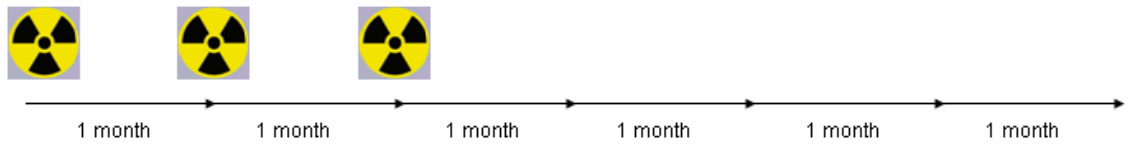


Figure 1. Schematic representation of irradiation cycles in the BM carcinogenesis model.

Statistical analysis

The data were analyzed using SigmaStat software from Jandell Corporation (San Rafael, CA, USA). Groups were compared using double comparison procedures (Rank Sum Test) and pairwise multiple comparison procedures (Dunn's method).

3) Results

Angiogenesis Profile of MDS

Increased progenitor cells in MDS bone marrow

To evaluate the hematopoietic and EPC content in MDS bone marrows FACS analysis was performed in order to detect single positive or double/triple positive $CD34^+$, $CD117^+$, $CD133^+$ and KDR^+ cells. As shown in Figure 1 there was a significant increase in $CD34^+$, $CD117^+$ and $CD133^+$ progenitor cells, $CD133^+KDR^+$ endothelial progenitor cells and KDR^+ endothelial cells in intermediate risk patients. These data suggest there is an increase in the BM progenitor cell pool, in particular EPC in the intermediate risk group the compared to the low risk category and also those undergoing some therapy. There is also the suggestion of increase vasculature (KDR^+ cells) with disease progression.

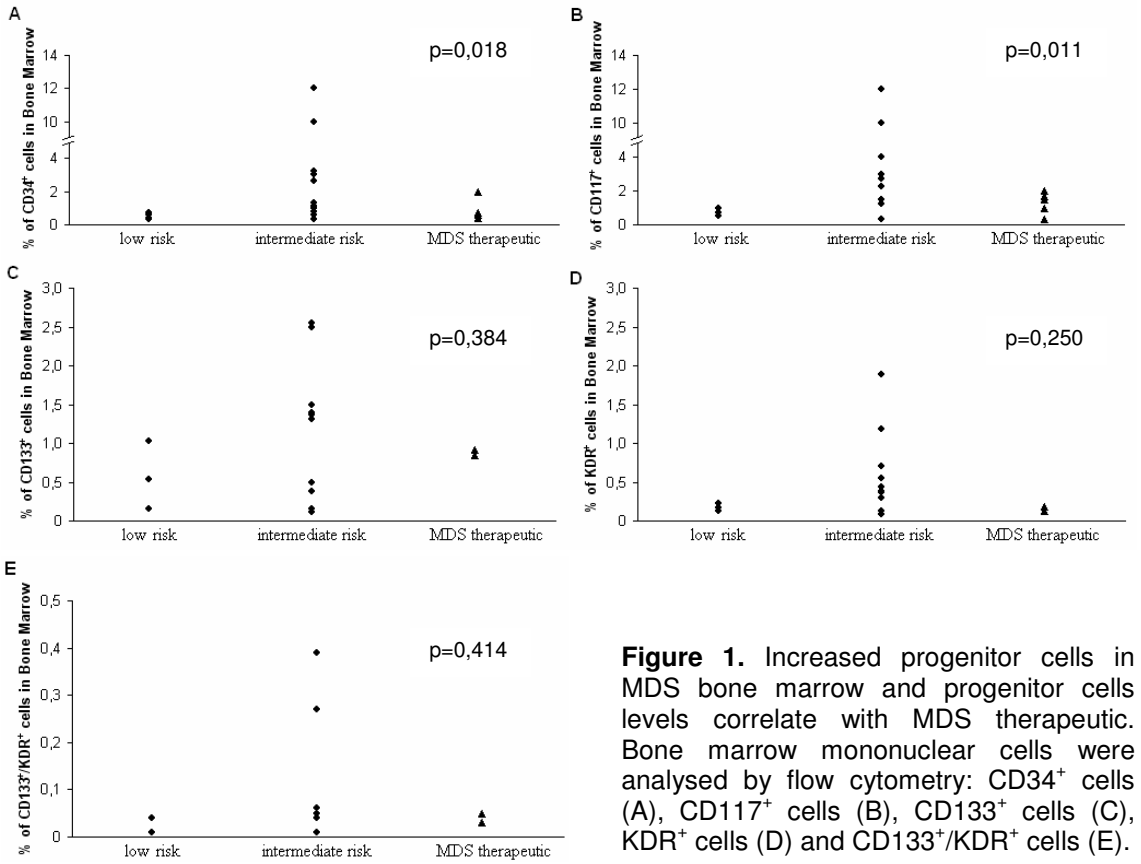


Figure 1. Increased progenitor cells in MDS bone marrow and progenitor cells levels correlate with MDS therapeutic. Bone marrow mononuclear cells were analysed by flow cytometry: $CD34^+$ cells (A), $CD117^+$ cells (B), $CD133^+$ cells (C), KDR^+ cells (D) and $CD133^+/KDR^+$ cells (E).

Progenitor cells levels are found to correlate with MDS treatment

We used the same analysis described previously in patients under MDS therapeutics, like Vidaza, Danazol and Thalidomide, to evaluate MDS bone marrow response to treatment and eventual recover during therapeutic intervention. The frequency of $CD34^+$, $CD117^+$ and $CD133^+$ progenitor cells, $CD133^+KDR^+$ endothelial progenitor cells and KDR^+ endothelial cells in these patients was significantly lower those detected in than intermediate risk patients. Although done in a small group of patients, MDS therapeutics seem to reduce BM angiogenesis (as determined by the number of KDR^+ cells) that was initiated by disease (Figure 1). This observation was exploited further by determining BM microvessel density in the different patient groups.

Apoptosis in MDS bone marrow cells

One of the most important characteristics of MDS BM is the presence of single or multiple cytopenias in mature and immature cells. We analysed the apoptotic index in $CD34^+$, $CD117^+$ and $CD133^+$ BM progenitor cells, $CD133^+KDR^+$ endothelial progenitor cells and KDR^+ endothelial cells using an apoptotic surface marker AnnexinV. There were a constantly higher apoptosis levels in intermediate risk patients both in $AC133^+$ progenitor cells and KDR^+ endothelial cells in MDS bone marrow (Figure 2). Therefore, this progenitor cell population is more frequent in intermediate risk patients (Figure 1), although these cells undergo higher turnover (increased apoptosis rate). Although we analysed only two patients under MDS treatment, it is interesting to notice that the overall BM apoptotic index is lower in treated patients when compared with risk groups.

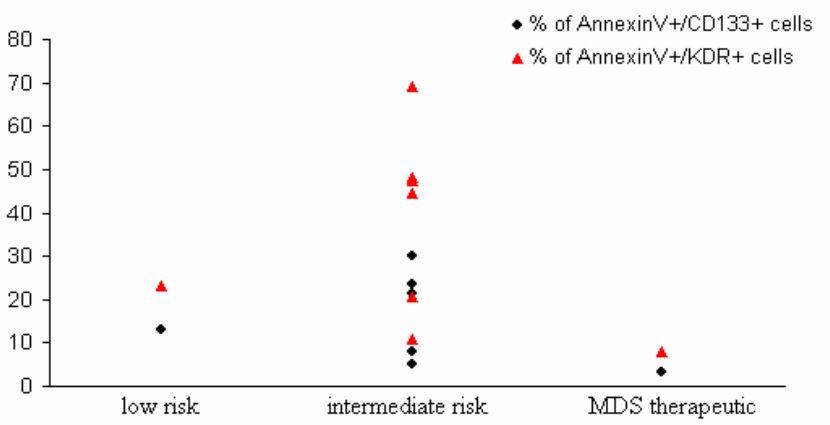
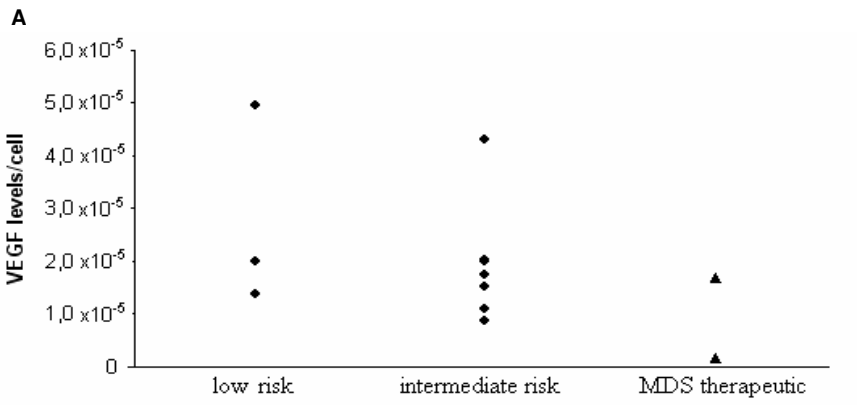


Figure 2. Apoptosis in MDS bone marrow cells. Apoptosis index was measured by flow cytometry with AnnexinV. Intermediate risk group has more apoptotic AnnexinV$^+$/CD133$^+$ cells (A) and AnnexinV$^+$/KDR$^+$ cells (B).

VEGF levels are reduced with MDS therapeutic

Having mesured an increase in CD133$^+$KDR$^+$ EPC and KDR$^+$ endothelial cells in MDS bone marrow we determined VEGF protein levels in bone marrow plasma samples as it is a main angiogenic factor. Surprisingly, VEGF levels among risk groups were similar but in patients under treatment were significantly lower (Figure 3A). These data although obtain from a very small number of treated patients, suggest the response of MDS patients to treatment involves reducing angiogenesis stimulation within the bone marrow. Nevertheless, vascular changes in MDS patients BM have been reported. Therefore, we also performed a more detailed study about mRNA expression of VEGF isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$ and $VEGF_{189}$). While $VEGF_{121}$ showed very weak variation, $VEGF_{189}$ appeared to be highest in MDS intermediate risk patients and the two patients treated with Danazol (Figure 3C).



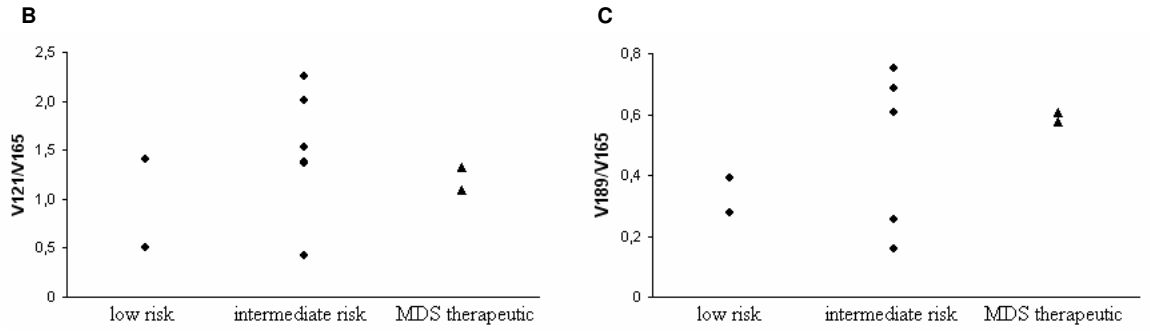


Figure 3. Total VEGF levels are lowered with MDS therapeutic. VEGF levels were measured by ELISA and mRNA expression of VEGF isoforms by quantitative Real-time PCR. MDS patients under treatment have a lower VEGF production per cell. Results are prescuted as the proportion between the less abundant isoforms, $VEGF_{121}$ and $VEGF_{189}$, and the most abundant isoform, $VEGF_{165}$.

TNF-α and TGF-β mRNA expression

In order to investigate the putative contribution of other angiogenic factors present in MDS BM, or their regulation in an abnormal BM, we analysed the levels of expression of TNF-α, TGF-β and PlGF (member of the VEGF family that binds only VEGFR1, Flt-1) mRNA. We observed a tendency for increased TNF-α and TGF-β mRNA in intermediate risk patients (Figure 4), but no relevant differences in PlGF mRNA expression (data not shown). These results support the idea of an abnormal BM microenvironment in MDS patients and suggest the higher apoptotic indexes in mature cells seen in more advanced diseased stages, may result from the action of TNF-α.

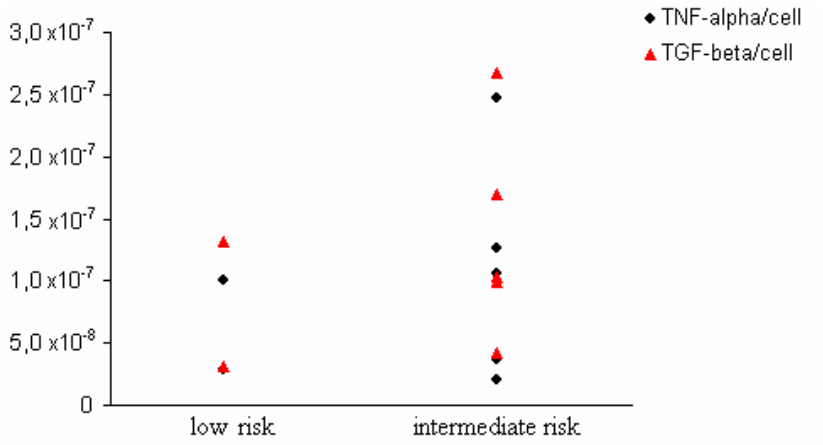


Figure 4. TNF-alpha, TGF-beta mRNA expression. TNF-α/cell and TGF-β/cell mRNA expression was determined by Real-time PCR.

Blood vessel quantification

To quantify blood vessels in BM of patients with MDS we used vwF and CD31 (vwF is produced in megakaryocytes and endothelium and CD31, PECAM-1, identifies platelet endothelial cell adhesion molecule) markers, and VEGF as angiogenesis indicators (Figure 5). As shown in figure 6, vessel number is higher in intermediate risk patients when compared with the lower risk. Patients under treatment have no significant decrease in this parameter. CD31 is an endothelial surface marker and it is augmented in the BM of MDS patients as we have seen through KDR^{+} cells. As mentioned before, these results suggest there is an increase in BM vasculature with disease progression.

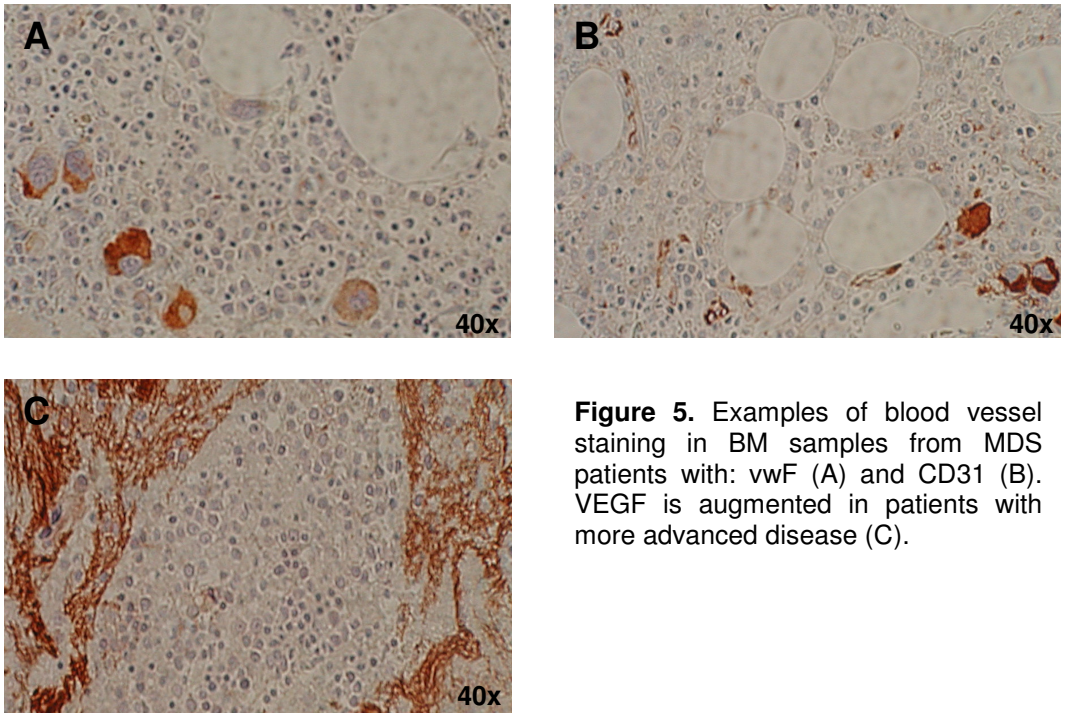


Figure 5. Examples of blood vessel staining in BM samples from MDS patients with: vwF (A) and CD31 (B). VEGF is augmented in patients with more advanced disease (C).

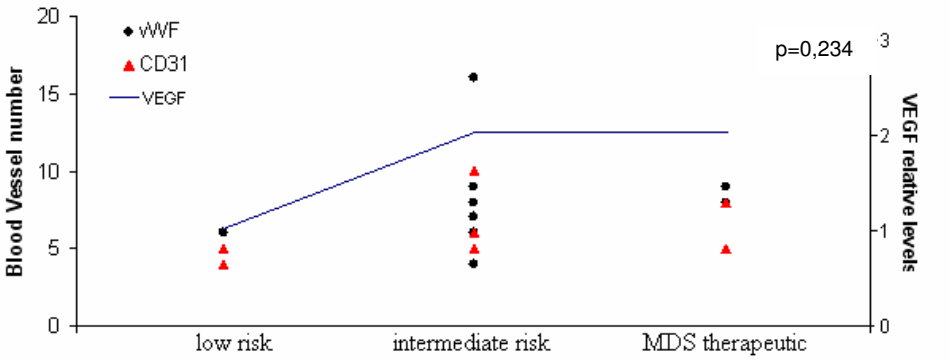


Figure 6. Blood vessel quantification MVD with angiogenic markers: vwF, CD31 and VEGF

Increased EPC in patients that received radiotheraphy

Secondary MDS is caused by chemotherapy or radiotherapy treatment for other diseases. In our group of MDS patients the ones treated previously with radiotherapy showed high levels of $CD133^+$ progenitor cells, $CD133^+KDR^+$ endothelial progenitor cells and KDR^+ endothelial cells (Figure 7). These results suggest that radiotherapy treatment changes BM microenviroment potentiating a secondary MDS and BM angiogenesis. Notably, unpublished data in our laboratory has shown that irradiation induces TNF-α production in BM cells.

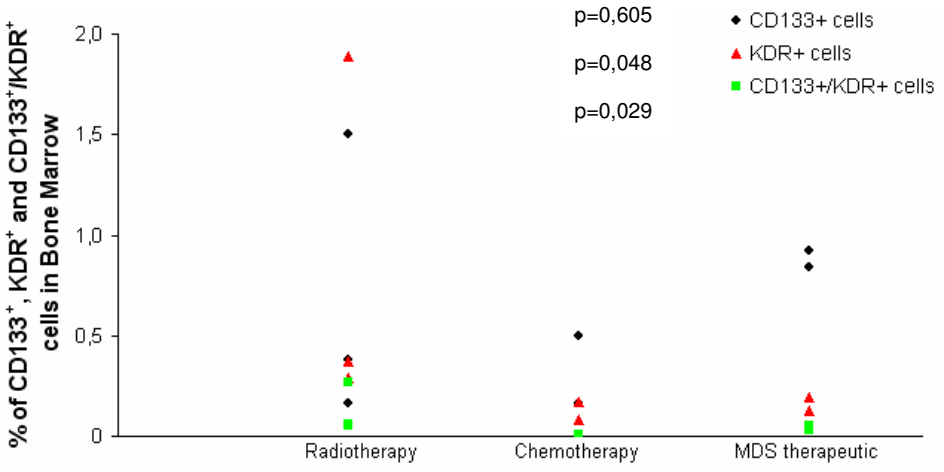


Figure 7. Increased EPC in patients that had received radiotherapy (alone or in combination with radiotherapy). Secondary MDS patients were analysed for $CD133^+$ cells, KDR^+ cells and $CD133^+/KDR^+$ cells in Bone Marrow.

Endothelial progenitor cells are malignant cells in MDS

Taken together, our data (although obtain from a small group of patients) suggests some of the cellular and molecular changes that take place in MDS BM involve modulation of the vascular compartment. Increased EPC and EC are detected in the intermediate risk patients; surprisingly, they do not completely correlate with total VEGF levels, but are accompanied by a specific isoform. As in other hematologic diseases, modulation of the BM vascular content in MDS may be interpreted in two ways: either EC and EPC increase in response to BM angiogenesis regulation/increase and sustain/support the expansion of malignant clones, or EPC/EC are part of the malignant transformation process.

To address the question if EPC are malignant cells in MDS BM we isolated $CD133^+$ cells from a sample of a low-risk patient with a cytogenetic abnormality del(20q). FISH analyses showed that $AC133^+$ cells are already transformed in early MDS stages. Immunohistochemistry analyses for endothelial marker HoxA9 were made in the same cells and, as shown in figure 8 and 9, suggest that EPC and possibly other progenitors ($AC133^+HoxA9^+$) are already transformed, ie, malignant, in MDS bone marrow.

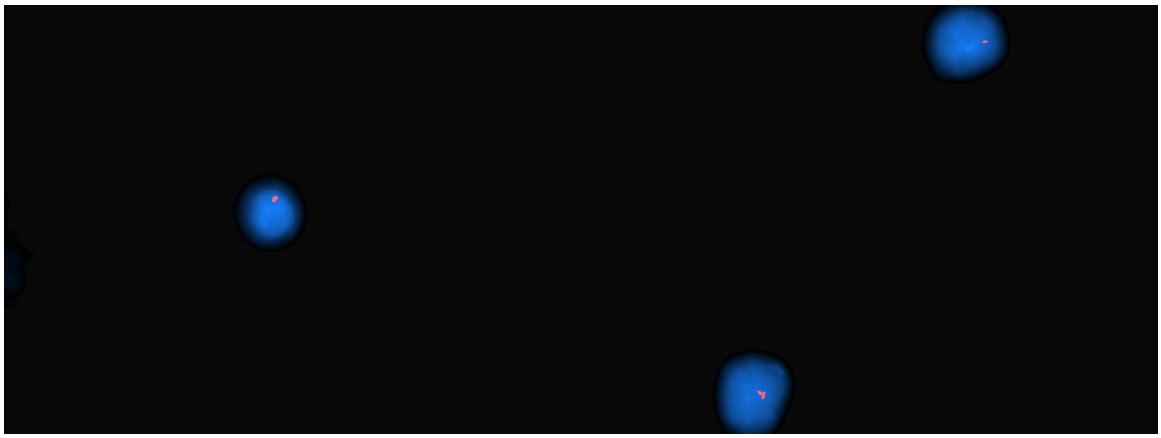


Figure 8. FISH for del(20q) in MDS $AC133^+$ cells. $AC133^+$ cells are malignant cells in MDS bone marrow.

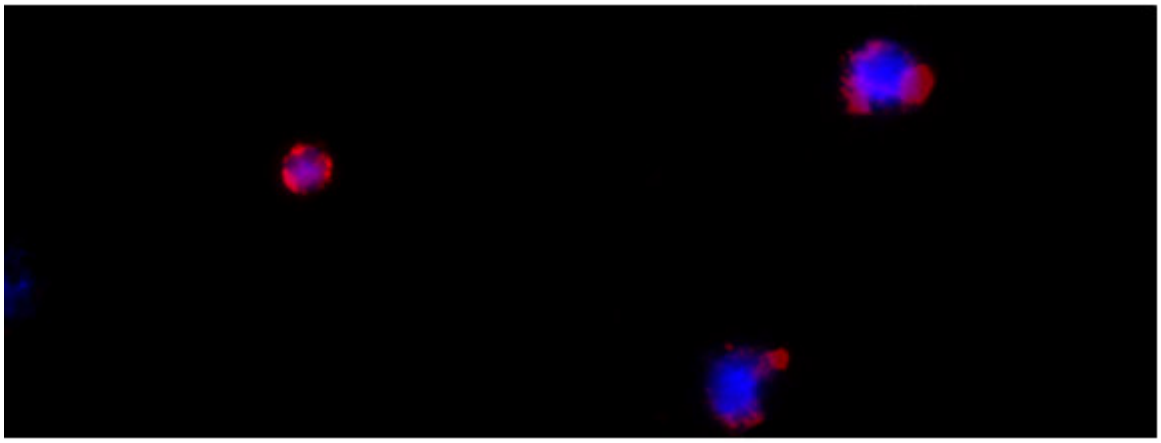


Figure 9. Immunohistochemistry for HoxA9 in MDS AC133^{+} cells.

Murine Carcinogenesis Model

In this mouse BM Carcinogenesis model established in our laboratory, BM leukemia incidence (at the end of the assay; 11 months after the last irradiation) was approximately 63% in the irradiated group with an equal thymoma and acute leukemia incidence (Figure 11A). About 37% of the irradiated mice presented signs of disease 2,5 months after the last irradiation (early disease group) while the other 26% presented signs of disease 7,2 months after the last irradiation (late disease group). Of importance to the work presented in this thesis (focussing on pre-leukemia), we analised the early disease group further. Regardless, the two groups of mice that developed BM disease had a decreased number of circulating EPC when compared with the no disease group. In the early disease group the decrease was more evident (Figure 12). In addition, mice with no disease presented low levels of VEGF compared with the two groups that developed tumors (Figure 11B). Taken together, these results suggest that BM EPC may modulate the onset of haematological diseases and BM microenvironment and correlate with the time of disease.

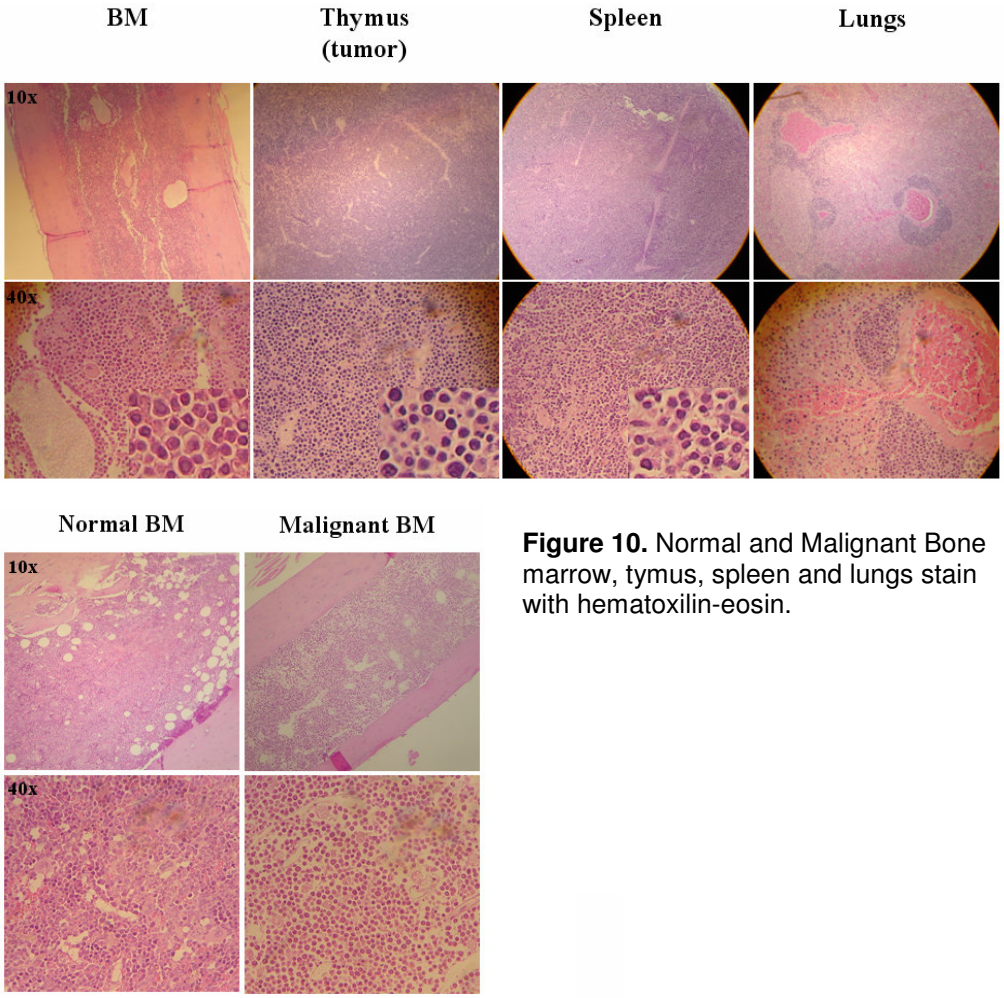


Figure 10. Normal and Malignant Bone marrow, tymus, spleen and lungs stain with hematoxilin-eosin.

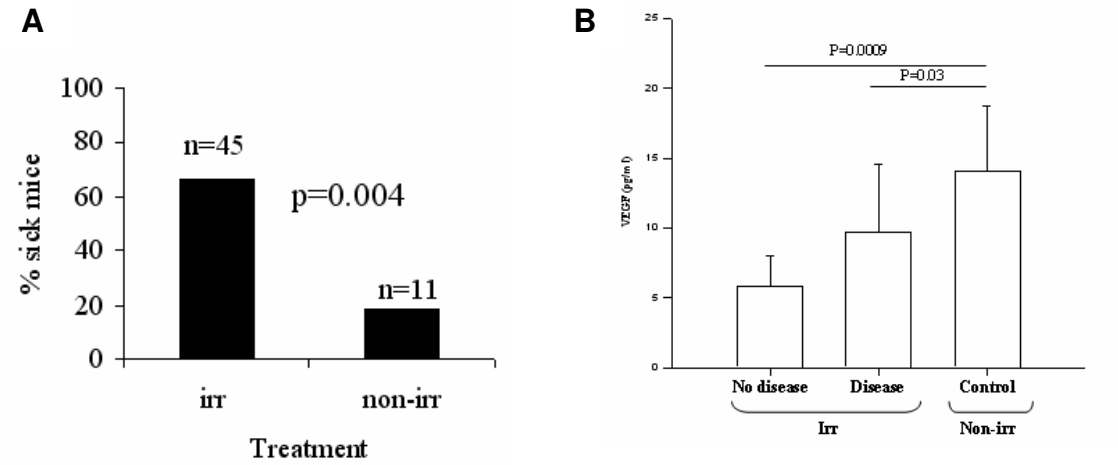


Figure 11. Tumor incidence (A) and VEGF levels in circulation (B) between irradiated and non-irradiated mice.

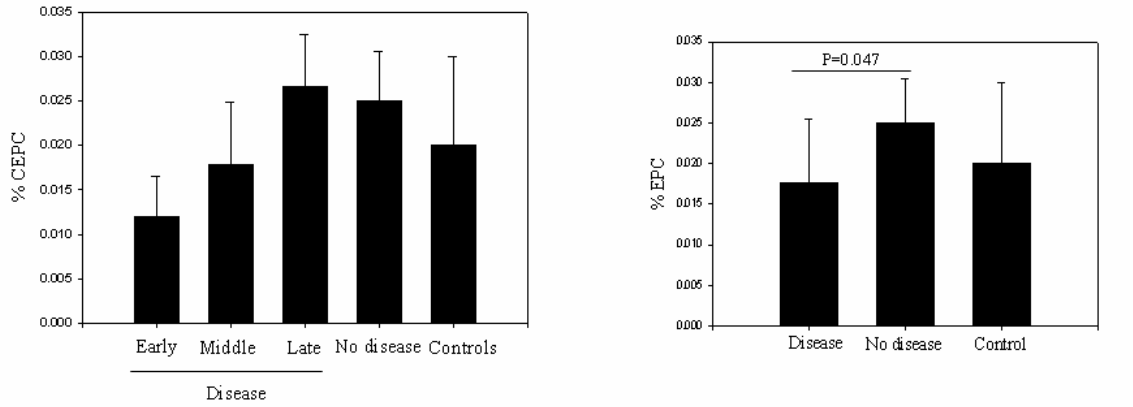


Figure 12. Circulating EPC levels correlate with time of disease onset.

4) Discussion

Angiogenesis induction is one of the crucial characteristics in tumors pathophysiology. In haematological diseases like MDS, angiogenesis and angiogenic inducers have an important contribution for disease development. Microvessel density (MVD) measures angiogenesis through immunohistochemical biological markers, giving considerable information about disease progression and prognosis. A significant increase in BM MVD in MDS and AML[32] and a higher expression in angiogenic factors such VEGF[44], bFGF, TNF-α[45], Ang1, Ang2 and VEGFR2[46] were demonstrated. Most of the factors are probably secreted by neoplastic hematopoietic cells.

Several studies have already demonstrated the importance of BM-derived EPC in tumor vasculature. In patients with AML, $AC133^+$ progenitors with known cytogenetic lesions are augmented in peripheral blood. This could indicate a possible role of $AC133^+$ progenitors, and subsequently EPC, in BM abnormal vasculogenesis and leukemia progression.[47] It was also reported that in peripheral blood of MDS patients circulating EP and circulating EC are augmented and there is a direct correlation with MVD in BM.[48] Only a few studies have suggest a contribution of EPC, particularly immature cells with the EPC marker AC133, in BM abnormal vasculature in MDS patients. MDS BM also expressed VEGF levels that correlate with these immature cells which revealed a probably contribution of these cells in disease dissemination.[49] Our results support these data. First, progenitor cell pool is augmented in patients with a more aggressive phenotype, in particular EPC and EC suggesting changes in vasculature. Immunohostochemistry analysis supported the idea of angiogenesis increase particularly with disease progression. Abnormal levels of angiogenic factors such total VEGF and $VEGF_{189}$, TGF-β and TNF-α change BM microenvironment, $VEGF_{189}$ appears to be the most important VEGF isoform in this malignancy and augmented levels of TNF-α in BM relate with higher apoptotic indexes are observed in progenitor and mature cells of advanced risk stages.

Our results revealed that $AC133^+/HoxA9^+$ immature cells have already a malignant transformation in MDS BM patients contributing for abnormal

vasculature and malignancy development. It is not yet clarified if apoptosis is affecting only normal or also transformed cells in MDS BM. Genetic lesions in BM-derived EPC can affect bone marrow vascularization in different ways contributing for the malignant process: EC differentiated from transformed EPC will affect vessels formation and structure; altered EC may also contribute for hematopoiesis deregulation though abnormal secretion of angiogenic factors or different signalling in cell-cell interaction. However, increase of transformed EPC and EC can be a response to support the malignant clone development.

More recently, our unpublished data suggests that Thalidomide may have the ability of induce EPC apoptosis. Thalidomide is known to have an anti-proliferative and pro-apoptotic effect in tumor cells by reducing cell synthesis and interfering with VEGF action.[50] We shown that MDS patients under MDS specific treatment, like Thalidomide, have reduced EPC an d EC, angiogenic factors and apoptosis. This anti-angiogenic drug reduces vascularization that was initiated by disease. In our group of patients under MDS specific treatment angiogenesis is reduced and probably the potential MDS progression to acute leukemia.

Interestingly, we observed that patients with secondary MDS caused by radiotherapy show a modulation of the vascular BM compartment by increased EPC and EC. This result is supported by our murine irradiation model in which almost 40% of mice present disease sings shortly after irradiation. Our group has shown that TNF-α is stimulated by BM radiation, which may be in the origin of increased apoptotic rates in BM cells changing BM microenvironment homeostasis.

The murine carcinogenesis model is extremely relevant to test the importance of all these findings in a true experimental setting, first, because it is an in vivo model and then because we can exploit changes that happen within BM since disease onset, allowing us to control disease progression thoughout time. After irradiation, mice BM cells suffer cellular and molecular damages that, in most cases, result in leukemia development. In initial months after radiation BM microenvironment is similar to MDS BM, a “pre-leukemic” stage: cell number is reduced, the number of circulating EPC decreases in the peripheral blood and there are increased levels of VEGF.

In conclusion our results suggest that BM EPC may act as a “pre-malignant” clone and may also have an important role in the progression of haematological diseases modulating BM angiogenesis. Particularly in MDS, the BM vasculature and angiogenic soluble factors regulation correlates with disease progression, hematopoietic deregulation and development to acute leukemia.

Future perspectives

The clinical relevance of this study relies in the importance of identifying EPC as a malignant cell in BM disease development, and according to our results in a murine carcinogenesis model, the possible use of measuring circulating EPC as a disease marker in some haematological diseases, with prognostic relevance.

It is also clear that angiogenesis is a conditional feature for MDS progression and its reduction, through anti-angiogenic drugs, may delay disease progression. It would be interesting to test the impact of Thalidomide in the murine carcinogenesis model, as well as the effect of blocking some angiogenic factors such as TNF-α and the specific isoform $VEGF_{189}$ during disease progression. Peripheral blood and BM analysis for EPC levels and other angiogenesis markers will answer how the BM is responding. This would allow us to clarify and understand BM regulation and the mechanisms that contribute to the development of haematological malignancies.

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