MECHANISMS THAT REGULATE CELL TURNOVER OR TRANSFORMATION IN THE BONE MARROW MICROENVIRONMENT

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The Bone marrow (BM) is the major hematopoietic organ in adulthood, 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 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. Hematopoietic tissue consists in a various type of mature blood cells and their precursors.

Bone marrow microenvironment consists in BM stroma cells and factors, growth factors and cytokines, provided from stroma and blood vessels cells. Stroma cells have been considered the responsible in maintaining BM microenvironment but vasculature is also important because it is the oxygen supplier and exerts other functions (see below). BM stroma consists in different types of cells: endothelial cells, macrophages, adypocytes, fibroblasts, osteoblasts and extracellular matrix elements like binding proteins and fibronectin. For hematopoiesis to take place it is necessary a stable microenvironment that produces/expresses factors suitable for their migration, differentiation and lineage commitment.

ENDOTHELIAL CELLS IN THE BM MICROENVIRONMENT

Similar to what is seen in other organs, endothelial cells (EC) exert crucial functions within the BM microenvironment, namely by modulating the trafficking and the terminal differentiation of hematopoietic cells. Several studies have focused on the identification and function of adhesion molecules and chemokines secreted by bone marrow endothelial cells (BMEC), including ICAM-1, E-selectin (adhesion molecules), SDF-1 (chemokines) among others; this way, BMEC have been shown to exert a crucial role in cell trafficking in and out of the BM microenvironment. Subsequent studies have exploited these properties in transplant settings, for instance.

FACTORS THAT PROMOTE ENDOTHELIAL TURNOVER WITHIN THE BM MICROENVIRONMENT

Angiogenic growth factors such as Vascular endothelial growth factor (VEGF) promote the survival and modulate the hematopoietic-supporting

functions of BM endothelia. Conversely, abnormal production of VEGF within the bone marrow may promote endothelial proliferation, and consequently affect the hematopoietic microenvironment.

The expansion of endothelial cells within the BM microenvironment may thus provide a source of nutrients and oxygen, needed for a transformed and highly proliferative leukaemia clone/s. Therefore, similar to solid tumor growth, activation of the angiogenic program within the BM is obviously critical for the progression of leukemias, and bone marrow diseases in general.

More recently, we have tested the hypothesis that BMEC turnover/apoptosis might condition BM function and could have a crucial role in BM carcinogenesis. First, we studied the importance of TNF-alpha, which is abundantly secreted in the BM microenvironment and has the capacity to induce hematopoietic and vascular cell apoptosis (depending on the dose). Interestingly, TNF-alpha levels increase and show a remarkable correlation with BM recovery following irradiation (Figure 1). In vitro experiments have shown that blocking TNF-alpha in total BM cell cultures decreases apoptosis incidence, most remarkably in the endothelial lineage (Figure 2). Therefore, it appears that TNF-alpha is induced in the BM microenvironment and promotes BMEC apoptosis following irradiation. Whether TNF-alpha is induced and plays a role in BM carcinogenesis is not known and is the recent subject of *in vivo* studies we are currently performing. In this regard, we have preliminary evidence that TNF-alpha deficient mice may be partially protected from the leukaemia-inducing effects of irradiation. The mechanisms whereby the absence of TNF-alpha may exert a protective effect against a leukaemia carcinogenic stimulus are not known and are currently being investigated in the laboratory, although the background hypothesis is that decreased turnover of the endothelium within the BM microenvironment may have protective effects.

BM ENDOTHELIUM IN PRE-LEUKEMIA AND DURING LEUKAEMIA ONSET

Bearing in mind the importance of BMEC in leukemic disease progression (angiogenesis) we have recently focused our attention in the group of diseases termed myelodisplastic syndromes (MDS). These are common BM complications in oncology patients treated with radio- or chemotherapy. MDS are interesting diseases to study the importance of the BM microenvironment in the regulation of homeostatic BM function and also during malignant transformation, in that they represent a "pre-leukemia" stage: first there is evidence of BM apoptosis (which may be quite

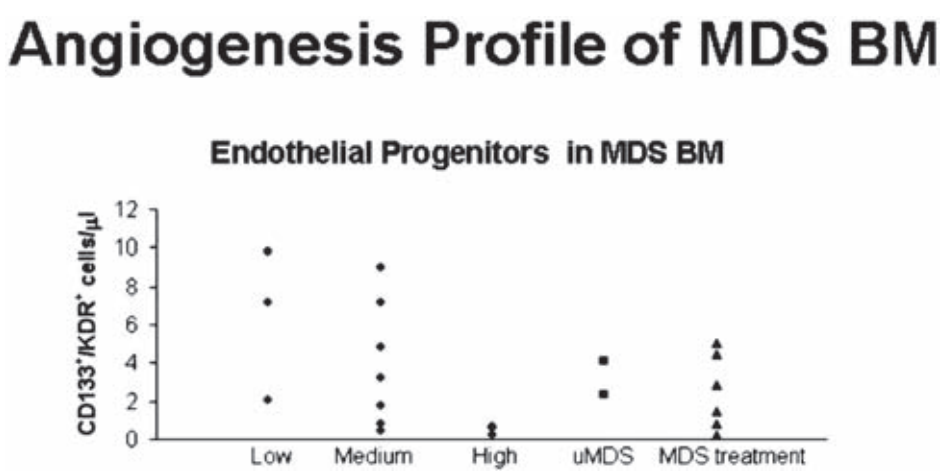

Fig. 1 – TNF-alpha levels increase in correlation with BM recovery following radiation.

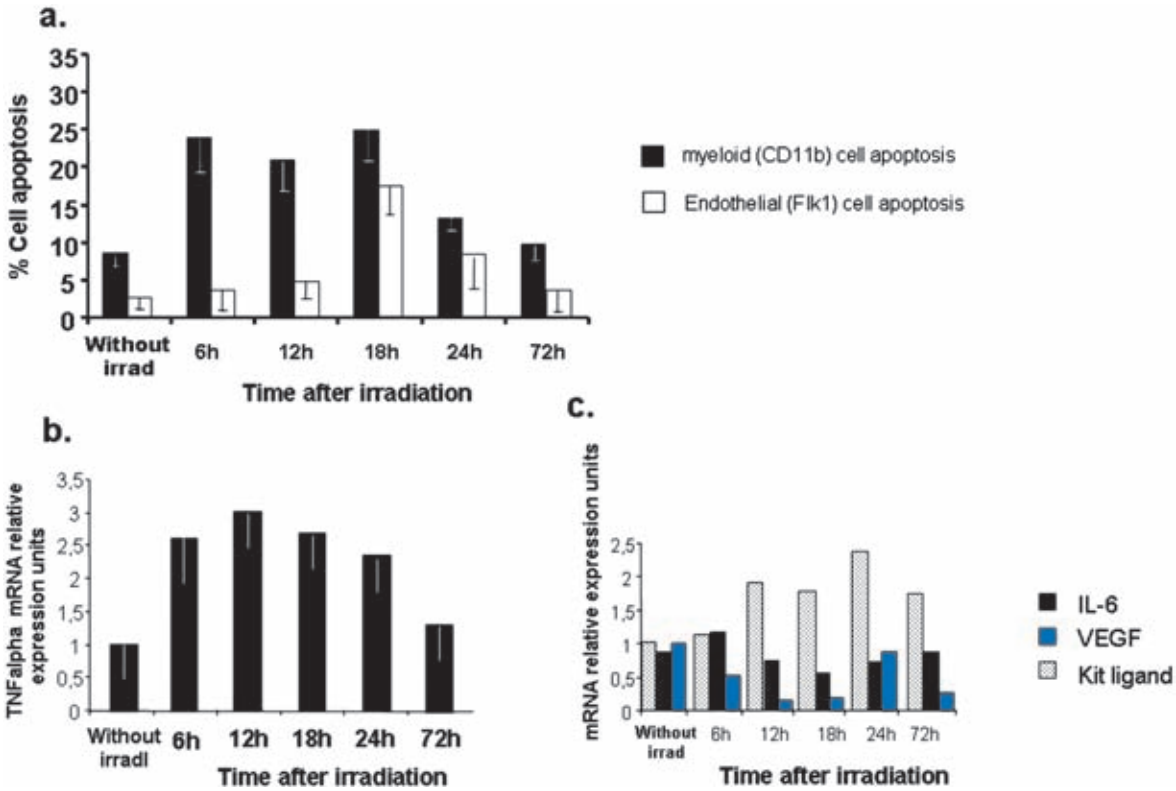

Fig. 2 – In vitro TNF-alpha blocking in total BM cell cultures decreases apoptosis incidence, most remarkably in the endothelial lineage

significant leading to cytopenias), subsequently BM turnover and selection of aggressive clones that will produce a very aggressive and un-curable acute leukemia. According to recent classifications suggested by the World Health Organization, high risk MDS have a high probability (over 80%) of progressing into acute leukaemia, while medium risk and low risk patients have reduced probabilities. Since the progression of MDS usually takes several years, these diseases provide a very interesting biological model where to study BM turnover, apoptosis and the involvement of different cell types in BM disease onset and/or progression. In our case, it was possible to isolate BM cells from patients at different stages of the disease, and in a few cases it was even possible to obtain samples from a same patient while in disease progression.

In this heterogenous group of patients, we have quantified and studied in detail the involvement of the BM vasculature (endothelial content) at the early stages of the affected BM and also during overt leukaemia progression. Interestingly, the BM endothelial progenitor (and mature endothelium) content is increased in low and medium-risk MDS patients, while the BM microvascular densities increases during the progression to leukaemia, accompanied by a significant increase in the levels (bone marrow and peripheral blood) of VEGF, and specifically of its VEGF 189 amino acids isoform (Figures 3-4).

The data obtained thus far suggests that MDS progression to leukaemia may involve (or be dependent on) an angiogenic outburst (angiogenic switch) within the BM. Nevertheless, we have evidence suggesting there is a "comsuption" of the BM endothelial content, namely in the number of endothelial progenitors, in early stages of these diseases, which is still

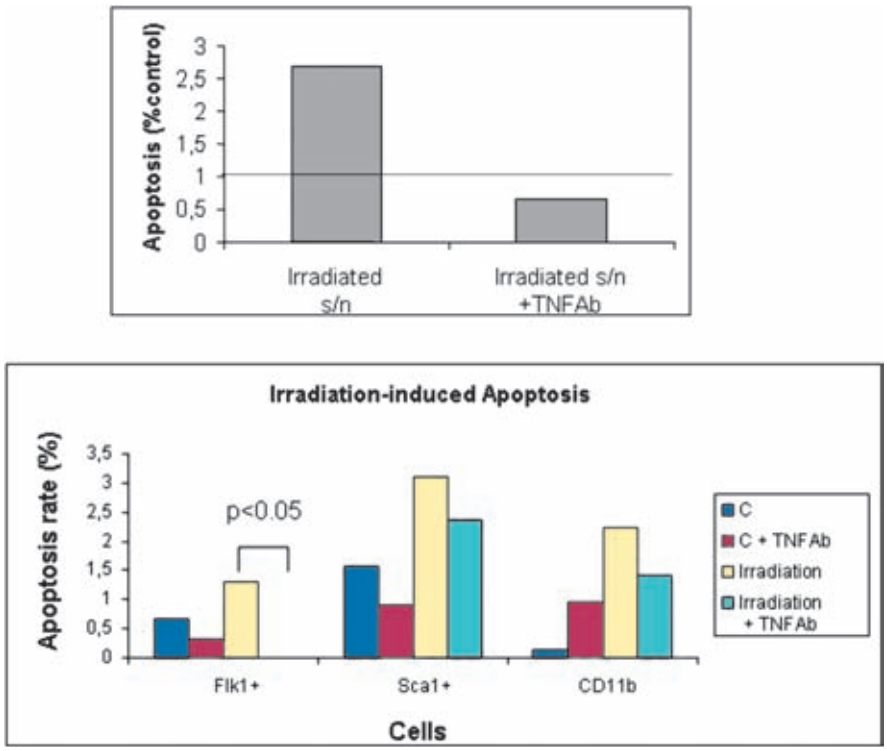

Fig. 3 – The BM endothelial progenitor (and mature endothelium) content is increased in low and medium-risk MDS patients.

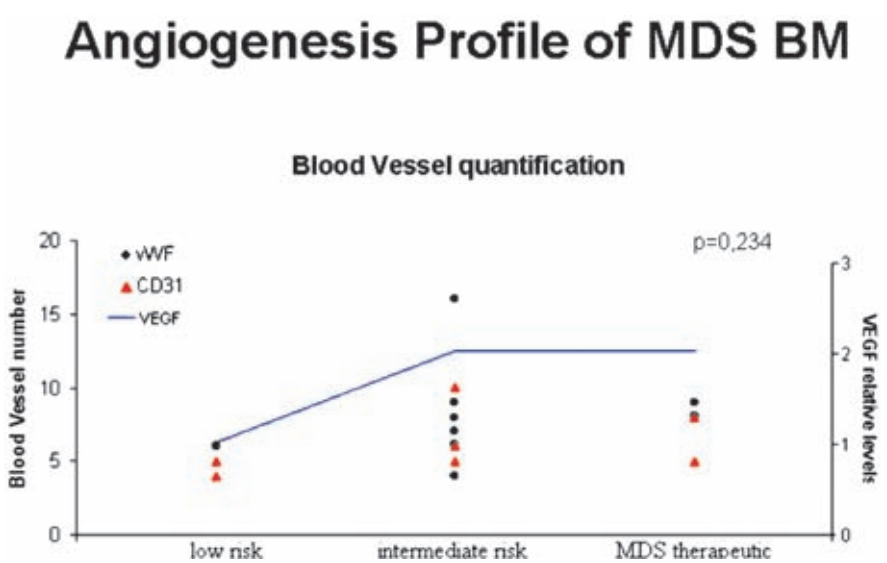

Fig. 4 – In MDS bone marrow there is an increase in vasculature with disease progression.

unexplained. Currently, we are attempting to determine the signals (hypoxia, acidosis, hypoglycaemia, etc) and the mechanisms involved in VEGF production and VEGF splicing within the BM microenvironment. In addition, recent data suggests that selective apoptosis (which may be triggered by TNF-alpha levels, for instance) of endothelial/endothelial progenitors in the BM microenvironment may facilitate leukaemia onset.

AUTOCRINE/PARACRINE STIMULATION OF LEUKAEMIA GROWTH BY VEGF

We have previously shown that acute leukemia blasts evidence an abnormal expression of VEGF receptors, whose stimulation modulates their survival, proliferation and migration. While the common expression of VEGF receptors on endothelial cells and malignant leukaemia blasts strongly suggests a putative "leukaemia-carcinogenesis hit" may be exerted at the level of a common precursor or stem cell, the fact that malignant bone marrow cells express and respond to the effects of this abundant pro-angiogenic growth factor, has been of great interest to the field of malignant hematology. In detail, we have shown that VEGF signalling on leukemia cells, via VEGFR-2 promoted their proliferation, migration while VEGFR-3 stimulation protected leukaemia cells from chemotherapy-induced apoptosis. We also demonstrated that the growth of subsets of acute leukemias is supported by both internal and external VEGF/VEGFR-2 autocrine loops, and that such loops lead to the activation of distinct signalling pathways.

More recently, we have shown acute lymphocytic leukemia expansion and the onset of extramedullary disease (EMD) involved migration of the leukemia cells, within the BM microenvironment and into the peripheral circulation. This migration was induced at least partly due to VEGF/PLGF stimulation of leukemia cells within the BM. Detailed biochemical analysis revealed that VEGF-induced leukemia migration within the BM microenvironment involved interaction between VEGFR-1, caveolae-like structures in the plasma membrane, and a close connection (activation and biochemical interaction) with the cell cytoskeleton of actin and tubulin.

Our data has revealed in detail the mechanisms whereby VEGF, produced in abundance within the bone marrow in pre-leukemia (MDS) and also during full blown leukaemia, induces transformation within the BM microenvironment suppressing selectively certain lineages, leading to the expansion of endothelial cells (and precursors) and promoting the survival (movement, proliferation and protection to apoptosis) of subsets of malignant clones.

Taken together we have been exploiting the hypothesis that sustained exposure of the bone marrow microenvironment to abnormal TNF-alpha and VEGF levels may result in selective apoptosis/turnover of endothelial cells/precursors in the BM, disruption of hematopoietic differentiation, and also promote the onset and subsequent expansion of subsets of acute leukemia. Future work includes revealing the mechanisms whereby BM endothelium may produce/establish a protective BM microenvironment to impede BM transformation in response to carcinogenesis stimuli including radiation.

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