Myeloma and other monoclonal gammopathies - Biology 2

P932

HIF-1A INHIBITION BLOCKS THE CROSS TALK BETWEEN MULTIPLE MYELOMA PLASMA CELLS AND TUMOR MICROENVIRONMENT

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Background: Multiple Myeloma (MM) is a clonal B-cell malignancy characterized by accumulation of malignant plasma cells (PCs) within the bone marrow (BM) in close contact with stromal cells (SCs) which secrete growth factors and cytokines, promoting tumor cell growth and survival. The rapid progression of MM is dependent upon cellular interactions within the BM microenvironment, and novel agents targeting this interaction appear to be promising therapeutic strategies for the treatment of MM tumor expansion.
Unlike most other organs, the BM microenvironment is physiologically hypoxic, a pre-requisite for normal BM hematopoiesis. It is well established that hypoxia is an important selective force in the evolution of tumor cells and a stabilization of HIF-1α protein has been documented in several human cancers. While the role of hypoxia in the pathogenesis of hematologic malignancies has yet to be elucidated, recent animal studies have shown that changes in oxygen levels within the BM microenvironment support the survival and expansion of MM cells. Furthermore, some drugs active in MM, such as Bortezomib and Lenalidomide, are believed to exert their effects in part by interfering with hypoxia-induced signaling cascades.
Aims: Given the importance of the BM microenvironment in MM pathogenesis, we investigated the possible involvement of HIF-1α in the PCs-BMSCs interplay.
Methods: A panel of MM cell lines (MM1.S, U266, OPM-2, RPMI8226) and primary samples from MM patients were cultured *in vitro* in the presence of clinically achievable doses of EZN-2968 (a small 3rd generation antisense oligonucleotide against HIF-1α, to inhibit HIF-1α functions) in normoxia ($p0_2$ 21%) culture conditions.
Results: We have already shown that EZN-2968 is highly specific for HIF-1α mRNA and it results in a long lasting and time dependent inhibition of HIF-1α protein level. Herein, we provide evidence that the interaction between MM cells and BMSCs is drastically reduced upon HIF-1α down-modulation. Notably, we showed that upon exposure to HIF-1α inhibitor, neither the incubation with IL-6 nor the co-culture with BMSCs were able to revert the anti-proliferative effect induced by EZN-2968. Moreover, we observed that EZN-2968 down-modulates cytokine-induced signaling cascades after a short incubation, and seems to induce a negative modulation of those transcripts previously shown to reflect the activation state of specific tumor cell pathways (cell proliferation and survival). This observation was also supported by gene expression profile experiments. One of the key finding of our study is that PC attachment to the extracellular matrix protein was markedly reduced in the presence of EZN-2968. The effects of HIF inhibition on MM cell adhesion are quite intriguing, since MM pathogenesis is dependent upon the interaction of MM cells with the SCs.
Summary and Conclusion: Taken together, these results strongly support the concept that HIF-1α plays a critical role in the interactions between BMSCs and PCs in MM. We conclude that HIF inhibition may be an attractive therapeutic target for MM.

P933

THE NOTCH PATHWAY CONTROLS MULTIPLE MYELOMA CROSSTALK WITH THE OSTEOCLASTOGENIC NICHE

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Background: Multiple myeloma (MM) is an incurable hematological tumor stemming from malignant plasma cells that accumulate in the bone marrow (BM) and establish interactions with BM stroma, promoting tumor survival and bone disease due to unbalanced bone deposition and resorption. The Notch family consists of 4 receptors, Notch1 to 4, which once activated, act as transcription factors. The activation is triggered by Jag1-2 and DLL1-3-4 ligands. Notch plays a key role in bone tissue remodeling and skeletal development. Notch signaling is deregulated in MM and plays a role in MM pathogenesis by modulating tumor cell biology, as well as pathological interactions with BM niche. The myeloma-associated alteration of Notch signaling consists in the aberrant expression of the ligands Jag1 and Jag2 by MM cells, resulting in Notch signaling activation in both tumor cells and BM stroma cells.
Aims: The aim of this work was to investigate the role of Notch signaling in MM-driven osteoclastogenesis. To address this issue we assessed:
• The role of Notch in osteoclast (OCL) differentiation and activity;
• The contribute of different Notch isoforms in OCL development;
• The involvement of Notch pathway in MM cell osteoclastogenic properties.
Methods: Cells were maintained in complete DMEM medium with 10% heat inactivated FBS. DAPT was added to the medium at a final concentration of 50μM. Recombinant mouse RANKL was used at the final concentration of 50ng/ml. Jagged1 recombinant peptide was used at 0.5μg/ml. anti-RANKL neutralizing antibody was used at 0.1μg/ml. OCL differentiation of Raw264.7 cells was induced by treating them with mRANKL or co-culturing with MM cells or their conditioned medium (CM). After 5-7days cells were stained using the TRAP Kit and counted. For bone resorption assay, Raw264.7 cells were cultured on Osteo Assay Surface plates under differentiation conditions. After 7-10 days, the plates were washed in 5% sodium hypochlorite solution. Images of the resorbed areas on the plates were captured and the percentage of resorbed area was measured by using the Wimasis image analysis software (Wimasis GmbH). Select RNAi™ siRNA system (Invitrogen) was used according to the manufacturer's guidelines for the selective knock-down of Jag1 and Jag2. Transfection was performed by electroporation using two plasmid carrying intracellular Notch1 (ICN1) and Notch2 (ICN2). Total RNA was isolated using TRI-Reagent. cDNA was prepared through MMLV reverse transcriptase, then quantitative PCR (qPCR) was performed by Maxima SYBR Green qPCR Master Mix. ELISA Assay was performed using biotin-conjugated goat anti-human RANKL (Merck-Millipore) and Streptavidin-HRP-labeled secondary antibody.
Results: We demonstrates that Notch signaling drives MM cell-induced osteoclastogenesis. The underlying molecular mechanisms is based on MM cell-derived Jagged ligands ability to efficiently drive osteoclastogenesis by contemporaneously activating Notch signaling on tumor cells and osteoclasts. Notch signaling activation in MM cell promote the release of the osteoclastogenic receptor activator of NF-kB ligand (RANKL). RANKL, in turn, promotes within OCL precursors Notch2 signaling which drives osteoclastogenesis completion by promoting the transcription of osteoclastogenic master genes, such as *Tartrate-resistant acid phosphatase* (TRAP) and *Receptor Activator of Nuclear Factor κ B (*RANK) and the autonomous secretion of RANKL by OCL precursors. Remarkably, MM-induced osteoclastogenesis can be disrupted by silencing Jagged1 and Jagged2 Notch ligands in MM cells.
Summary and Conclusion: Our findings make Jagged1 and Jagged2 new promising therapeutic targets to hamper MM-associated bone disease and co-morbidities, lacking the toxicity of the currently used drugs which contemporaneously affect the signaling of all Notch receptors.

P934

JAGGED-INDUCED NOTCH SIGNALING PROMOTES ENDOGENOUS AND BONE MARROW-MEDIATED DRUG RESISTANCE IN MULTIPLE MYELOMA

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Background: Multiple myeloma (MM) represents 10% of all hematological malignancies. Malignant plasma cells accumulate in the bone marrow. Although in the last 10 years new drugs such as immunomodulators or proteasome inhibitors increased patients' survival, MM remains still incurable mainly due to the development of endogenous or BM mediated drug resistance. Therefore it is crucial to find new therapeutic targets. The deregulated expression of two Notch ligands, Jagged1 and 2, activates Notch pathway both in MM cells and in bone marrow stromal cell (BMSC) which express Notch receptors. Several Notch downstream effectors are involved in MM cell growth, survival and proliferation, *i.e.* IL-6, SDF-1alfa, CXCR4, NF-kB, VEGF and IGF.
Aims: The aim of this study was to evaluate the role of Notch signaling in endogenous and BMSC-promoted drug resistance in MM, focusing on the contribution of the chemokine axis CXCR4/SDF-1alfa.
Methods: The human MM cell lines, U266 and OPM2, were cultured alone in complete RPMI-1640 medium or co-cultured with murine (NIH3T3) or human (HS5) BMSC cell lines in DMEM medium supplemented with 10% V/V FBS. U266 and OPM2 cells were either kept in suspension for 24 hours at 3 x 10^5/mL or plated on BMSC monolayer for 24h, then treatments with drugs (1-2 mM Mitoxantrone, 5-8 nM Bortezomib or 100-30 mM Melphalan) or 50μM AMD3100 were applied for additional 24 hours. Apoptosis assay: HS5 cells were colored with PKH26 red fluorescent dye (Sigma-Aldrich) before co-culturing to allowed flow-cytometric detection of MM cells co-cultured with BMSCs. At the end of the treatment, cells were stained with Annexin V-FITC (ImmunoTools) and processed with Cytomics FC500 software (Beckman Coulter). RNA interference: specific knock-down of Jagged1 and 2 was obtained by transient expression of specific siRNAs for Jagged1-2 (Stealth Select RNAiTM siRNA system, Life Technologies). MM cell lines were seeded at 350.000 cells/ml and,

after 24h, Jagged1 and 2 genes were simultaneously silenced. Every 48h cells were diluted and transfected again. Quantitative PCR reactions were carried out on a 7500 Fast Real-time PCR system (Applied Biosystems) using the Maxima™ SYBR Green/ROX qPCR Master Mix (Dasit).
Results: RNA interference for Jagged-1 and 2 in OPM-2 and U266 cells resulted in the reduced expression of anti-apoptotic genes such as SDF-1alfa, CXCR4, Bcl-XL, Bcl-2, Survivin and ABCC1. At the same time, MM cells with reduced levels of Jagged-1 and 2 showed an increased sensivity to different drugs commonly used in MM therapy such as Bortezomib, Mitoxantrone and Melphalan. By co-culturing MM cell lines and BMSCs in the presence or the absence of chemotherapeutic agents we observed that BMSCs were able to protect MM cells from apoptosis. We investigated the underlying mechanism showing that MM cells and BMSC interaction resulted in the activation of Notch signaling in both cell types. MM cells-driven Notch signaling activation in BMSCs resulted in the increased expression of soluble growth factors relevant for MM cell growth and survival, such as SDF-1alfa and VEGF. On the other side, BMSCs increased in MM cells the expression of several anti-apoptotic genes, *i.e.* Bcl-XL, Bcl-2, Survivin and ABCC1. Interestingly, Jagged-1 and 2 silencing in MM cells could reverse all gene expression changes and BMSC protective effect. Finally, the CXCR4 antagonist AMD3100 could partially reverse the protective effect of BMSCs to drugs-induced apoptosis in MM cells, suggesting that Jagged ligands deregulation observed in MM is necessary to BM-promoted drug resistance by activating the SDF1alfa/CXCR4 chemokine signaling.
Summary and Conclusion: The evidence that anti-Jagged-1 and 2 siRNAs affect endogenous and BMSC-induced drug resistance in MM cells suggests that a Jagged-directed approach could be effective in MM therapy alone or in a combined treatment with commonly used drugs.

P935

NOTCH PATHWAY PROMOTES MULTIPLE MYELOMA CELL IL-6 INDEPENDENCE

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Background: Multiple myeloma (MM) is a malignant plasma cells (PC) disorder accounting for approximately 10% of hematologic cancers. Even though advanced chemotherapeutic regimens have increased the median time of survival to 5 years after diagnosis, myeloma remains incurable. Once immortalized, the survival and proliferation of myeloma cells strictly depend on a complex interaction with the bone marrow (BM) microenvironment, which is mediated both by adhesion molecules and production of several cytokines, especially interleukin-6 (IL-6). Following MM progression, at the stage of plasma cell leukemia, the malignant PC acquires autonomous proliferative ability, becomes indipendent on growth factors like IL-6 and is no longer confined in the BM. Several recent evidences point to a possible role for Notch signaling in mediating critical events in MM progression. The Notch pathway is highly conserved and plays a crucial role in cell-fate decision, tissue patterning and morphogenesis. Recently, Notch receptors and ligands have been shown to be upregulated during MM progression and their signaling positively regulates cell proliferation, drug resistance and BM infiltration.
Aims: The ability of Notch signaling to regulate proliferation and survival pathways (*i.e.* NF-kB, AKT, Myc, and the same IL-6) prompted us to study if its up-regulation during MM progression may play a role in the acquirement of IL-6 independence. To this end we used two opposite approaches. Specifically, we verified if Notch signaling upregulation in IL-6 dependent cell lines promotes their independence and assessed if, upon Notch inihibition, IL-6 independent MM cell lines lost self-sufficient proliferation.
Methods: Cell culture and cell growth analysis: HMCL CMA03, INA-6 and XG-1 were maintained in complete RPMI-1640 medium supplemented with 10% V/V FBS and IL-6 10, 2.5 or 1 ng/mL, respectivetly. OPM2, CMA03/06 and U266 cell lines were cultured in the same conditions without IL-6 addition. The number and viability of cells were assessed by means of trypan blue exclusion assay. The Notch inihibitor, DAPT, was added to the medium at the final concentration of 50mM. Soluble Jagged1 was used at 5mg/mL.
Flow cytometry analysis: Apoptosys analysis was perfomed by AnnexinV-FITC/Propidium Iodide staining. Cell cycle analysis was performed by Propidium Iodide staining.
Real time-PCR: Quantitative PCR reactions were carried out using the Maxima™ SYBR Green/ROX qPCR Master Mix.
Results: To evaluate if Notch pathway upregulation is involved in the development of IL-6 independence in MM cells, we activated the Notch signaling in three MM cell lines, CMA03, INA-6 and XG-1, strictly dependent on IL-6. At this purpose, MM cells were cultured with the soluble form of the Notch ligand Jagged1. We demonstrated that Jagged1 stimulation partially rescued the reduced cell growth due to IL-6 withdrawal. On the other hand, three different IL-6 independent cell lines, CMA03/06, OPM2 and U266, treated with a gamma-secretase inhibitor (DAPT) which causes Notch pathway blockade, displayed a significant decrease in cell growth. Remarkably, this effect could be reverted by the addition of IL-6 in the culture medium. The mechanisms underlying Notch-IL-6 crosstalking was partially investigated. Preliminary results indicate that Notch signalling is required for MM cell autonomous IL-6 production.
Summary and Conclusion: The present results suggest that Notch pathway activation may contribute to the transition from IL-6-dependent to IL-6-independent MM cell growth. Furthermore, the inhibition of the Notch patwhay may lead to a decrease in MM cells proliferation in part due to the reduction of IL-6 expression. Even though studies are necessary to identify further mechanisms of IL-6 independence possibly involving other Notch downstrem pathways, these preliminary results support the rationale for a Notch-directed approach in plasma cell dyscrasias.

P936

PROTEASOME INHIBITORS MODULATE OSTEOCYTE DEATH AND AUTOPHAGY IN MULTIPLE MYELOMA

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Background: Cell death and autophagy are the main cellular processes involved in the regulation of bone remodeling by osteocytes. Recently we have demonstrated that an increased osteocyte death is involved in multiple myeloma (MM)-induced osteolysis through the upregulation of osteoclast recruitment.
Aims: Because proteasome inhibitors including Bortezomib (BOR) are known to be able to target osteoblasts in this study we have investigated the potential effect of these drugs on osteocytes and their cell death and autophagy.
Methods: Firstly the effect of the proteasome inhibitors BOR and MG262 on osteocyte viability was evaluated *in vitro* in murine osteocytic cell line MLO-Y4 and in the human pre-osteocytic one HOB-01. Both cell lines were co-coltured for 48 hours in the presence or absence of the human myeloma cell lines (HMCLs) RPMI8226 and JJN3, placed in a transwell insert in the presence or the absence of BOR or MG262. Moreover the effect of proteasome inhibitors on dexamethasone (DEX)-induced MLO-Y4 death, obtained at high doses (10^{-5}-10^{-6}M), was checked in combination with PTH(1-34). To evaluate the presence of autophagy and apoptosis in osteocytes, we checked the expression of both autophagic marker LC3 and apoptotic marker APAF-1 by confocal microscopy in the co-colture system with MLO-Y4 and RPMI-8226. Finally we performed a retrospective histological evaluation on bone biopsies of a cohort of 31 newly diagnosis MM underwent to different treatments including BOR-based regimen. Bone biopsies were obtained at the diagnosis and after an average time of 12 months of treatment. Osteocyte viability was evaluated in a total of 500 lacunae per histological sections.
Results: The *in vitro* treatment with BOR or MG262 significantly blunted MLO-Y4 and HOB-01 cell death. Similarly, DEX-induced MLO-Y4 death was reduced by proteasome inhibitors. Interestingly, we found that both proteasome inhibitors potentiated the PTH (1-34) short-term effects on DEX-induced osteocyte death. Prevalence of autophagic cell death compared to apoptosis was observed in this system. In line with these data, we showed that neither the HMCLs nor treatment with DEX increase the apoptotic death and caspase 3 activation in both MLO-Y4 and HOB-01 cell lines. BOR treatment increased the basal level of LC3 indicating a pro-survival and protective function of autophagy against the BOR-induce stress. On the contrary, when the cells undergo to a stronger stress such as in the presence of HMCLs or by treatment with high dose of DEX we found that both proteasome inhibitors blocked autophagic cell death in osteocytes. In the *in vivo* study we found a significant increase of the number of viable osteocytes in MM patients treated with BOR-based regimen as compared to those treated without BOR (% median increase: +6% *vs*. +1.30%; p=0.017). Patients treated with BOR alone showed the highest increase of osteocyte viability, as compared to those either treated without BOR (+11.6% *vs*. +1.3%, p=0.0019) or treated with BOR plus DEX (+11.6% *vs*. +4.4%, p=0.01). On the other hand, any significant difference was not observed in patients treated with Thalidomide (THAL) or Immunomodulatory drugs (IMiDs) than in those untreated with these drugs (p=0.7). A multiple regression non-parametric analysis showed that BOR had a significant positive impact on osteocyte viability (p=0.042) whereas THAL/IMiDs as well as Zoledronic acid (ZOL) treatments have not (p=0.2). BOR also counterbalanced the negative effect of DEX treatment (p=0.035).
Summary and Conclusion: Our data suggest that proteasome inhibitors blunted osteocyte cell death induced by MM cells and DEX through the modulation of the autophagy and potentiated the effect of PTH. Overall our *in vitro* and *in vivo* data support the use of BOR to improve bone integrity in MM patients.