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.

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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.

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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.