Heparan sulfate proteoglycans in B cell maturation and myeloma plasma cell survival
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Appendix

Loss of heparan sulfate expression sensitizes myeloma cells to drug-induced cell death

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**Introduction**

Despite the recent advances made in the treatment of MM, the patients’ prognosis is still poor, with an median survival of 3-5 years. A major problem in the treatment is that the malignant plasma cells, although initially sensitive, inevitably develop drug resistance irrespective of the drug dosage used (Hideshima et al., 2007). An additional problem is that high drug dosages, which are often required, will unavoidably lead to side-effects, such as neutropenia, thrombocytopenia, peripheral neuropathy, acute respiratory failure, and pulmonary embolism (Raab et al., 2009). Our previous findings imply that targeting of the HS biosynthesis may be a promising therapeutic strategy, since it can induce apoptosis of MM cells (Reijmers et al., 2010). Here, we show that EXT1 knock down sensitizes myeloma cells to drug-induced cell death.

**Materials and methods**

**Anti-myeloma drugs**
Lenalidomide was obtained from Celgene Corporation (Summit, NJ, USA) and bortezomib was from Millennium Pharmaceuticals (Cambridge, MA, USA), concentrations used were 0.1 μM and 9.0 nM, respectively. The concentrations were based on a titration, which resulted in the most enhanced effect compared to the control, and are in range with concentrations applied in the clinic (Richardson et al., 2010).

**Cell lines and culture**
The human MM cell line L363 was cultured as described (Rozemuller et al., 2008).

**Generation of inducible cell lines**
Doxycycline-inducible cell lines were generated as described (Reijmers et al., 2010). To induce shRNA expression, 1 μg/ml doxycycline (Sigma-Aldrich) was used.

**In vitro growth and apoptosis measurements**
Cells were plated (10^4) in 96-well plates. Cells were quantified by fluorescence-activated cell sorter (FACS; BD Biosciences), using TO-PRO-3-‘iodide to exclude dead cells. Apoptotic cells were identified by annexin V and TO-PRO-3-‘iodide.

**Statistical analysis**
The unpaired 2-tailed Students’-t test was used to determine the significance of differences between means, unless stated otherwise.
Results and discussion

Our previous findings indicated that HS expression is crucial for MM cell survival (Reijmers et al., 2010). In the current study, we have investigated the effect of targeting the HS biosynthesis on the sensitivity to drug-induced cell death, and the impact on the growth rate of MM cells. For this purpose, we used the L363-shEXT1a cells. Upon treatment with doxycycline for a 5 day period, the expression of cell surface HS was reduced by ~90%, as determined by FACS analysis (Figure 1A).

To directly assess the effect on cell viability by chemotherapeutic drugs either or not after EXT1 knockdown, we performed an experiment in which we incubated L363-shEXT1a cells with lenalidomide or bortezomib, as these chemotherapeutics demonstrate the most promising effects in current phase I/II trials (Raab et al., 2009; Richardson et al., 2010). In line with our previous findings (Reijmers et al., 2010), knockdown of EXT1 only resulted in a marked reduction in viable cell number (Figure 1B, no drugs). In addition, both lenalidomide and bortezomib clearly affected the viable cell number reducing it to ~68% and ~31%, respectively (Figure 1B). Interestingly, the doxycycline-induced knockdown of EXT1 caused an additional effect on the percentage of viable MM cells by lenalidomide (~24%) and bortezomib (~9%), demonstrating a synergism of drug-induced cell death and the removal of cell surface HS (Figure 1B). Of note, the control L363-TetR cells lacking shRNA against EXT1 that were pre-treated with doxycycline, only demonstrated a drug-induced effect on viable cell number, excluding a role for doxycycline in the results obtained (data not shown). These findings suggest that targeting HS expression sensitizes the MM cells to drug-induced cell death, which is in particularly striking for bortezomib (Figure 1B), and provide a strategy to lower the concentration of current chemotherapeutics that could result in similar, or even enhanced cell death, but prevents the development of secondary events, such as neutropenia, thrombocytopenia, peripheral neuropathy, acute respiratory failure, and pulmonary embolism (Raab et al., 2009).

To further explore the role of EXT1 knockdown on drug-induced cell death in MM, we performed a growth assay in the presence of the same chemotherapeutics. As anticipated, loss of HS expression on L363-shEXT1a cells significantly affected the cell growth. Similarly, treatment with lenalidomide and bortezomib clearly diminished the cell growth, however, EXT1 knockdown already resulted in a growth rate similar (bortezomib) or even lower (lenalidomide) than the control cells treated with the chemotherapeutic drugs (Figure 1C). Moreover, whereas L363-shEXT1a cells expressing cell surface HS could still proliferate in the presence of bortezomib and lenalidomide, knockdown of EXT1 (+dox) almost completely inhibited the cell growth during the 7-day culture period when incubated with a similar drug dosage (Figure 1C). Hence, these findings reveal that in vitro MM cell growth is attenuated in the presence of bortezomib and lenalidomide, and that loss of cell surface HS dramatically increases that effect by nearly completely inhibiting cell growth (Figure 1C).

In conclusion, our current study demonstrates that the HS chains are crucial for the growth and survival of MM cells. Furthermore, by targeting the HS chain biosynthesis, thereby preventing the MM cells to accumulate proliferation and
survival inducing soluble factors, MM cells are sensitized to drug-induced cell death. These findings provide interesting new opportunities in the treatment of MM patients, since the cell viability after treatment with the chemotherapeutic drugs lenalidomide and bortezomib was dramatically reduced upon loss of surface HS. For this reason, drug-induced side effects could be prevented or minimized, or treatment regimens could be shortened.

**Figure 1.** Knockdown of EXT1 results in loss of HS and sensitizes L363 MM cells to drug-induced cell death leading to a strongly impaired growth. L363-shEXT1a cells were incubated with (+dox) or without doxycycline (-dox) for 5 days before each experiment to allow for optimal knockdown of EXT1. All experiments were performed in the presence of 10% FCS. (A) Expression of cell surface heparan sulfate (HS) on L363-shEXT1a measured by FACS using antibody 10E4 (Seikagaku America) after RNAi-mediated knockdown of EXT1. The expression of untreated samples was normalized to 100%. Bars represent mean ± SEM of 3 independent experiments. (B) Viable L363-shEXT1a cells after a 5-day incubation period with or without lenalidomide or bortezomib. Untreated samples (no drugs) were normalized to 100%. Bars represent mean ± SEM of two independent experiments, performed in triplicate. len, lenalidomide; bor, bortezomib. (A-C) * p<0.05, **p<0.01, *** p<0.001.
References


