Targeting EXT1 reveals a crucial role for heparan sulfate in the growth of multiple myeloma

Rogier M. Reijmers, Richard W. J. Groen, Henk Rozemuller, Annemieke Kuil, Anneke de Haan-Kramer, Tamás Csikós, Anton C. M. Martens, Marcel Spaargaren, and Steven T. Pals

Expression of the heparan sulfate proteoglycan syndecan-1 is a hallmark of both normal and multiple myeloma (MM) plasma cells. Syndecan-1 could affect plasma cell fate by strengthening integrin-mediated adhesion via its core protein and/or by accommodating and presenting soluble factors via its HS side chains. Here, we show that inducible RNAi-mediated knockdown of syndecan-1 in human MM cells leads to reduced growth rates and a strong increase of apoptosis. Importantly, knockdown of EXT1, a co-polymerase critical for HS chain biosynthesis, had similar effects. Using an innovative myeloma xenotransplantation model in Rag-2<sup>-/-</sup>γc<sup>-/-</sup> mice, we demonstrate that induction of EXT1 knockdown in vivo dramatically suppresses the growth of bone marrow localized myeloma. Our findings provide direct evidence that the HS chains of syndecan-1 are crucial for the growth and survival of MM cells within the bone marrow environment, and indicate the HS biosynthesis machinery as a potential treatment target in MM.

Introduction

Within the lymphoid system, expression of the heparan sulfate proteoglycan syndecan-1 is characteristic for terminally differentiated B cells, that is, plasma cells, and their malignant counterpart multiple myeloma (MM), a plasma cell neoplasm, that is, plasma cells, and their malignant counterpart multiple myeloma (MM), a plasma cell neoplasm. Syndecan-1 could affect plasma cell fate by strengthening integrin-mediated adhesion via its core protein and/or by accommodating and presenting soluble factors via its HS side chains. Here, we show that inducible RNAi-mediated knockdown of syndecan-1 in human MM cells leads to reduced growth rates and a strong increase of apoptosis. Importantly, knockdown of EXT1, a co-polymerase critical for HS chain biosynthesis, had similar effects. Using an innovative myeloma xenotransplantation model in Rag-2<sup>-/-</sup>γc<sup>-/-</sup> mice, we demonstrate that induction of EXT1 knockdown in vivo dramatically suppresses the growth of bone marrow localized myeloma. Our findings provide direct evidence that the HS chains of syndecan-1 are crucial for the growth and survival of MM cells within the bone marrow environment, and indicate the HS biosynthesis machinery as a potential treatment target in MM.

Methods

Cell lines and culture

The human MM cell lines RPMI-8226 and L363 were cultured as described.

Generation of inducible cell lines

Doxycycline-inducible cell lines were generated using the T-Rex System (Invitrogen). L363 and RPMI-8226 were transfected by electroporation (Gene Pulser Apparatus; Bio-Rad) with the pcDNA6/TR construct alone (TeiR), or in combination with a construct (pTER) containing an shRNA directed against either syndecan-1 (shSYN1) or EXT1 (shEXT1a, b, or c). shRNA sequences are given in the supplemental Methods (available on the Blood website; see the Supplemental Materials link at the top of the online article), and the position of the target sites is shown in supplemental Figure 1A. 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 fluorescence-activated cell sorter (FACS; BD Biosciences), using TO-PRO-3-iodide to exclude dead cells. Apoptotic cells were identified by fluorescence-activated cell sorter (FACS; BD Biosciences), using TO-PRO-3-iodide to exclude dead cells. Apoptotic cells were identified by fluorescence-activated cell sorter (FACS; BD Biosciences), using TO-PRO-3-iodide to exclude dead cells. Apoptotic cells were identified by fluorescence-activated cell sorter (FACS; BD Biosciences), using TO-PRO-3-iodide to exclude dead cells.

Transplantation of MM cells in mice

Rag-2^-/-γc^-/- mice (9-14 weeks) were bred and housed as described. Transplantation of GFP-luciferase transduced L363 or RPMI-8226 cells into Rag-2^-/-γc^-/- mice was performed essentially as described, except that 1 × 10^6 MM cells were injected intracardially. During the experiment, the mice were supplied with water ad libitum, containing 5% sucrose with or without 1 mg/mL doxycycline (Sigma-Aldrich). In addition, mice receiving doxycycline were injected intraperitoneally, twice a week, with 125 μg doxycycline to sustain adequate serum levels.

Bioluminescent imaging

Mice were anesthetized by isoflurane inhalation before they received an intraperitoneal injection of 100 μL of 7.5mM D-luciferine (Synchron Chemie). Bioluminescence images were acquired using a third generation cooled GaAs intensified charge-coupled device camera, controlled by the Photo Vision software and analyzed with M3Vision software (all from Photon Imager; Biospace Laboratory).

Statistical analysis

The unpaired 2-tailed Student t test was used to determine the significance of differences between means, unless stated otherwise.

Results and discussion

In this study, we have directly explored the impact of HS modification of syndecan-1 on MM growth in vivo, using a recently developed, innovative, xenotransplantation model. Key features of this model are that it uses immunodeficient Rag-2^-/-γc^-/- mice as recipients of human MM cells transduced with GFP-luciferase to allow noninvasive real-time monitoring of MM cell growth in vivo. Rag-2^-/-γc^-/- mice completely lack B, T, and NK cells and therefore permit highly reproducible engraftment of human lymphocytes. As we recently demonstrated, these Rag-2^-/-γc^-/- mice are also highly permissive to grafting of human MM cell lines, several of which displayed a strikingly selective tumor outgrowth in recipient’s BM compartment on intravenous injection. For our current studies, 2 of these MM-cell lines (L363 and RPMI-8226) were selected and stably transfected with doxycycline-inducible shRNAs against either the syndecan-1 core protein or the HS copolymerase EXT1 (shSYN1 and shEXT1, respectively). To rule out off-target effects of the shRNAs, 3 different L363-shEXT1 lines, each containing a different nonoverlapping targeting sequence for EXT1, were generated (shEXT1a, b, and c; supplemental Methods; supplemental Figure 1A-B).

Doxycycline treatment of L363-shSYN1 cells led to an approximately 75% reduction of syndecan-1 expression (Figure 1A), whereas treatment of L363-shEXT1a, b, or c or RPMI-shEXT1a cells was even more effective and reduced cell-surface HS expression by approximately 90% (Figure 1B). In control (TetR) cells, doxycycline did not affect syndecan-1 or HS levels (Figure 1A-B). Interestingly, the doxycycline-induced knockdown of either syndecan-1 or EXT1 led to a marked reduction of the in vitro growth rate of the myeloma cells (Figure 1C-D), which was accompanied by a prominent increase in apoptosis (Figure 1E), as well as a minor increase in the percentage of cells in phase G0/G1 of the cell cycle (data not shown). Importantly, the expression of the syndecan-1 core protein was unaffected by EXT1 knockdown (Figure 1A). Therefore, our results underscore the importance of...
HS side chains, rather than the proteoglycan core protein per se, in the growth and survival of MM cells in vitro.

To directly assess the role of HS on tumor growth in vivo, the L363-shEXT1a and RPMI-shEXT1a MM cells were retrovirally transduced to express a GFP-luciferase fusion protein and injected intracardially into irradiated Rag-2<sup>−/−</sup>γc<sup>−/−</sup> mice. Consistent with our previous observations using untransfected L363 cells,22 mice that did not receive doxycycline displayed a rapid exponential tumor growth (Figure 2A-B; supplemental Figure 3A), localized in various parts of the skeleton, preferentially in the large bones, including the femurs (Figure 2A) and the lower spinal cord (supplemental Figure 2). Immunohistochemical analysis of the isolated bones confirmed the presence of highly proliferative tumor foci (Figure 2C-D). In striking contrast, mice injected with L363-shEXT1a that received doxycycline from the first day remained completely tumor free throughout the entire experiment (Figure 2A-D). Interestingly, in mice in which these tumors were allowed to form before doxycycline treatment, doxycycline administration from week 6 onward led to either growth arrest or even a reduction in tumor size. Histologic studies revealed the presence of areas of extensive necrosis within these tumors, which were found to be devoid of HS expression (Figure 2E), confirming EXT1 knockdown in vivo. In line with these findings, knockdown of EXT1 in RPMI-8226 cells also markedly inhibited the in vivo MM tumor growth, resulting in a significantly extended survival (supplemental Figure 3A-B). Importantly, doxycycline did not influence the tumor growth of L363-TetR or RPMI-TetR control cells in vivo (Figure 2B; supplemental Figure 3A).

In conclusion, although mutations in essential growth control genes underlie MM development, signals from the BM microenvironment are also essential for driving tumor growth.2,3 As targets for intervention, these signals may be equally important as mutated oncogene products. Our current study demonstrates that the HS chains decorating syndecan-1 are crucial for the growth and survival of MM cells within the BM environment and indicates these HS chains and their biosynthesis machinery5 as potential treatment targets in MM.

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Authorship

Contribution: R.M.R. and R.W.J.G. equally performed research, analyzed data, and wrote the paper; H.R and A.C.M.M. provided the xenotransplant human MM mouse model, provided technical assistance, and analyzed data; A.K., A.d.H.-K., and T.C. performed research; and M.S. and S.T.P. equally designed and supervised the research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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