Published in final edited form as:

Leukemia. 2014 November ; 28(11): 2178–2187. doi:10.1038/leu.2014.121.

Heparanase enhances myeloma progression via CXCL10 down regulation

Uri Barash¹, Yaniv Zohar², Gizi Wildbaum², Katia Beider³, Arnon Nagler³, Nathan Karin², Neta Ilan¹, and Israel Vlodavsky^{1,*}

¹Cancer and Vascular Biology Research Center, Technion, Haifa 31096, Israel

²Department of Immunology, Rappaport Faculty of Medicine, Technion, Haifa 31096, Israel

³Division of Hematology and Bone Marrow Transplantation, Chaim Sheba Medical Center, Tel Aviv University, Tel Hashomer, Israel

Abstract

In order to explore the mechanism(s) underlying the pro-tumorigenic capacity of heparanase we established an inducible Tet-on system. Heparanase expression was markedly increased following addition of doxycycline (Dox) to the culture medium of CAG human myeloma cells infected with the inducible heparanase gene construct, resulting in increased colony number and size in soft agar. Moreover, tumor xenografts produced by CAG-heparanase cells were markedly increased in mice supplemented with Dox in their drinking water compared with control mice maintained without Dox. Consistently, we found that heparanase induction is associated with decreased levels of CXCL10, suggesting that this chemokine exerts tumor suppressor properties in myeloma. Indeed, recombinant CXCL10 attenuated the proliferation of CAG, U266 and RPMI-8266 myeloma cells. Similarly, CXCL10 attenuated the proliferation of human umbilical vein endothelial cells (HUVEC), implying that CXCL10 exhibits anti-angiogenic capacity. Strikingly, development of tumor xenografts produced by CAG-heparanase cells over expressing CXCL10 was markedly reduced compared with control cells. Moreover, tumor growth was significantly attenuated in mice inoculated with human or mouse myeloma cells and treated with CXCL10-Ig fusion protein, indicating that CXCL10 functions as a potent anti-myeloma cytokine.

Keywords

Heparanase; myeloma; CXCL10; tumor suppressor

*To whom correspondence should be addressed: Israel Vlodavsky, Cancer and Vascular Biology Research Center, Rappaport Faculty of Medicine Technion, P. O. Box 9649, Haifa 31096, Israel, Tel. 972-4-8295410; Fax. 972-4-8523947, Vlodavsk@mail.huji.ac.il.

The authors have no conflict of interest to declare.

Contribution: U. B., G. W., and K. B., designed and performed experiments, analyzed and interpreted data; Y. Z., N. K., and A. N. provided valuable reagents, designed, analyzed and interpreted data; N. I. codirected the study, designed, analyzed and interpreted data and wrote the manuscript; I. V. directed the study, designed, analyzed and interpreted data and co-wrote the manuscript.

Supplementary information is available at *Leukemia's* website.

Introduction

Heparanase is an endoglucuronidase that cleaves heparan sulfate (HS) chains of proteoglycans. These macromolecules are most abundant in the sub-epithelial and sub-endothelial basement membranes and their cleavage by heparanase leads to disassembly of the extracellular matrix (ECM) that becomes more susceptible to invasion and dissemination of metastatic tumor cells. Heparanase expression is increased in many types of tumors and this elevation is often associated with more aggressive disease and poor prognosis due to advanced local and distant metastases¹⁻⁵. In addition, heparanase up regulation in primary human tumors correlates in some cases with tumors larger in size and with enhanced micro vessel density^{3, 5}. Likewise, cells engineered to over-express heparanase are endowed with more rapid expansion of tumor xenografts^{1, 2, 6-8}, while heparanase inhibitors attenuate tumor growth in pre-clinical settings⁹⁻¹². The molecular mechanism exerted by heparanase to promote tumor development is incompletely understood and likely combines enzymatic activity-dependent and -independent aspects. Heparanase activity can release HS-bound growth factors stored in the ECM as reservoir and thereby promote angiogenesis^{13, 14}, while inactive heparanase facilitates the survival and proliferation of tumor cells by activation of signaling molecules such as Akt, EGFR, Src and STAT^{3, 15-19}. Moreover, heparanase induces the transcription of pro-angiogenic (i.e., *VEGF-A*, *VEGF-C*, *COX-2*), pro-thrombotic (i.e., *TF*), mitogenic (*HGF*), and osteolytic (*RANKL*) genes²⁰⁻²⁴, thus significantly expanding the functional repertoire and mode of action of heparanase. In order to further explore the tumorigenic capacity of heparanase we established an inducible Tet-on system. Heparanase expression and activity were markedly increased following addition of doxycycline (Dox) to the culture medium of CAG myeloma cells infected with the inducible heparanase gene construct, resulting in increased colony number and size in soft agar. Moreover, tumor xenografts produced by CAG-heparanase cells were noticeably increased in mice supplemented with Dox in their drinking water compared with control mice maintained without Dox. Consistently, we found that heparanase induction by Dox is associated with decreased levels of CXCL10, suggesting that this chemokine exerts tumor suppressor properties. We provide compelling evidence that CXCL10 exerts anti-cancer properties in myeloma, attenuating cell proliferation, colony formation, and tumor growth.

Materials and methods

Cells and cell culture

CAG myeloma cells were kindly provided by Dr. Ben-Zion Katz (Tel Aviv Sourasky Medical Center, Tel Aviv, Israel)²⁵. U266, RPMI-8226 human and MPC-11 mouse myeloma cells were kindly provided by Dr. Ralph Sanderson (University of Alabama at Birmingham, Birmingham, AL)²⁶. Cells were grown in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum and antibiotics. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured essentially as described²⁷. Isolation, culturing, and FACS analysis of CD138-positive primary myeloma cells from patients with plasma cell leukemia was carried out essentially as described²⁸.

Antibodies and reagents

Anti-Src, anti-Erk, anti-phospho Erk, anti-caspase 3 and anti-syndecan-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Anti-phospho-Src (Tyr416), anti-phospho-STAT3 and anti-cleaved caspase 3 antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Anti-V5 antibody was purchased from Invitrogen (Carlsbad, CA), anti BrdU monoclonal antibody was from Roche (Indianapolis, IN), and anti actin monoclonal antibody was from Sigma (St. Louis, MO). Recombinant CXCL10, anti CXCR3 monoclonal antibody and Quantikine® ELISA for human CXCL-10 were purchased from R&D Systems (Minneapolis, MN) and anti CXCL10 antibody was from PeproTech (Rocky Hill, NJ). Rabbit polyclonal antibody #1453 was prepared against purified 65 kDa heparanase²⁷. Anti-mouse platelet endothelial cell adhesion molecule (PECAM)-1 (CD31) polyclonal antibody was kindly provided by Dr. Joseph A. Madri (Yale University, New Haven, CT)²⁹. HRP-conjugated goat anti-rabbit/mouse antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Human CXCL10 cDNA was purchased from OriGene (Rockville, MD) and sub-cloned into NSPI-CMV-MCS-myc-HIS viral plasmid¹⁵. Anti-heparanase and anti-CXCL10 shRNAs (GIPZ lentiviral shRNA) were purchased from Thermo Scientific (Waltham, MA).

Construction and expression of CXCL10-Ig

Mouse IgG1 Fc (Hinge CH2-CH3) constant region was cloned from RNA extracted from mouse splenocytes that were cultured for 96 h in the presence of LPS and mouse IL-4. The cDNA was ligated into the mammalian expression/secretion vector pSecTag2/Hygro B (Invitrogen), essentially as described³⁰. A different set of primers, 5' ATGAACCCAAGTGCTGCCGTCATTTT 3' (sense) and 5' AGGAGCCCTTTTAGACCTTTTTT 3' was used to amplify cDNA encoding mouse CXCL10. The original κ chain leader sequence of pSecTag2/Hygro B vector was replaced by a mCXCL10 leader and coding sequences, resulting in CXCL10-Ig fusion protein. The fusion protein was purified from medium conditioned by Chinese hamster ovary dhfr^{-/-} (DG44) cells (kindly provided by Dr. L. Chasin, Columbia University, New York, NY) over expressing CXCL10-Ig fusion protein by a High-Trap protein A affinity column (GE Healthcare), as described³⁰. The biological activity of CXCL10-Ig fusion protein was evaluated by standard T-cell migration assay²⁹.

Cell lysates, immunoblotting and heparanase activity assay

Cell extracts were prepared using a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, supplemented with a cocktail of protease inhibitors (Roche). Protein concentration was determined (Bradford reagent, BioRad) and 30 μ g protein was resolved by SDS-PAGE under reducing conditions using 12% gels. After electrophoresis, proteins were transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was probed with the appropriate antibody followed by HRP-conjugated secondary antibody and a chemiluminescent substrate (Pierce, Rockford, IL), as described¹⁵. Preparation of dishes coated with ECM and determination of heparanase enzymatic activity was carried out essentially as described^{15, 16, 31}.

Tet-on system

The Tet-on expression system (ViraPower HiPerform T-rex Gateway Vector Kit) was established according to the manufacturer's (Invitrogen) instructions utilizing the following primers: Heparanase Forward -5'

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTGCTGCGCTC GAAG 3'; Reverse- 5'

GGGGACCACTTTGTACAAGAAAGCTGGGTAGATGCAAGCAGCAACTT TG3' T5 Forward- 5'

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTGCTGCGCTC GAAG 3', Reverse- 5'

GGGGACCACTTTGTACAAGAAAGCTGGGTATTTCTTACTTGAGTAGGT G 3' 8C Forward- 5'

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGAGACAGACA CACTC 3' Reverse- 5'

GGGGACCACTTTGTACAAGAAAGCTGGGTAGATGCAAGCAGCAACTT TG 3'.

Briefly, CAG cells were infected with pLenti3.3/TR virus, which encodes the tetracycline repressor gene. High expressing clones were selected and infected with pLenti6.3/TO/V5-DEST viruses expressing heparanase, 8C¹⁶, or T5¹⁵ variants. Cells were selected with Blasticidin (10 µg/ml; Invitrogen), and expanded. Gene expression was induced by adding doxycycline (Sigma) to the cell culture medium (1 µg/ml), or to the mice drinking water (2 mg/ml). Drinking water was also supplemented with 5% Sucrose (Sigma).

Real time-PCR

Real time-PCR analyses were performed using ABI PRISM 7000 Sequence Detection System employing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The following primers were used: *Actin* F: 5'-CGCCCCAGGCACCAGGGC-3', R: 5'-GCTGGGGTGTGAAGGT-3'; *HPSE* F: 5'-CCCTTGCTATCCGACACCTT-3', R: 5'-CACCATTCTATTCCCTTTTCG-3'; *CXCL10* F: 5'-TCCACGTGTTGAGATCATTGC -3', *CXCL10* R: 5'-TCTTGATGGCCTTCGATTCTG-3'.

Colony formation in soft agar

Dulbecco's modified Eagle's medium (DMEM) (3 ml) containing 0.5% low-melt agarose (Bio-Rad) and 10% FCS was poured into 60-mm Petri dishes. The layer was covered with cell suspension (2×10^3 cells) in 1.5 ml DMEM containing 0.3% low-melt agarose and 10% FCS, followed by addition of 2 ml DMEM containing 10% FCS. Medium was exchanged every 3 days. Colonies were visualized and counted under a microscope 2–5 weeks after seeding, as described previously¹⁵.

MTT assay

The number of viable cells was evaluated by thiazolyl blue tetrazolium bromide (MTT; Sigma) that measures the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, yielding a purple color. Cells (5×10^3 well) were grown in 96 wells plate for the time indicated. MTT (20 µl of 5 mg/ml) was then added to each well for 2-3

hours, followed by centrifugation. The cell pellet was re-suspended in 150 μ l of isopropanol and absorbance was measured at 570nm using an ELISA plate reader.

Tumorigenicity and immunohistochemistry

Cells of control-, heparanase-, heparanase C-terminal domain (8C)-, and T5-infected CAG myeloma cultures were detached with trypsin/EDTA, washed with PBS, and brought to a concentration of 1×10^7 cells/ml. Cell suspension ($1 \times 10^6/0.1$ ml) was inoculated subcutaneously at the right flank of 5-wk-old female SCID mice ($n=6$). Drinking water was supplied with sucrose or sucrose and doxycycline (Dox; 2 mg/ml) and was replaced twice weekly. Xenograft size was determined by externally measuring tumors in 2 dimensions using a caliper. At the end of the experiment, mice were administrated with BrdU (10 μ l/gr; Amersham, GE Healthcare, Buckinghamshire, UK) and were sacrificed 2 hours thereafter. Tumor xenografts were then removed, weighed, and fixed in formalin. Paraffin-embedded 5 μ m sections were subjected to immunostaining with anti-CXCL10, anti BrdU, and anti-PECAM (CD31) antibodies using the Envision kit (Dako) according to the manufacturer's instructions, as described previously¹⁵. Similar experiments were performed with CAG myeloma cells over expressing heparanase and infected with CXCL10 or control vector. Balb/C and SCID mice were also inoculated with MPC-11 or CAG-heparanase myeloma cells, respectively and treated with purified CXCL10-Ig fusion protein (200 μ g/mouse, three times a week). Tumor infiltrating lymphocytes were isolated by a dissociation kit according to the manufacturer's (Miltenyi Biotec; Auburn, CA) instructions. Briefly, tumor samples were carefully washed with Hank's solution containing 2% fetal calf serum and 1% ethylenediaminetetraacetic acid (EDTA) to remove peripheral blood and whittled into small pieces. Cell suspension was then obtained by gentle mechanical dissociation (MACS Dissociator, Miltenyi Biotec). Dissociated cell suspensions was kept on ice for 15 minutes; The upper layer was carefully recovered, passed through a 70- μ m cell strainer (BD Labware), and layered onto Ficoll-Hypaque separation solution (Lymphoprep; Axis-Shield, Oslo, Norway). Cells were then isolated by density gradient centrifugation and subjected to FACS analysis using anti-CD8 and anti-NK (BioLegend, San Diego) antibodies. The Animal Care Committee of the Technion (Haifa, Israel), approved all animal experiments.

Illumina human GX arrays

All RNA samples for gene expression analysis had a RNA Integrity Number (RIN) value above 7.5 using Experion system (Biorad). Microarray expression profiling was performed in the Genomics Core Facility of the Rappaport Research Institute at the Technion. The RNA was amplified into cRNA and biotinylated by *in vitro* transcription using the Illumina TargetAmp-Nano Labeling Kit according to the manufacturer's (Epicentre, Illumina) protocol, using 100 ng of total RNA as input material. Biotinylated cRNAs was purified, fragmented, and subsequently hybridized to an Illumina Human HT-12 v4 Bead Chip according to the Direct Hybridization assay (Illumina Inc.). The hybridized chip was stained with streptavidin-Cy3 (AmershamTM) and scanned with an Illumina bead array reader. The scanned images were imported into GenomeStudio (Illumina Inc.) for extraction and quality control. The biostatistics analysis was performed using JMP-Genomics[®] Version 5.0., Cary, NC, 1989-2007 (SAS). Probes below the background levels were filtered out. The expression measures were then log transformed base 2, requiring no further normalization

since Illumina gene expression results are extremely robust. Differentially expressed genes (DEG) were identified using 1-way analysis of variance (ANOVA) for time point. Significant DEG was defined as transcript that has as at least two fold changes in expression at p-value of 0.05 after false discovery rate correction (FDR).

Statistics

Data are presented as mean $\pm$ SE. Statistical significance was analyzed by two-tailed Student's t test. The value of $p < 0.05$ was considered significant. All experiments were repeated at least three times with similar results.

Results

Establishment of an inducible (Tet-on) system of heparanase variants

In order to investigate the significance of heparanase for tumor development, we established an inducible model system. In this system, gene expression is constantly repressed; gene induction is obtained following the addition of tetracycline or its analog, doxycycline (Dox), to the cell culture medium or mice drinking water. CAG myeloma cells were infected with inducible wild type heparanase, heparanase C-terminal domain (8C)¹⁶ or T5 (a heparanase splice variant)^{15, 32} gene constructs and expression levels were examined in cells grown in the absence or presence of Dox by immunoblotting. Expression of heparanase variants was not detected in the absence of Dox (Fig. 1A, left upper panel, -) but was markedly enhanced in cell grown in its presence (Fig. 1A, left upper panel, +). Similarly, heparanase enzymatic activity was noticeably increased in cells infected with Tet-on heparanase constructs while no heparanase activity was observed following 8C or T5 induction (Suppl. Fig. 1A), as expected. Moreover, heparanase induction was associated with decreased levels of syndecan-1 on the cell membrane (Fig. 1A, right lower panel), likely representing syndecan-1 shedding reported in myeloma cells over-expressing heparanase²⁶. In order to examine the reversibility of the system, Dox was added to CAG cells for 24 hours and then removed. Cells were grown in the absence of Dox for additional 1, 2, 3, or 4 days and protein expression was revealed by immunoblotting. While Dox efficiently stimulated the expression of heparanase variants (Fig. 1B, 1), its withdrawal resulted in a marked decrease in protein levels. Densitometry analysis revealed distinct kinetics of protein elimination. Thus, while the decline in 8C and T5 appeared rapid and the proteins became undetectable 2 days after Dox withdrawal (Fig. 1B, 3), heparanase decrease was slower and could be detected even three days following the removal of Dox (Fig. 1B, Hepa, 4).

In order to reveal the consequences of heparanase variants induction, we first examined anchorage-independent growth capacity of heparanase-, 8C- and T5-infected CAG cells. Colony formation in soft agar in term of number and size was increased markedly following heparanase, 8C and T5 induction by Dox (Suppl. Fig. 1B, C), in agreement with previous results¹⁵. Likewise, the development of tumor xenografts produced by heparanase-, 8C-, and T5-induced (+Dox) cells was prominently enhanced compared with xenografts generated in the absence of Dox (-Dox). On termination of the experiment on day 34, noticeable differences in tumor xenograft development were observed (Fig. 2A,B); average weights of heparanase xenografts were 552 $\pm$ 175 mg in the presence Dox compared with

77+43 in its absence, representing 7-fold increase in tumor weight (Fig. 2B). Even greater increase in tumor weight was obtained following 8C (352+123 vs. 42+19 mg) and T5 (695+130 vs. 12+12 mg) induction, differences that are statistically highly significant ($P=0.01$ for heparanase vs. control, $P=0.01$ for 8C vs. control, and $P=0.0002$ for T5 vs. control; Fig. 2B).

Heparanase induction is associated with CXCL10 down regulation

In order to appreciate alteration in gene expression associated with heparanase induction we applied gene array methodology. Total RNA was extracted from CAG-heparanase clone 13 cells grown in the absence of Dox (0) or 8, 16, and 24 h after its addition, representing gradual increase in heparanase expression and secretion (Suppl. Fig. 1D 1st and 2nd panels) and associating with enhanced Src phosphorylation (Suppl. Fig. 1D, fourth panel) shown previously to be affected by heparanase^{15, 17, 29}. Analyzing the results at high stringency ($p=0.001$) we found that the expression of 21 genes was altered following heparanase induction, 10 genes were up regulated while 11 were down regulated. We next validated (qPCR) the induction (i.e., ATF5, SDF2L1) or repression (i.e., CXCL10) of selected genes in clone 13 cells and a pool of CAG cells over expressing heparanase (Fig. 3A, B). Regulation of ATF5 and SDF2L1 by heparanase was restricted to clone 13 cells selected for the Tet-on system, but is not common in myeloma. In contrast, we consistently observed a 2-4-fold decrease in CXCL10 (IP10) expression by heparanase in both the CAG clone and pool cells (Fig. 3A, B, right panels) that was statistically highly significant ($p<0.01$). We further substantiated CXCL10 mRNA down regulation in another CAG-heparanase inducible clone (#1; Fig. 4A). Moreover, immunostaining of CAG clone #13 revealed a marked decrease in CXCL10 staining in response to Dox (Fig. 4B, upper panels), as also verified by ELISA determination of CXCL10 (94.3 + 12.2 ng/ml vs. 31.1 + 17 ng/ml, without or with Dox treatment, respectively) (Fig. 4B, lower panel; $p=0.0005$). Likewise, reduced CXCL10 mRNA expression comparable in magnitude was observed in U266 and RPMI-8266 myeloma cells over expressing heparanase vs. control (Vo) cells (Fig. 4C) or following exogenous addition of heparanase (Fig. 4D). Moreover, heparanase gene silencing (Suppl. Fig. 2A) was associated with increased CXCL10 expression (Fig. 4C, sh-Hepa), collectively implying that heparanase down regulates CXCL10 expression in myeloma cells.

CXCL10 attenuate proliferation, colony formation and tumor development by myeloma cells

Since reduced CXCL10 expression is associated with heparanase induction and tumor development (Fig. 2), we rationalized that CXCL10 may exhibit tumor suppressor properties in myeloma. In order to examine this possibility we employed MTT assay to assess cell proliferation. Addition of recombinant CXCL10 to CAG (Fig. 5A, upper panel), RPMI-8266 (Fig. 5A, second panel), and U266 (Fig. 5A, third panel) cells resulted in significant decrease in cell number. Cell proliferation was similarly attenuated in myeloma cells engineered to over express CXCL10 (Fig. 5B; Suppl. Fig. 2B, C). CAG-heparanase cells readily form colonies in soft agar and are highly tumorigenic¹⁵. Over expressing CXCL10 in these cells resulted in significantly fewer and smaller colonies in soft agar compared with control cells infected with an empty vector (Vo; Fig. 5C; Suppl. Fig. 2D), clearly depicting its anti-proliferative capacity. Applying FACS analyses we confirmed that our myeloma

cells express CXCR3, the high affinity receptor for CXCL10 (Fig. 5D), which appears functional because the decrease in cell number following CXCL10 treatment was prevented by neutralizing anti-CXCR3 antibody (Fig. 5E, upper and middle panels). Notably, reduced viability was also observed in cells collected from a patient with plasma cell leukemia treated with CXCL10, and this effect was prevented by anti-CXCR3 neutralizing antibody (Fig. 5E, lower panel), thus supporting a clinical relevance of CXCL10 in myeloma. Likewise, anti CXCL10 neutralizing antibody enhanced the proliferation of CAG (Fig. 5F, upper panel) RPMI-8266 and U266 cells (Suppl. Fig. 2F, G) cells, and CXCL10 gene silencing in CAG cells (Suppl. Fig. 2E) resulted in increased colony formation in soft agar (Fig. 5F, middle and lower panels), further supporting the notion that CXCL10 attenuates myeloma cell proliferation. In order to further explore the anti-myeloma feature of CXCL10, CAG-heparanase cells over expressing CXCL10 or control empty vector (Vo) were inoculated subcutaneously and tumor development was inspected. Nine out of ten mice inoculated with the CAG-heparanase control cells developed tumor xenografts with an average weight of 0.41 ± 0.08 gr (Fig. 6A, Vo). In striking contrast, only three out of nine mice inoculated with CAG-heparanase cells over expressing CXCL10 developed tumor xenografts that were remarkably smaller (0.02 ± 0.01 gr; Fig. 6A, CXCL10).

Reduced tumor burden in cells over expressing CXCL10 (Suppl. Fig. 2H and Suppl. Fig. 3A) was associated with a two-fold decrease in BrdU incorporation (Fig. 6B) and tumor angiogenesis (Fig. 6C), decrease that was statistically highly significant ($p=0.001$ for BrdU-positive cells in Vo vs. CXCL10 tumors and $p=0.002$ for blood vessel density in Vo vs. CXCL10 tumors). The latter is supported by attenuation of endothelial cell proliferation by CXCL10 (Fig. 5A, lower panel), thus implying that CXCL10 hampers myeloma progression by decreasing the proliferation rate of both the tumor and endothelial cells. CXCL10 has a very short half-life *in vivo* and hence its potential use as a drug is limited. To overcome this, we constructed a chimeric protein composed of CXCL10 fused to IgG1 (Fc)²⁹. The fusion protein was expressed as a disulphide-linked homodimer, similar to IgG1, yielding a molecular mass of ~ 72 kDa when analyzed under non-reducing conditions, consisting of two identical 36 kDa subunits (Suppl. Fig. 3B). Next, we confirmed that the CXCL10-Ig fusion protein maintains its functional property to attract activated T cells, comparable to recombinant CXCL10 (Suppl. Fig. 3C). Treatment of mice inoculated with CAG-heparanase cells with the CXCL10-Ig fusion protein significantly attenuated tumor development (Fig. 6D). On termination of the experiment on day 21, noticeable differences in tumor xenograft development were observed; average weights of control PBS treated tumors were 460 ± 44 mg compared with 260 ± 58 mg for CXCL10-Ig treated tumors, representing an average decrease of 43% in tumor weight (Fig. 6D). Administration of CXCL10-Ig to mice inoculated with MPC-11 mouse myeloma cells, resulted in even more efficient inhibition of tumor growth (Fig. 6E). On termination of the experiment (day 11), tumors developed in PBS treated mice had an average weight of 343 ± 40 mg vs. 118 ± 61 mg in the CXCL10-Ig treated mice, representing an impressive 66% average decrease in tumor weight (Fig. 6E). Moreover, half of the CXCL10-Ig treated mice failed to develop tumors at all (Fig. 6E). Notably, treatment with CXCL10-Ig was associated with a marked increase in the amount of anti-tumor CD8⁺ (12 fold) and NK (6 fold) cells in the tumor lesion (Fig. 6F), reflecting the ability of CXCL10-Ig to chemoattract anti-tumor immune cells *in vivo*. This may provide

another path by which CXCL10 suppresses myeloma tumor growth, and altogether indicating that CXCL10 and to a higher extent its Ig-fusion protein, is a potential new therapeutic modality for myeloma.

Discussion

Compelling evidence have shown that heparanase is up regulated in various primary solid tumors (i.e., carcinomas and sarcomas) and hematological malignancies^{3, 5, 33}. Mechanisms responsible for heparanase induction are poorly defined and appear to combine epigenetic, systemic and local mediators that operate at the transcriptional and post-transcriptional levels^{3, 34}. The consequence of heparanase induction most often associates with disease progression and bad prognosis due to increased tumor metastasis, thus providing strong clinical support for the pro-metastatic function of heparanase and encouraging the development of heparanase inhibitors^{4, 10, 35}. Heparanase appears nonetheless more versatile and promotes the development of primary human tumors via activation of signaling molecules (i.e. Akt, Src, EGFR, IR, STATs)^{16-18, 29, 36} and induction of pro-tumorigenic gene transcription (i.e. VEGF, HGF, MMP9)^{24, 29, 37}. The molecular mechanism(s) exerted by heparanase to promote tumor progression is still incompletely understood. Applying an inducible model system we clearly demonstrate that heparanase induction markedly promotes colony formation and tumor development by CAG myeloma (Fig. 2; Suppl. Fig. 1B, C), in agreement with the decisive role of heparanase in myeloma progression^{38, 39}. Many of the pro-tumorigenic effects of heparanase in myeloma have been shown to relay upon increased expression and shedding of syndecan-1³⁸. This proteoglycan is expressed on most myeloma tumor cells and is a critical determinant of myeloma cell survival and growth⁴⁰. Cell surface syndecan-1 promotes adhesion of myeloma cells; Syndecan-1 remains biologically active after it is shed from cells and can modulate the activity of heparin-binding growth factors⁴⁰. Heparanase enhances syndecan-1 shedding by up-regulating the expression of matrix metalloproteinase 9 (MMP9) and urokinase plasminogen activator (uPA) that are recognized as syndecan sheddases³⁷. In addition, heparanase enhances the expression of HGF that can associate with shed syndecan-1 and facilitate paracrine signaling via its high affinity receptor, c-Met²⁴. Unlike the induction of MMP9 and uPA, enhanced expression of HGF does not require heparanase enzymatic activity²⁴. Marked stimulation of tumor development by the heparanase C-terminal domain (8C) and the heparanase splice variant T5 (Fig. 2) that lack enzymatic activity but exhibit signaling properties^{15, 16} further implies that enzymatically active and inactive heparanase cooperate to drive myeloma progression.

Utilizing gene array methodology we have found that heparanase induction associates with CXCL10 down-regulation. CXCL10 repression by heparanase was demonstrated in three myeloma cell lines (CAG, U266 and RPMI-8266) following over-expression or exogenous addition of heparanase (Fig. 4C, D). Reduced CXCL10 level as a consequence of heparanase induction was also evident at the protein level in CAG cells grown in the presence of Dox (Fig. 4B) and in corresponding tumor xenografts (Suppl. Fig. 3D). Moreover, heparanase gene silencing resulted in elevation of CXCL10 transcription (Fig. 4C, sh-Hepa), collectively implying that heparanase levels inversely associate with CXCL10 expression. CXCL10 is therefore a new member in a growing list of genes regulated by

heparanase and associated with tumorigenesis (i.e., VEGF, VEGF-C, TF, RANKL, HGF, MMP9). A novel set of genes under heparanase regulation has recently been characterized in T cells⁴¹. In this context, nuclear heparanase was shown to regulate the transcription of a cohort of inducible immune response genes by controlling histone H3 methylation⁴¹, further expanding the transcriptional potential of heparanase. Whether a similar mechanism is also exerted by heparanase in the framework of tumor progression is yet to be revealed. Noteworthy, heparanase activity is dramatically increased in patients with rheumatoid arthritis, and this induction was associated with a 2.5-fold decrease in CXCL10⁴², further strengthening the inverse correlation between heparanase and CXCL10 in the context of autoimmunity.

The mechanism by which heparanase down-regulates CXCL10 transcription is not entirely clear but associates with activation of Src (Suppl. Fig. 1D), Erk and STAT3 (Suppl. Fig. 4E) and appears not to relay on heparanase enzymatic activity. This is concluded because CXCL10 repression was observed also when heparanase was added together with potent inhibitors of its catalytic activity (heparin and SST0001; Suppl. Fig. 4A)⁴, and because reduced CXCL10 expression was noted by heparanase variants [C-terminal domain (8C) and T5 splice variant] (Suppl. Fig. 4B, C) that lack catalytic activity^{15, 16}. Down regulation of CXCL10 in the course of tumor progression suggests that this protein exerts tumor suppressor properties in myeloma. Indeed, recombinant CXCL10 attenuated the proliferation of myeloma cells (Fig. 5A), in agreement with previous results⁴³, and even greater inhibition of proliferation was observed in cells over expressing CXCL10 (Fig. 5B). Over expression of CXCL10 was also associated with higher levels of cleaved caspase 3 (Suppl. Fig. 4D), suggesting that CXCL10 may promote apoptosis. Importantly, CXCL10 reduced the viability of myeloma cells collected from a patient with plasma cell leukemia (Fig. 5F), further strengthening its clinical relevance.

CXCL10 over expression resulted in significantly fewer and smaller myeloma colonies in soft agar (Fig. 5C), while CXCL10 gene silencing was associated with increased colony formation in soft agar (Fig. 5F). Moreover, CXCL10 gene silencing resulted in tumor xenografts that were 60% bigger than control myeloma tumors (0.29 ± 0.05 and 0.47 ± 0.09 gr for shCon and shCXCL10 tumors, respectively), differences that approached significance ($p=0.06$; data not shown). Strikingly, infection of CXCL10 to CAG cells that over express heparanase and hence are endowed with high tumorigenic capacity¹⁵ resulted in a marked decrease in tumor development (Fig. 6A). Reduced tumor development following CXCL10 over expression was associated with a 2-fold decrease in cell proliferation (Fig. 6B) and tumor angiogenesis (Fig. 6C), clearly depicting anti-cancer properties of CXCL10 in myeloma. Decreased tumor development in mice treated with the CXCL10-Ig fusion protein (Fig. 6D-F) not only supports the notion that CXCL10 exerts anti-myeloma activity, but also provides a more practical means of implementing this chemokine for the treatment of myeloma. CXCL10 (= interferon- γ inducible protein of 10 kDa; IP-10) is an interferon-inducible chemokine with potent chemotactic activity on activated effector T cells and other leukocytes expressing its high affinity G protein-coupled receptor CXCR3^{44, 45}. Indeed, administration of CXCL10-Ig to mice inoculated with MPC-11 mouse myeloma cells significantly increased the number of NK and T cytotoxic cells in the myeloma microenvironment, resulting in smaller or no tumors (Fig. 6E, F). In addition to its role in

directing the trafficking of activated T lymphocytes and other leukocytes, CXCL10 acts on other cell types. In particular, CXCL10 exhibits anti-proliferative effect on endothelial cells and inhibits wound healing and tumor angiogenesis⁴⁴⁻⁴⁷. Consequently, over expression of CXCL10 inhibits the progression of tumor xenografts produced by renal, breast, cervical, and lung carcinoma, fibrosarcoma, melanoma, and Burkitt lymphoma cells^{44, 48-51}, resulting in tumor necrosis⁵². Moreover, administration of CXCL10 prolonged the survival of mice inoculated with lung carcinoma cells due to decreased lung metastasis⁵³. In other model systems and contrary to its tumor suppressor properties, CXCL10 exhibits tumor-promoting function thought to be mediated by enhanced expression of CXCR3⁴⁴. CXCL10 is up-regulated in many human cancers, but is down regulated in others⁴⁴. In myeloma patients, CXCL10 appears to be down regulated (<https://www.oncomine.org>), yet this aspect requires in depth investigation. Studies aimed at this direction are currently underway.

Taken together, we describe a novel molecular mechanism that underlines the pro-tumorigenic function of heparanase in myeloma and involves down regulation of CXCL10. This chemokine appear to operate in three independent manners to suppress myeloma progression, directly attenuating myeloma cell proliferation; attenuating endothelial cell proliferation and tumor angiogenesis; and chemoattracting anti-tumor immune cells. Hence, CXCL10 can be considered as a tumor suppressor in this malignancy. Down regulation of such a tumor suppressor provides growth advantage associated with heparanase induction in myeloma. CXCL10, and especially the CXCL10-Ig fusion protein, may thus possibly be considered as a novel therapeutics in myeloma, along with other molecular determinants that have recently been identified^{54, 55}, offering new therapeutic modalities for this incurable disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge the devoted help of Dr. Liat Linde and Dr. Boaz Kigel (Rappaport Faculty of Medicine) in performing the gene array methodology and purification of the CXCL10-Ig fusion protein, respectively. This study was supported (in part) by research funding from the Israel Science Foundation (grant 549/06); National Cancer Institute, NIH (grant CA106456); the Israel Cancer Research Fund (ICRF); and the Rappaport Family Institute Fund to I. Vlodavsky.

I. Vlodavsky is a Research Professor of the ICRF.

References

1. Arvatz G, Shafat I, Levy-Adam F, Ilan N, Vlodavsky I. The heparanase system and tumor metastasis: is heparanase the seed and soil? *Cancer Metastasis Rev.* 2011 Jun;30:253–268. [PubMed: 21308479]
2. Barash U, Cohen-Kaplan V, Doweck I, Sanderson RD, Ilan N, Vlodavsky I. Proteoglycans in health and disease: new concepts for heparanase function in tumor progression and metastasis. *FEBS J.* 2010; 277:3890–3903. [PubMed: 20840586]
3. Ilan N, Elkin M, Vlodavsky I. Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. *Intl J Biochem & Cell Biol.* 2006; 38:2018–2039.

4. Vlodavsky I, Ilan N, Naggi A, Casu B. Heparanase: structure, biological functions, and inhibition by heparin-derived mimetics of heparan sulfate. *Curr Pharm Des.* 2007; 13:2057–2073. [PubMed: 17627539]
5. Vreys V, David G. Mammalian heparanase: what is the message? *J Cell Mol Med.* 2007; 11:427–452. [PubMed: 17635638]
6. Cohen I, Pappo O, Elkin M, San T, Bar-Shavit R, Hazan R, et al. Heparanase promotes growth, angiogenesis and survival of primary breast tumors. *Intl J Cancer.* 2006; 118:1609–1617.
7. Fux L, Ilan N, Sanderson RD, Vlodavsky I. Heparanase: busy at the cell surface. *Trends Biochem Sci.* 2009; 34:511–519. [PubMed: 19733083]
8. Lerner I, Baraz L, Pikarsky E, Meirovitz A, Edovitsky E, Peretz T, et al. Function of heparanase in prostate tumorigenesis: potential for therapy. *Clin Cancer Res.* 2008; 14:668–676. [PubMed: 18212251]
9. Cassinelli G, Lanzi C, Tortoreto M, Cominetti D, Petrangolini G, Favini E, et al. Antitumor efficacy of the heparanase inhibitor SST0001 alone and in combination with antiangiogenic agents in the treatment of human pediatric sarcoma models. *Biochemical Pharmacol.* 2013; 85:1424–1432.
10. Dredge K, Hammond E, Handley P, Gonda TJ, Smith MT, Vincent C, et al. PG545, a dual heparanase and angiogenesis inhibitor, induces potent anti-tumour and anti-metastatic efficacy in preclinical models. *Br J Cancer.* 2011; 104:635–642. [PubMed: 21285983]
11. Ritchie JP, Ramani VC, Ren Y, Naggi A, Torri G, Casu B, et al. SST0001, a chemically modified heparin, inhibits myeloma growth and angiogenesis via disruption of the heparanase/syndecan-1 axis. *Clin Cancer Res.* 2011; 17:1382–1393. [PubMed: 21257720]
12. Shafat I, Ben-Arush MW, Issakov J, Meller I, Naroditsky I, Tortoreto M, et al. Pre-clinical and clinical significance of heparanase in Ewing's sarcoma. *J Cell Mol Med.* 2011; 15:1857–1864. [PubMed: 21029368]
13. Elkin M, Ilan N, Ishai-Michaeli R, Friedmann Y, Papo O, Pecker I, et al. Heparanase as mediator of angiogenesis: mode of action. *FASEB J.* 2001; 15:1661–1663. [PubMed: 11427519]
14. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I. A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane. *Am J Pathol.* 1988; 130:393–400. [PubMed: 3277442]
15. Barash U, Cohen-Kaplan V, Arvatz G, Gingis-Velitski S, Levy-Adam F, Nativ O, et al. A novel human heparanase splice variant, T5, endowed with protumorigenic characteristics. *FASEB J.* 2010; 24:1239–1248. [PubMed: 20007507]
16. Fux L, Feibish N, Cohen-Kaplan V, Gingis-Velitski S, Feld S, Geffen C, et al. Structure-function approach identifies a COOH-terminal domain that mediates heparanase signaling. *Cancer Res.* 2009; 69:1758–1767. [PubMed: 19244131]
17. Cohen-Kaplan V, Doweck I, Naroditsky I, Vlodavsky I, Ilan N. Heparanase augments epidermal growth factor receptor phosphorylation: correlation with head and neck tumor progression. *Cancer Res.* 2008; 68:10077–10085. [PubMed: 19074873]
18. Cohen-Kaplan V, Jrbashyan J, Yanir Y, Naroditsky I, Ben-Izhak O, Ilan N, et al. Heparanase induces signal transducer and activator of transcription (STAT) protein phosphorylation: preclinical and clinical significance in head and neck cancer. *J Biol Chem.* 2012; 287:6668–6678. [PubMed: 22194600]
19. Riaz A, Ilan N, Vlodavsky I, Li JP, Johansson S. Characterization of Heparanase-induced Phosphatidylinositol 3-Kinase-AKT Activation and Its Integrin Dependence. *J Biol Chem.* 2013; 288:12366–12375. [PubMed: 23504323]
20. Cohen-Kaplan V, Naroditsky I, Zetser A, Ilan N, Vlodavsky I, Doweck I. Heparanase induces VEGF C and facilitates tumor lymphangiogenesis. *Intl J Cancer.* 2008; 123:2566–2573.
21. Nadir Y, Brenner B, Zetser A, Ilan N, Shafat I, Zcharia E, et al. Heparanase induces tissue factor expression in vascular endothelial and cancer cells. *J Thromb Haemost.* 2006; 4:2443–2451. [PubMed: 16970801]
22. Yang Y, Ren Y, Ramani VC, Nan L, Suva LJ, Sanderson RD. Heparanase enhances local and systemic osteolysis in multiple myeloma by upregulating the expression and secretion of RANKL. *Cancer Res.* 2010; 70:8329–8338. [PubMed: 20978204]

23. Okawa T, Naomoto Y, Nobuhisa T, Takaoka M, Motoki T, Shirakawa Y, et al. Heparanase is involved in angiogenesis in esophageal cancer through induction of cyclooxygenase-2. *Clin Cancer Res.* 2005; 11:7995–8005. [PubMed: 16299228]
24. Ramani VC, Yang Y, Ren Y, Nan L, Sanderson RD. Heparanase plays a dual role in driving hepatocyte growth factor (HGF) signaling by enhancing HGF expression and activity. *J Biol Chem.* 2011; 286:6490–6499. [PubMed: 21131364]
25. Nadav L, Katz BZ, Baron S, Cohen N, Naparstek E, Geiger B. The generation and regulation of functional diversity of malignant plasma cells. *Cancer Res.* 2006; 66:8608–8616. [PubMed: 16951174]
26. Purushothaman A, Uyama T, Kobayashi F, Yamada S, Sugahara K, Rapraeger AC, et al. Heparanase-enhanced shedding of syndecan-1 by myeloma cells promotes endothelial invasion and angiogenesis. *Blood.* 2010; 115:2449–2457. [PubMed: 20097882]
27. Gingis-Velitski S, Zetser A, Flugelman MY, Vlodavsky I, Ilan N. Heparanase induces endothelial cell migration via protein kinase B/Akt activation. *J Biol Chem.* 2004; 279:23536–23541. [PubMed: 15044433]
28. Beider K, Begin M, Abraham M, Wald H, Weiss ID, Wald O, et al. CXCR4 antagonist 4F-benzoyl-TN14003 inhibits leukemia and multiple myeloma tumor growth. *Exp Hematol.* 2011; 39:282–292. [PubMed: 21138752]
29. Zetser A, Bashenko Y, Edovitsky E, Levy-Adam F, Vlodavsky I, Ilan N. Heparanase induces vascular endothelial growth factor expression: correlation with p38 phosphorylation levels and Src activation. *Cancer Res.* 2006; 66:1455–1463. [PubMed: 16452201]
30. Meiron M, Zohar Y, Anunu R, Wildbaum G, Karin N. CXCL12 (SDF-1 α) suppresses ongoing experimental autoimmune encephalomyelitis by selecting antigen-specific regulatory T cells. *J Exp Med.* 2008; 205:2643–2655. [PubMed: 18852294]
31. Vlodavsky, I. Preparation of extracellular matrices produced by cultured corneal endothelial and PF-HR9 endodermal cells. In: Bonifacino, JS., MD; Hartford, JB.; Lippincott-Schwartz, J.; Yamada, KM., editors. *Protocols in Cell Biology*. Vol. 1. John Wiley & Sons; New York: 1999. p. 10.14.11–10.14.14.
32. Barash U, Arvatz G, Farfara R, Naroditsky I, Doweck I, Feld S, et al. Clinical significance of heparanase splice variant (t5) in renal cell carcinoma: evaluation by a novel t5-specific monoclonal antibody. *PLoS One.* 2012; 7(12):e51494. [PubMed: 23251556]
33. Sanderson RD, Yang Y, Kelly T, Macleod V, Dai Y, Theus A. Enzymatic remodeling of heparan sulfate proteoglycans within the tumor microenvironment: Growth regulation and the prospect of new cancer therapies. *J Cell Biochem.* 2005; 96:897–905. [PubMed: 16149080]
34. Arvatz G, Barash U, Nativ O, Ilan N, Vlodavsky I. Post-transcriptional regulation of heparanase gene expression by a 3' AU-rich element. *FASEB J.* 2011; 24:4969–4976. [PubMed: 20798248]
35. Miao HQ, Liu H, Navarro E, Kussie P, Zhu Z. Development of heparanase inhibitors for anti-cancer therapy. *Curr Med Chem.* 2006; 13:2101–2111. [PubMed: 16918340]
36. Purushothaman A, Babitz SK, Sanderson RD. Heparanase enhances the insulin receptor signaling pathway to activate extracellular signal-regulated kinase in multiple myeloma. *J Biol Chem.* 2012; 287:41288–41296. [PubMed: 23048032]
37. Purushothaman A, Chen L, Yang Y, Sanderson RD. Heparanase stimulation of protease expression implicates it as a master regulator of the aggressive tumor phenotype in myeloma. *J Biol Chem.* 2008; 283:32628–32636. [PubMed: 18812315]
38. Ramani VC, Purushothaman A, Stewart MD, Thompson CA, Vlodavsky I, Au JL, et al. The heparanase/syndecan-1 axis in cancer: mechanisms and therapies. *The FEBS J.* 2013; 280:2294–2306.
39. Sanderson RD, Iozzo RV. Targeting heparanase for cancer therapy at the tumor-matrix interface. *Matrix Biol.* 2012; 31:283–284. [PubMed: 22655968]
40. Sanderson RD, Yang Y. Syndecan-1: a dynamic regulator of the myeloma microenvironment. *Clin Exp Metastasis.* 2008; 25:149–159. [PubMed: 18027090]
41. He YQ, Sutcliffe EL, Bunting KL, Li J, Goodall KJ, Poon IK, et al. The endoglycosidase heparanase enters the nucleus of T lymphocytes and modulates H3 methylation at actively

- transcribed genes via the interplay with key chromatin modifying enzymes. *Transcription*. 2012; 3:130–145. [PubMed: 22771948]
42. Li RW, Freeman C, Yu D, Hindmarsh EJ, Tymms KE, Parish CR, et al. Dramatic regulation of heparanase activity and angiogenesis gene expression in synovium from patients with rheumatoid arthritis. *Arthritis Rheum*. 2008; 58:1590–1600. [PubMed: 18512775]
 43. Giuliani N, Bonomini S, Romagnani P, Lazzaretti M, Morandi F, Colla S, et al. CXCR3 and its binding chemokines in myeloma cells: expression of isoforms and potential relationships with myeloma cell proliferation and survival. *Haematologica*. 2006; 91:1489–1497. [PubMed: 17082008]
 44. Liu M, Guo S, Stiles JK. The emerging role of CXCL10 in cancer (Review). *Oncology Letters*. 2011 Jul.2:583–589. [PubMed: 22848232]
 45. Rosenkilde MM, Schwartz TW. The chemokine system - a major regulator of angiogenesis in health and disease. *Apmis*. 2004; 112:481–495. [PubMed: 15563311]
 46. Bodnar RJ, Yates CC, Rodgers ME, Du X, Wells A. IP-10 induces dissociation of newly formed blood vessels. *J Cell Sci*. 2009; 122:2064–2077. [PubMed: 19470579]
 47. Luster AD, Greenberg SM, Leder P. The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. *J Exp Med*. 1995; 182:219–231. [PubMed: 7790818]
 48. Feldman AL, Friedl J, Lans TE, Libutti SK, Lorang D, Miller MS, et al. Retroviral gene transfer of interferon-inducible protein 10 inhibits growth of human melanoma xenografts. *Intl J Cancer*. 2002; 99:149–153.
 49. Man K, Ng KT, Xu A, Cheng Q, Lo CM, Xiao JW, et al. Suppression of liver tumor growth and metastasis by adiponectin in nude mice through inhibition of tumor angiogenesis and downregulation of Rho kinase/IFN-inducible protein 10/matrix metalloproteinase 9 signaling. *Clin Cancer Res*. 2010; 16:967–977. [PubMed: 20103676]
 50. Sun Y, Finger C, Alvarez-Vallina L, Cichutek K, Buchholz CJ. Chronic gene delivery of interferon-inducible protein 10 through replication-competent retrovirus vectors suppresses tumor growth. *Cancer Gene Therapy*. 2005; 12:900–912. [PubMed: 15905858]
 51. Tannenbaum CS, Tubbs R, Armstrong D, Finke JH, Bukowski RM, Hamilton TA. The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. *J Immunol*. 1998; 161:927–932. [PubMed: 9670971]
 52. Sgadari C, Angiolillo AL, Cherney BW, Pike SE, Farber JM, Koniaris LG, et al. Interferon-inducible protein-10 identified as a mediator of tumor necrosis in vivo. *Proc Natl Acad Sci USA*. 1996; 93:13791–13796. [PubMed: 8943014]
 53. Arenberg DA, White ES, Burdick MD, Strom SR, Strieter RM. Improved survival in tumor-bearing SCID mice treated with interferon-gamma-inducible protein 10 (IP-10/CXCL10). *Cancer Immunol Immunother*. 2001; 50:533–538. [PubMed: 11776375]
 54. Lamy L, Ngo VN, Emre NC, Shaffer AL 3rd, Yang Y, Tian E, et al. Control of autophagic cell death by caspase-10 in multiple myeloma. *Cancer Cell*. 2013; 23:435–449. [PubMed: 23541952]
 55. Veitonmaki N, Hansson M, Zhan F, Sundberg A, Lofstedt T, Ljungars A, et al. A human ICAM-1 antibody isolated by a function-first approach has potent macrophage-dependent antimyeloma activity in vivo. *Cancer Cell*. 2013; 23:502–515. [PubMed: 23597564]

Figure 1.

Inducible expression of heparanase, 8C and T5. **A**. CAG myeloma cells were infected with Tet-inducible heparanase, 8C, or T5 gene constructs. Cells were left untreated (-) or incubated with Dox (1µg/ml; +) for 24 h. Cell lysates were then prepared and subjected to immunoblotting applying anti-V5-tag (left upper panels) and anti-actin (left lower panels) antibodies. Tet-inducible control (Vo) and heparanase CAG cells were grown in the absence (0) or presence of Dox for 24 or 48 h and were then subjected to FACS analysis utilizing anti-CD138 (syndecan-1) antibody (**A**, right panels). (**B**). Elimination of heparanase, 8C and T5 protein levels following Dox withdrawal. CAG cells were left untreated (0) or incubated with Dox for 24 h and harvested (1). Dox was then removed, and cells were harvested on days 2-5 thereafter. Lysate samples were immunoblotted with anti V5-tag (upper panel) and anti-actin (second panel) antibodies, and protein levels were further quantified by densitometry analysis (lower panels).

Figure 2.

Heparanase, 8C and T5 induction enhances tumor xenograft development. **A.** Tumor volume. Heparanase-, 8C-, and T5-infected CAG myeloma cells were injected subcutaneously ($1 \times 10^6/0.1$ ml) and tumor development in mice supplemented (red) or not supplemented (blue) with Dox in their drinking water was calculated from external caliper tumor measurements. At the end of the experiment on day 34, tumors were resected, photographed (**insets**) and weighed (**B**).

Figure 3.

Validation of gene array results. Total RNA was extracted from CAG cell clone 13 carrying Tet-inducible heparanase before (0) and 8, 16, and 24 hours after the addition of Dox and subjected to gene array analysis as described under 'Materials and Methods'. Expression of selected genes that appeared to be induced (ATF5, SDF2L1) or repressed (CXCL10) by Dox was validated by real-time PCR (A). Similar analysis was carried out on RNA samples extracted from pool of CAG cells transfected with heparanase (Hepa), T5, or control empty vector (Vo) (B). Note that only CXCL10 repression is consistent in the clone and pool cell populations.

Figure 4.

Heparanase down-regulates CXCL10 expression. **A.** Total RNA was extracted from CAG-heparanase Tet-on clone 1 cells before (0) and 8, 16, and 24 hours after the addition of Dox, and CXCL10 expression was quantified by real time PCR. **B.** CAG-heparanase clone 13 cells were grown in the absence (-Dox) or presence of Dox (+Dox) for 24 hours. Cells were then fixed and subjected to immunostaining applying anti-CXCL10 antibody (upper panels). CXCL10 levels were quantified by ELISA in the corresponding conditioned medium (lower panel). Note marked decrease of CXCL10 levels following heparanase induction by Dox. **C.** CAG cells were transfected with anti-heparanase shRNA (sh-Hepa) or control shRNA (sh-Con) and CXCL10 expression was quantified by real time PCR. CXCL10 was similarly quantified in U266 and RPMI-8266 myeloma cells infected with heparanase (Hepa) or empty vector (Vo). Note increased CXCL10 expression following heparanase gene silencing and decreased CXCL10 levels following heparanase over expression. **D.** Exogenous addition. Heparanase (1 µg/ml) was added to the indicated cell lines; Total RNA was extracted after 24 hours and CXCL10 expression was quantified by real time PCR. Data is presented as expression relative to control cells set arbitrarily to a value of 1.

Figure 5.

CXCL10 attenuates myeloma and endothelial cell proliferation. **A.** Exogenous addition. 5×10^3 CAG (upper panel), RPMI-8226 (second panel), U266 (third panel) and human umbilical endothelial cells (HUVEC, lower panel) were grown in the absence (0) or presence of the indicated concentration of recombinant CXCL10. Cell number was evaluated by the MTT assay after 3 days as described under 'Materials and Methods'. **B.** Over expression. Tet-on CAG-heparanase clone 13 (upper panel), CAG (second panel) and RPMI-8226 (third panel) myeloma cells were infected with CXCL10 or control empty vector (Vo) and cell proliferation was evaluated by MTT assay. Note decreased cell proliferation following CXCL10 over expression. **C.** Colony formation. CAG-heparanase cells were infected with CXCL10 or control empty vector (Vo) and were seeded ($2 \times 10^3/35$ mm dish) in soft agar. Shown are representative photomicrographs of colonies at low (upper panel) and high magnification (lower panel). Note that colony number and size are prominently decreased following CXCL10 over expression. **D.** FACS analysis. CAG, RPMI-8226, and U266 myeloma cells were subjected to FACS analysis applying anti CXCR3 antibody. **E.** CXCL10 inhibits cell proliferation via CXCR3. CAG (upper panel), U266 (middle panel) and cells freshly isolated from a patient with plasma cell leukemia (PCL; representing myeloma cells that grow in the circulation; lower panel) were cultured in

the absence (Con) or presence of CXCL10 (5 μ g/ml) without or with anti CXCR3 neutralizing antibody (CXCL10+anti CXCR3; 50 μ g/ml) or control mouse IgG (CXCL10+Mo. IgG). Cell viability was evaluated after 2 (PCL) or 3 (CAG, U266) days by FACS or MTT assay, respectively. **F.** CAG cells were cultured in the presence rabbit IgG (Rb. IgG) or anti-CXCL10 neutralizing antibody (anti-CXCL10; 20 μ g/ml) for three days and cell proliferation was evaluated by MTT assay as above (upper panel). CAG cells were infected with control (sh-Con) or anti-CXCL10 shRNA (sh-CXCL10) and were seeded (2 $\times$ 10³/35 mm dish) in soft agar and grown for three weeks. Shown are representative photomicrographs of colonies at low (second panel) and high magnification (third panel). Colony number in shCXCL10 vs. control shRNA is shown graphically in the lower panel. Note that colony number and size are increased following CXCL10 gene silencing.

Figure 6.

CXCL10 attenuates tumor xenograft development. CAG-heparanase cells were infected with CXCL10 or control empty vector (Vo) and inoculated (1×10^6) subcutaneously into SCID mice ($n=9-10$) and tumor development was calculated from external caliper measurements (A, upper panel). At the end of the experiment on day 31, tumors were resected, photographed (A, inset) and weighed (A, lower panel). B, C. Immunohistochemical analysis. Paraffin-embedded 5 micron sections of tumor xenografts produced by control (Vo) and CXCL10 over expressing cells were stained with anti-BrdU (B) and anti-CD31 (C) antibodies. Quantification of BrdU-positive cells and blood vessel density is shown in the lower panels. Note prominent decrease of tumor development, tumor angiogenesis and BrdU incorporation in xenografts produced by CXCL10 infected cells. D.

CXCL10-Ig fusion protein. CAG heparanase cells (5×10^6) were implanted subcutaneously in NOD-SCID mice and treated with PBS (n=6) or CXCL10-Ig fusion protein (n=5; 200 μ g/mouse every other day) and tumor development was inspected over time (upper panel). At the end of the experiment on day 21, tumors were collected and weighed (lower panel). **E.** MPC-11 mouse myeloma cells (1×10^6) were inoculated subcutaneously in BALB/c mice and were treated with PBS (n=7) or CXCL10-Ig fusion protein (n=6; 200 μ g/mouse every other day). At the end of the experiment on day 11, tumors volume (upper panel) and weight (lower panel) were determined. Tumor cell suspension was prepared as described in 'Materials and Methods', and cells were subjected to FACS analysis applying antibodies specific for CD8 (**F**, left panels) and NK cells (**F**, right panels).