Enoxaparin Attenuates Mouse Colon Cancer Liver Metastases by Inhibiting Heparanase and Interferon-γ-inducible Chemokines

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Abstract. *Background/Aim: Low-molecular-weight heparin (LMWH) has been suggested to reduce the risk of cancer progression in both preclinical and clinical studies but the underlying mechanisms remain poorly explored. The aim of the study was to investigate the anti-metastatic role of enoxaparin, a clinically-used LMWH, in a murine model of colon cancer and to explore its underlying mechanisms. Materials and Methods: Using a reproducible mouse model of colon carcinomas, we assessed the capacity of enoxaparin, a LMWH, to affect tumor metastasis of colon carcinoma cell lines in mice. Results: The hepatic growth of colon carcinoma metastases was strongly inhibited by enoxaparin compared to (Ctrl) group (p=0.001). This effect was associated to an inhibition of heparanase mRNA expression and protein production both in vivo and in vitro. In addition, enoxaparin inhibited the liver and serum production of interferon gamma (Ifnγ)-inducible chemokine receptor ligands. Overexpression of heparanase prompted proliferation, migration and growth of colon carcinoma in vitro and in vivo to a point that was not affected by enoxaparin in vivo anymore. Conclusion: Enoxaparin decreased liver metastases in a mouse model of colon carcinoma. These results suggest that enoxaparin may benefit patients with cancer and deserves further laboratory and clinical investigations.*

Colorectal cancer (CRC) is one of the most common cancers world-wide with liver metastases present in approximately 25% of newly diagnosed patients (1). One critical step of metastasis is the ability of cancer cells to degrade the extracellular matrix (ECM) and the basement membrane (BM), including their main components, the heparan sulfate proteoglycan (HSPG) and the heparan sulfates (HS) (2, 3). HSPG consists of a protein core to which linear HS side chains are covalently linked (4). They also act as storage for a variety of HS-binding proteins, including cytokines, chemokines and HSPG-degrading enzymes (5-8).

Heparanase is the only mammalian endo-β-glycosidase that cleaves HS side chains at specific sites of the HSPG (9, 10). This enzymatic cleavage contributes to cancer cell migration, at least in part by liberating the HS-bound active proteins (11). Heparanase is up-regulated in virtually all mouse and human tumors (12, 13), is associated to an increased intra-tumoral microvessel density and has been suggested to contribute to the growth of metastases (14, 15). Moreover, a higher level of heparanase is associated with poorer patient survival (16-18).

In addition to heparanase, cytokines and chemokines play an important role in the development of metastasis (19, 20). Among them, Cxcr3 (chemokine (C-X-C motif) receptor 3) is highly expressed by many colorectal cell lines, promotes colon cancer metastasis and is associated to a poorer outcome (21-23). Cxcl9 (chemokine (C-X-C motif) ligand 9) and Cxcl10 (chemokine (C-X-C motif) ligand 10) are two Cxcr3 ligands that are expressed by a large variety of cells and function as major chemoattractants. They are associated to an improved natural killer (NK) cell survival and a better response to chemotherapy in a variety of cancer types (24, 25). Moreover, Cxcl10 can promote the invasiveness of human colorectal carcinoma cells (26). Based on these observations, we hypothesized that the inhibition of heparanase is associated with a lower level of inflammatory cytokines and chemokines, including Cxcl9 and Cxcl10.

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Low-molecular-weight heparin has been widely used as anticoagulant to treat cancer-associated thromboembolisms (27). In addition to this effect, they have been observed to improve patient survival (28, 29). The mechanism behind this effect remains unclear but it may implicate the inhibition of heparanase (30).

The aim of the present study was to assess the capacity of enoxaparin, a LMWH, to affect tumor metastasis of colon carcinoma cell lines in mice. Cell lines expressing heparanase constitutively or modified to overexpress it were used. To identify the mechanism(s) implicated in the beneficial effect of heparanase on tumor growth, we investigated a possible impact of heparanase on interferon-γ-inducible chemokines, such as Cxcl9 and Cxcl10.

Materials and Methods

Mice. Wild-type C57BL/6 mice were obtained from Charles River Laboratories (Chatillon-sur-Chalaronne, France). Animals were maintained in the conventional housing facility at the Geneva University Medical School.

Tumor cells. Mouse colon carcinoma cells (MCA38) were provided by Dr. Bertrand Huard (Immunology and Pathology Department, Geneva University). Mouse melanoma cells (B16-F10 BL6) were provided by Prof. Imohf Beat (Immunology and Pathology Department, Geneva University). They were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and glutamine (Invitrogen, Carlsbad, CA, USA).

Experimental metastasis. MCA38 and MCA38HPA cell lines were harvested after trypsinization, re-suspended in Hanks' balanced salt solution and injected intra-portally through a median laparotomy (5×10^5 cells/mouse). Treated mice received 200 μl of enoxaparin intravenously (200 μg/mouse) 4 h before tumor cell followed by daily injection. Control animals received the same volume of phosphate buffered saline (PBS). Such a dose of enoxaparin has been previously tested in mice and has been shown to be safe and non-hemorrhagic (30, 31). Mice were sacrificed after eight or fifteen days. Liver sections were analyzed and images were acquired using an Axioskop microscope (per magnification (10X); Zeiss, Schweiz, Switzerland). Perpendicular tumor diameters were measured and the tumor volume was determined by integration of the following formula: $\Sigma t_1 + t_2 + \ldots . t_n$ ($t=a^2 \times b \times 0.52$; a=smaller tumor diameter, b=larger tumor diameter) as described previously (32).

Western blot analysis. After euthanasia, tumoral and non-tumoral liver biopsy fragments were removed from all liver lobules and mixed, because of the small size of the tumor nodules. Liver fragments were frozen immediately at –80˚C. Protein extracts were prepared as described previously (33). Twenty-five μg of total liver proteins were separated by electrophoresis in a 12% sodium dodecyl sulfate polyacrylamide gel (Invitrogen, Taastrup, Denmark). The membranes were then incubated overnight at 4˚C with one of the following antibodies diluted in the blocking buffer: for heparanase, rabbit polyclonal antibody: HPA1 (Santa Cruz, Biotechnology, Inc. Heidelberg, Germany) diluted 1:500; for Cyclin D1, rabbit polyclonal antibody diluted 1:500 (Santa Cruz Biotechnology, Inc.). After rinsing in TBS-Tween, the membranes were incubated for 1 h at room temperature with a horse radish peroxidase conjugated-goat anti-rabbit antibody (Hercules, CA, USA) diluted 1:6,000 in the blocking buffer. For all blots, the amount of loaded proteins was controlled by probing the same membranes with a rabbit polyclonal antibody directed against Gapdh (Santa Cruz Biotechnology, Inc) diluted 1:1,000. Densitometric quantification of each band was performed using Quantity One software (PDI, Inc. Huntington Station, NY, USA) and normalized to the expression of Gapdh in the re-probed blot.

In vitro protein expression. For cell lines, 5×10^5 cells were plated and serum-starved for 24h. The following day, FCS was added to the medium containing enoxaparin doses for 24 h of incubation. Adherent cells were lysed in a standard lysis buffer. Forty μg of total proteins were subjected to Western blot analysis as described above.

Flow cytometry analysis. MCA38 cells were treated for 24 h with enoxaparin or PBS, then harvested and stained with APC-Annexin-V and 7-amino-actinomycin D (7AAD) (BD Biosciences, New Jersey, USA) in binding buffer for 15 min. Cells were immediately subjected to flow cytometry analyses using a Calibur flow cytometer (BD Biosciences, New Jersey, USA). Three populations of cells were observed: viable cells (negatively stained, lower left quadrant); early apoptotic cells (annexin-V-positive and 7-AAD-negative, lower right quadrant) and cells in the late stages of apoptosis (annexin-V- and 7-AAD-positive, upper right quadrant).

Apoptosis analysis. For *in vivo* apoptosis analysis, cryosections from non-treated and treated murine livers were submitted to immunofluorescence for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (red) and nucleus (blue). Sections were fixed for 20 min with paraformaldehyde 4% and rinsed in PBS. Liver sections were permeabilized with Triton 0.5% X-100 in 0.1% sodium citrate (10 min at room temperature), rinsed in PBS and submitted to TUNEL, using the *In Situ* Cell Death Detection kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Finally, liver sections were incubated 15 min with Hoechst at 1:1,000 at room temperature. After a last rinse in PBS, liver sections were analyzed and images were acquired using an Axioskop microscope (Zeiss). The number of TUNEL-positive cells (red) was assessed per high magnification.

Bioplex analysis. Serum inflammatory cytokines/chemokines were detected with the use of a multiplex immunoassay (20-BioPlex assay; Invitrogen-Life Technologies, Switzerland), according to the manufacturer's instructions. Each experiment was performed in duplicate. The cytokines' concentration was determined from the standard curve by analysis of mean fluorescent intensity values using a Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA, USA) with software (20-Bio-Plex Manager™ 6.0). For the *in vitro* experiments, cells were plated (1.5×10^5/well) and starved. After overnight culture, enoxaparin-containing fetal calf serum (FCS) was added for 24 h incubation. Supernatants were collected for cytokines'/chemokines' assessment.

Analysis of gene expression by real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from treated and non-treated mouse livers (day 8) utilizing the Qiagen RNeasy Midi kit (Qiagen, San Diego, CA, USA), according to manufacturer's instructions. For

cell lines, total RNA was extracted with the Qiagen RNeasy Micro kit. Cells were plated (5×10^5/well) and serum-starved for 24 h. The following day, serum was added for a subsequent 24 h incubation and total RNA was extracted from adherent cells.

For both liver and cell RNA extracts, cDNA was synthesized from 0.5 μg of total RNA using PrimeScript RT reagent Kit (Takara Bio Inc, Saint-Germain-en-Laye, France) following the supplier's instructions. For quantitative PCR, amplification of genes was performed from 2 ng cDNA and 300 nM of forward and reverse oligonucleotides using the Power SYBR Green PCR Master Mix (Applied Biosystems Inc, Carlsbad, CA, USA) and a SDS 7900 HT machine (Applied Biosystems Inc.). Specific oligonucleotides were used (Invitrogen): *Hpse* (forward 5'-CCGCGCTTCCTCACCTT-3' and reverse 5'-CAAGTATGCAGGAGATAAGCCTCTAG-3'), Cyclin D1 (*Ccnd1*) (forward 5'-GCCCTCTGTGCCACAGATG-3' and reverse 5'-CCACGCTCCCAGCAGCTA-3'), *Cxcl9* (forward 5'-GAA CCCTAGTGATAAGGAATGCA-3' and reverse 5'-CTGTTTGAG GTCTTTGAGGGATT-3'), *Cxcl10* (forward 5'-TCTGAGTGGAC TCAAGGGATC-3' and reverse 5'-CAGTTGCAGCGA CCGTC-3'), *Ifn-γ* (forward 5'-ATC TGG AGG AAC TGG CAA AA-3' and reverse 5'-TTC AAG ACT TCA AAG AGT CTG AGG TA-3'). Control genes used for normalization are *rps9, Gapdh, Actin b* and *36B4R*. mRNA levels were represented as arbitrary units (A.U).

Lentiviral transductions. HPA cDNA was amplified by PCR from genomic DNA using the primers (forward 5'-ATGACGTTTAAA CGCCACCATGTTCCTGAGGAGCAGC-3' and reverse 5'-ATG ACGTTTAAACCTAAATTCTCACACATTCTTC-3') and cloned into the lentiviral pWPI vector, which contains a green fluorescent protein (GFP) expression cassette for assessing transduction efficiency. Transduction of MCA38 was performed as previously described and assessed on the basis of GFP expression (34).

Trans-well migration assay. The migration assay was performed and analyzed using Costar Transwell migration chambers (6.5-mm diameter inserts) with 8.0-μm pore size, as previously described (35). Cells were cultured for at least 24 h in RPMI containing 5% FCS. Cells were trypsinized and 2×10^4 cells in 200 μl were put in the upper compartment, while the lower chamber was supplied with 500 μl of RPMI containing 5% FCS alone or containing recombinant platelet-derived growth factor BB (PDGF-BB) (20 ng/μl) as a control and incubated at 37˚C for 48 h (36). The medium was removed from the upper chamber and the remaining non-migrating cells were scraped off with a cotton swab. Cells that have migrated in the other side of the membrane were fixed with methanol and stained with Hoechst. The number of migrating cells was counted by counting blue nuclei using Metamorph software (Molecular devices (UK) Limited). Each test was performed in triplicate.

5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. EdU labeling was performed with Alexa Fluor 488 using (Click-iT™ EdU cell proliferation assay, Invitrogen). Twenty thousand cells were plated on a chamber slide, cultured for at least 24 h in RPMI 5% FCS and then incubated with 10 μM EdU for 2 h. After rinsing three times with PBS containing 1% (w/v) BSA, cells were fixed and EdU incorporation into the nucleus was detected with azide conjugated with Alexa Fluor™ 488 according to the manufacturer's protocol. Cells were rinsed with PBS between incubations and mounted with ProLong Gold with 4',6-diamidino-2-phenylindole (DAPI).

Statistical analysis. Survival was analyzed by the Kaplan–Meier method and differences between groups were tested by log-rank. Groups were compared using the Mann–Whitney or the Student's *t*-test. Figures report medians and quartiles. Analyses were conducted using of GraphPad PRISM version 5 software (GraphPad, San Diego, CA, USA). Statistical significance was set at $p<0.05$.

Results

Mouse MCA38 colorectal carcinoma cells express heparanase and are affected by enoxaparin treatment. The expression level of heparanase in the colorectal carcinoma cell line, MCA38, and in melanoma cells, B16-F10, was analyzed. MCA38 colorectal carcinomas and B16-F10 melanoma cells both expressed significant levels of heparanase (Figure 1). Enoxaparin at 200 μg/mouse, significantly inhibited the heparanase expression in MCA38 cells (1.051 (Enoxaparin) *vs*. 3.752 (Ctrl), $p<0.02$, n=4) (Figure 1a). In addition, the heparanase protein production was also decreased after enoxaparin treatment in MCA38 cells with a dose-dependent effect (0.212 (Enoxaparin)) *vs*. 1.013 (ctrl), $p<0.03$, n=4, Figure 1c). In B16-F10 cells, enoxaparin decreased the heparanase production, (1.253 (Enoxaparin) *vs*. 2.821 (ctrl), $p<0.03$, n=4, (Figure 1d), whereas no significant effect was noted at the mRNA level (Figure 1b).

Significantly, more dead MCA38 cells (Annexin V+, 7-AAD+) were detected following enoxaparin treatment (67% (Enoxaparin) *vs*. 45% (Ctrl), $p<0.02$, n=6, Figure 1e), but not in B16-F10 after treatment with enoxaparin (Figure 1f).

The enoxaparin effect was, at least in part, linked to a proliferation inhibition; the *cyclin D1* expression was tested in MCA38 and B16-F10 cells. We noted a significant decrease of *cyclin D1* expression in MCA38 cells (0.864 (Enoxaparin) *vs*. 1.547 (Ctrl)), $p<0.02$ n=4), Figure 1g) but not in B16-F10 after treatment with enoxaparin (Figure 1h).

Enoxaparin attenuates hepatic metastasis of MCA38 cells. To mimic the hematogenous phase of tumor cell dissemination during the metastatic process, MCA38 cells were injected into the portal vein. In the enoxaparin-treated group, all mice (n=12) survived until day eight, whereas, in the control group, 75% of mice survived ($p=0.069$, n=12 (Enoxaparin) *vs*. n=9 (Ctrl), as shown in Figure 2a. Increased survival was still observed in the enoxaparin-treated group when animals were kept until 24 days (data not shown). Liver tumor growth was attenuated after enoxaparin treatment. On day eight, tumors were detectable on histology (Figure 2c); enoxaparin significantly prevented tumor growth by 70% (284.3×10^3 mm^3 (Ctrl) (n=9) *vs*. 69.14×10^3 mm^3 in treated group (n=12), $p<0.001$) (Figure 2b). Insignificant differences in tumor volume were observed when mice were treated for 24 days (data not shown).

Enoxaparin affects heparanase expression, cell cycle and tumor cell viability in hepatic metastases. To test whether the

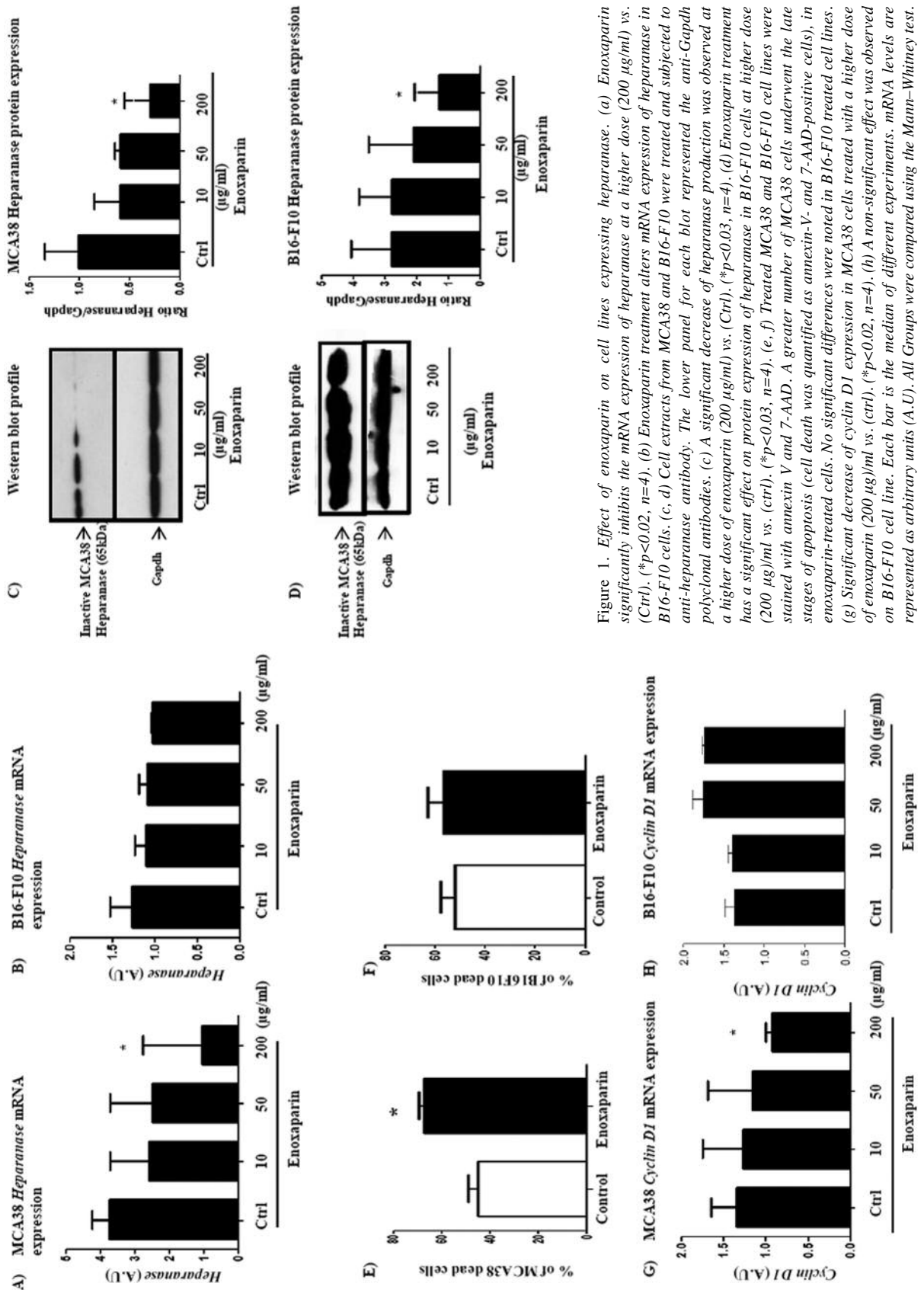

Figure 1. *Effect of enoxaparin on cell lines expressing heparanase. (a) Enoxaparin significantly inhibits the mRNA expression of heparanase at a higher dose (200 μg/ml) vs. (Ctrl). (*p<0.02, n=4). (b) Enoxaparin treatment alters mRNA expression of heparanase in B16-F10 cells. (c, d) Cell extracts from MCA38 and B16-F10 were treated and subjected to anti-heparanase antibody. The lower panel for each blot represented the anti-Gapdh polyclonal antibodies. (c) A significant decrease of heparanase production was observed at a higher dose of enoxaparin (200 μg/ml) vs. (Ctrl). (*p<0.03, n=4). (d) Enoxaparin treatment has a significant effect on protein expression of heparanase in B16-F10 cells at higher dose (200 μg)/ml vs. (ctrl). (*p<0.03, n=4). (e, f) Treated MCA38 and B16-F10 cell lines were stained with annexin V and 7-AAD. A greater number of MCA38 cells underwent the late stages of apoptosis (cell death was quantified as annexin-V- and 7-AAD-positive cells), in enoxaparin-treated cells. No significant differences were noted in B16-F10 treated cell lines. (g) Significant decrease of cyclin D1 expression in MCA38 cells treated with a higher dose of enoxaparin (200 μg)/ml vs. (ctrl). (*p<0.02, n=4). (h) A non-significant effect was observed on B16-F10 cell line. Each bar is the median of different experiments. mRNA levels are represented as arbitrary units (A.U). All Groups were compared using the Mann–Whitney test.*

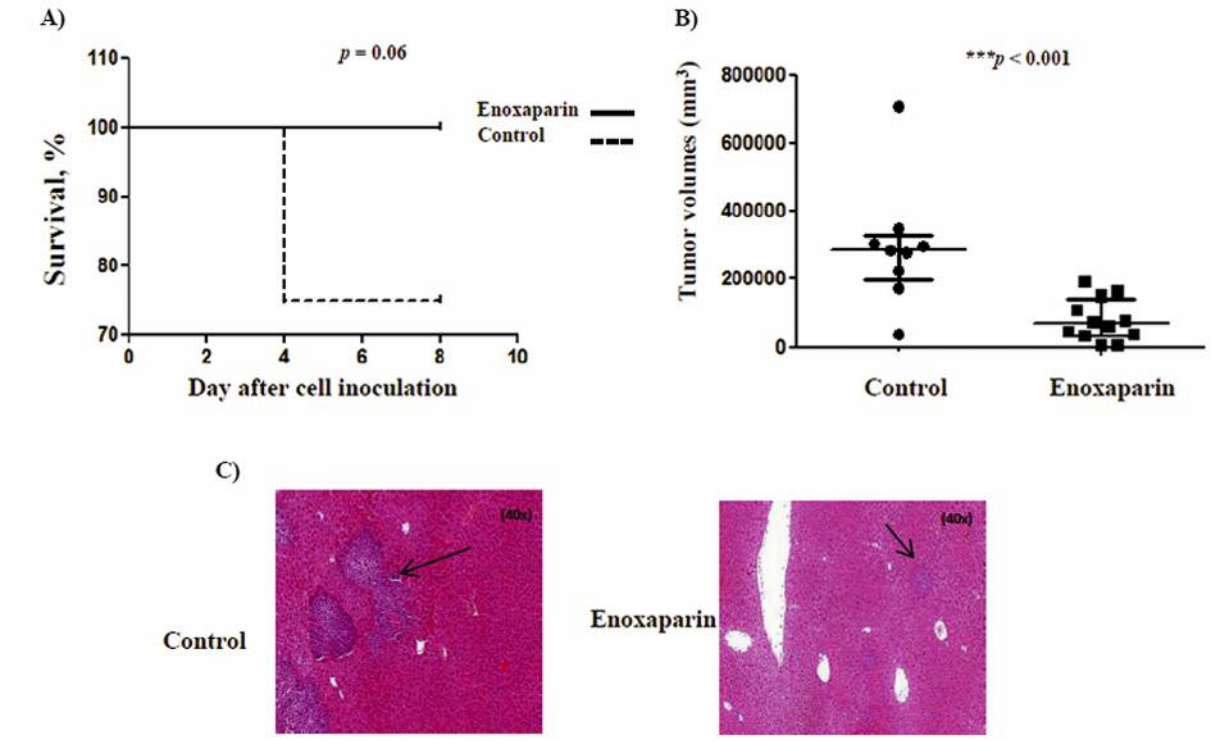

Figure 2. *Inhibition of hepatic metastasis. (a) Survival and/or time to euthanasia in tumor-bearing mice after MCA38 cell infusion. The totality of mice that received treatment (continued line) survived longer than control (Ctrl) mice group (discontinued line) in which only 75% survived. ($p=0.069$, n=12 (treated) vs. n=9 (Ctrl)). (b) Daily doses of enoxaparin reduced significantly liver tumor formation in enoxaparin-treated mice compared to Ctrl group (**$p<0.001$, n=12 (Enoxaparin) vs. n=9 (Ctrl)). Each bar is the median of tumor volume of 12 different mice (enoxaparin) and 9 (Ctrl). (c) Representative photographs (40× magnification) were taken from HE-stained liver sections at day 8. Arrows point to intra-hepatic metastatic nodules. Groups were compared using the Mann–Whitney test.*

observed effects were due to a heparanase inhibition, liver mRNA and proteins were extracted at day 8. Enoxaparin treatment (200 μg/ml) significantly decreased the *Heparanase* mRNA expression (0.474 (Enoxaparin) *vs*. 0.913 (Ctrl), $p<0.05$, n=8) (Figure 3a) and protein production (0.357 (Enoxaparin) *vs*. 0.714 (Ctrl), $p<0.004$, n=4) (Figure 3c and d). *Cyclin D1* expression was analyzed to determine whether the delay and reduction of tumor development was the consequence of an alteration in the cell cycle. Indeed, enoxaparin (200 μg/ml) decreased cyclin D1 (*Ccnd1*) mRNA expression compared to controls (0.438 (Enoxaparin) *vs*. 0.723 (Ctrl), $p<0.004$, n=8) (Figure 3b). On Western blot, Cyclin D1 production was also decreased in enoxaparin-treated mouse livers (0.307 (Enoxaparin) *vs*. 2.191 (Ctrl), $p<0.004$, n=6) (Figure 3c (middle panel) and 3e).

To further characterize the influence of enoxaparin on MCA38 cells, the presence of apoptotic cells in the tissues was analyzed. Cryo-sections from non-treated and treated murine livers were submitted to immunofluorescence for TUNEL (white) and nucleus (grey). Higher number of TUNEL-positive cells were observed in enoxaparin-treated tumors compared to controls (95 *vs*. 43 (Ctrl), n=7) (Figure 3f); this result was consistent with the cell death analysis by FACS and confirmed the deleterious effect of enoxaparin on MCA38 cells *in vitro* and *in vivo*.

Enoxaparin affects chemokine and growth factor production in vivo. To determine whether enoxaparin has an impact on relevant growth factors, cytokine and chemokine levels, we examined sera from euthanized mice at day eight. The secretion of inflammatory cytokines, such as vascular endothelial growth factor (Vegf) (14.66 *vs*. 20.01 (Ctrl), $p=0.6$, n=7), fibroblast growth factor beta (Fgf-b) (48.93 *vs*. 58.84 (Ctrl), $p=0.9$, n=6), interleukin (Il)-12p40 (40.40 *vs*. 68.96 (Ctrl), $p=0.09$, n=9), interleukin Il-1β (5.51 *vs*. 8.78 (Ctrl), $p=0.6$, n=9), interleukin Il-2 (5.43 *vs*. 6.09 (Ctrl), $p=0.2$, n=7) and monocyte chemoattractant protein-1 (Mcp-1) (5.07 *vs*. 9.90 (Ctrl) ($p=0.32$, n= 9) were non-significantly reduced in treated mice (Figure 4a). Whereas blood levels of macrophage inflammatory protein 1 alpha (Mip1-α) (7.09 *vs*. 8.04 (Ctrl), $p=0.2$, n=9) were not affected in enoxaparin-treated mice. Strong effects of enoxaparin were observed at

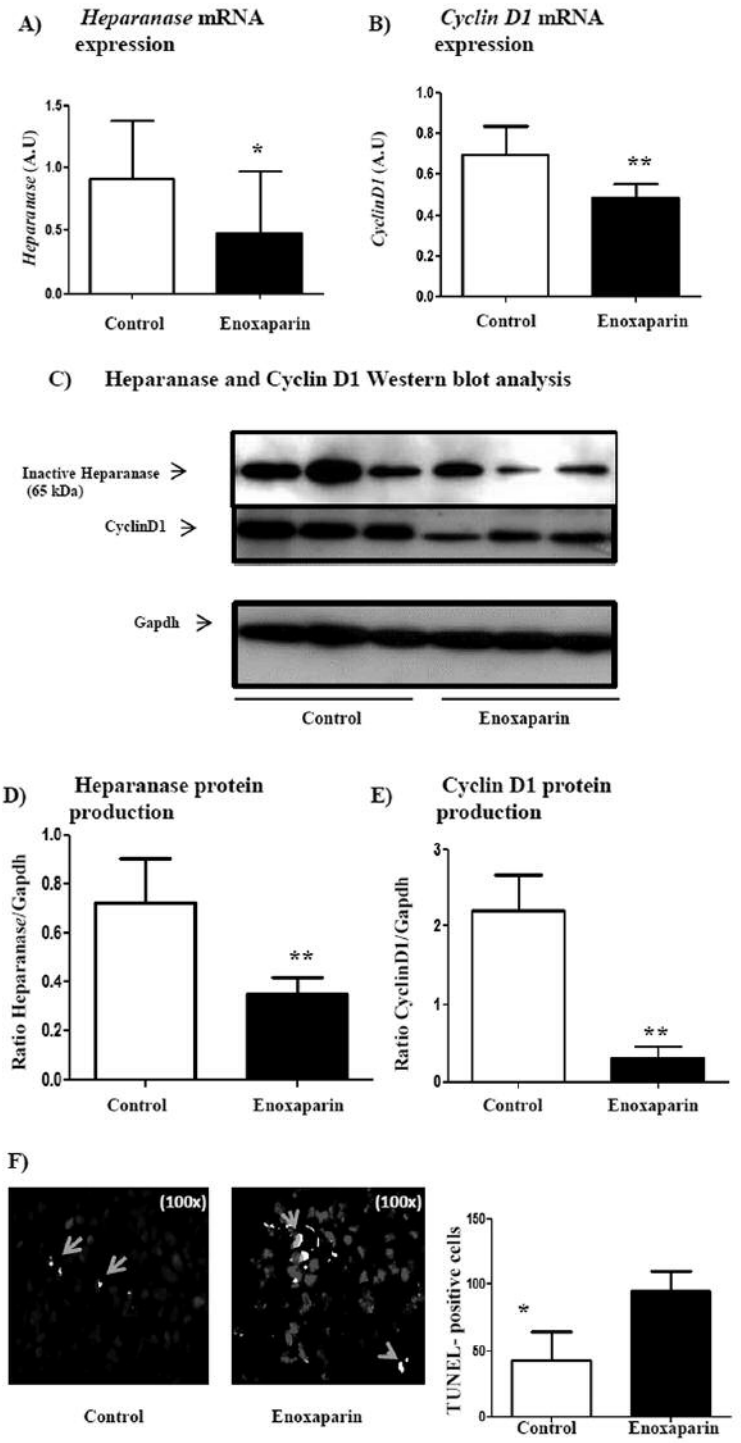

Figure 3. *Effects of enoxaparin treatment on heparanase and cyclin D1 expression in liver. (a) Enoxaparin treatment decreases significantly the mRNA expression of heparanase compared to control (Ctrl) group (*p<0.05, n=8). (b) Enoxaparin treatment decreased cyclin D1 expression compared to control group (**p<0.004, n=8). (c) Western blots analysis of liver extracts using anti-HPA antibodies (upper panel) and anti-cyclin D1 antibodies (middle panel). The anti-Gapdh polyclonal antibodies (lower panel) were used as an internal control for protein loading. Each lane represents an individual mouse. (d) Decreased expression of heparanase was detected in mouse liver treated by enoxaparin compared to control mice (**p<0.004, n=6). (e) Significant reduction of cyclin D1 production in enoxaparin-treated murine livers, compared to control mice (**p<0.004, n=6). (f) Higher number of TUNEL-positive cells in liver tumors of enoxaparin-treated mice compared to Ctrl mice. Arrows point to the TUNEL-positive cells (*p<0.03, n=7) (100× magnification). Groups were compared using the Mann–Whitney test. Each bar is the median of different animals and each reaction was repeated 3 times. mRNA levels are represented as arbitrary units (A.U).*

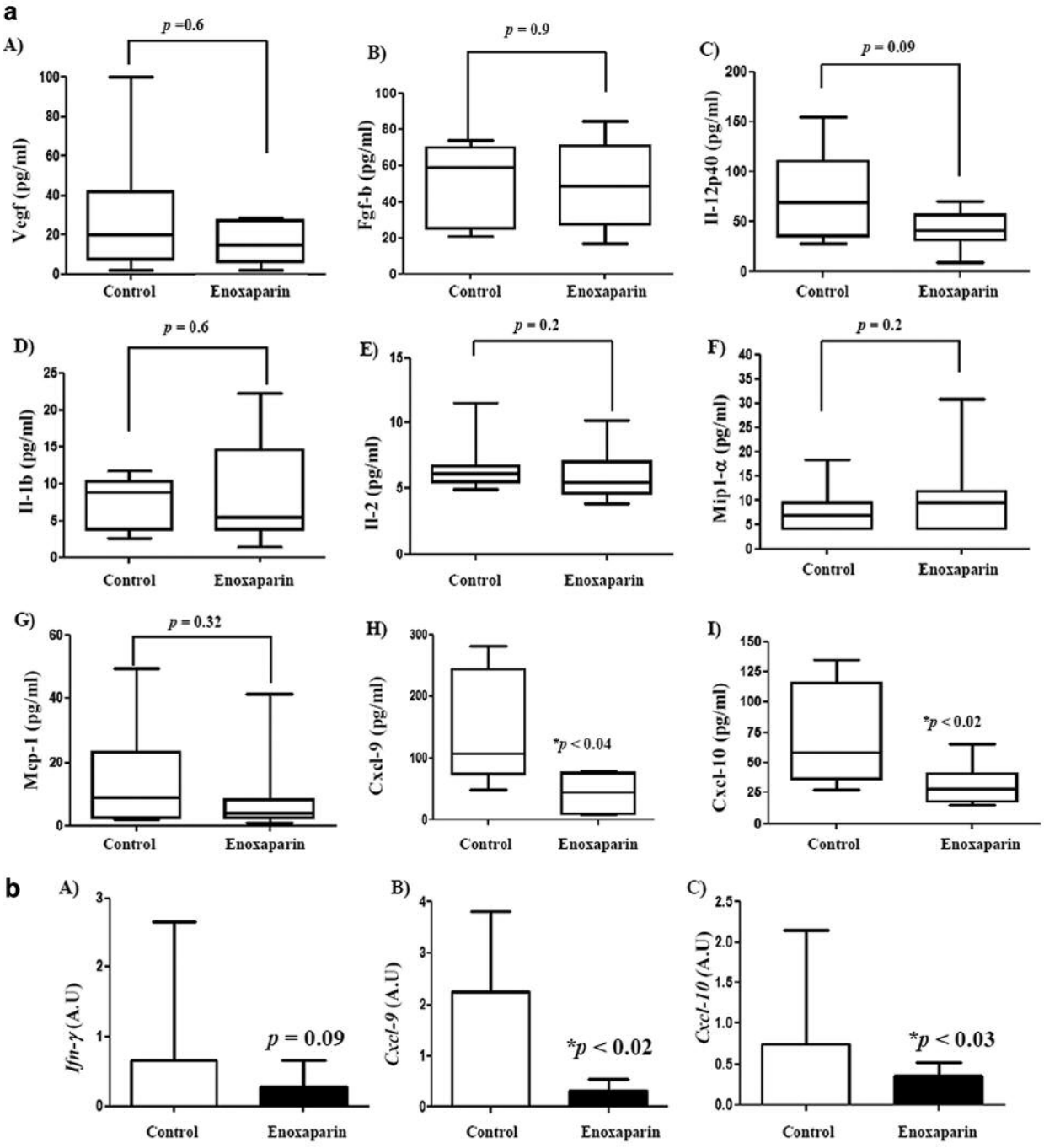

Figure 4. *a. Enoxaparin affects chemokine and growth factor production in vivo. Multiplex immunoassay (20-BioPlex assay) was used to assess serum from euthanized mice in treated and control group. (A, B) Enoxaparin treatment seems to affect non-significantly the release of the angiogenic factor Vegf and Fgf-b ((p=0.6, n=7) and (p=0.9, n=6)), respectively. (C-G) In treated mice, a decreasing trend was observed in inflammatory cytokine production, such as Il-12p40 (p=0.09, n=9), Il-1β (p=0.6, n=9) and Il-2 (p=0.2, n=7). No significant differences were observed for Mip1-α (p=0.2, n=9) and Mcp-1 (p=0.32, n= 9) production. (H, I) Chemokine release (Cxcl9 and Cxcl10) was significantly decreased in mice treated with enoxaparin compared to controls (*p<0.04, n=8 and *p<0.02, n=8 respectively). Each box represents the median of serum levels of 8 mice per group and (ns) means not significant. The values (picogram per ml) were plotted with box-and-whisker graphs and the line in the middle is the median. Each analysis was repeated 3 times. Groups were compared using the Mann–Whitney test. b. Enoxaparin affects chemokines' and cytokines' mRNA in vivo. Liver mRNA extracts from mice was analyzed for the expression of Ifn-γ, Cxcl9 and Cxcl10. (A) Higher but non-significant Ifn-γ expression was observed in livers of control mice compared to treated mice. (p=0.09, n=8). (B, C) The treatment reduced significantly the expression of Cxcl9 and Cxcl10 (*p<0.02, n=8, and *p<0.03, n=9, respectively). Each bar is the median (n=8 for Cxcl10 and n=9 for Cxcl9) different animals. mRNA levels are represented as arbitrary units (A.U). Groups were compared using the Mann–Whitney test.*

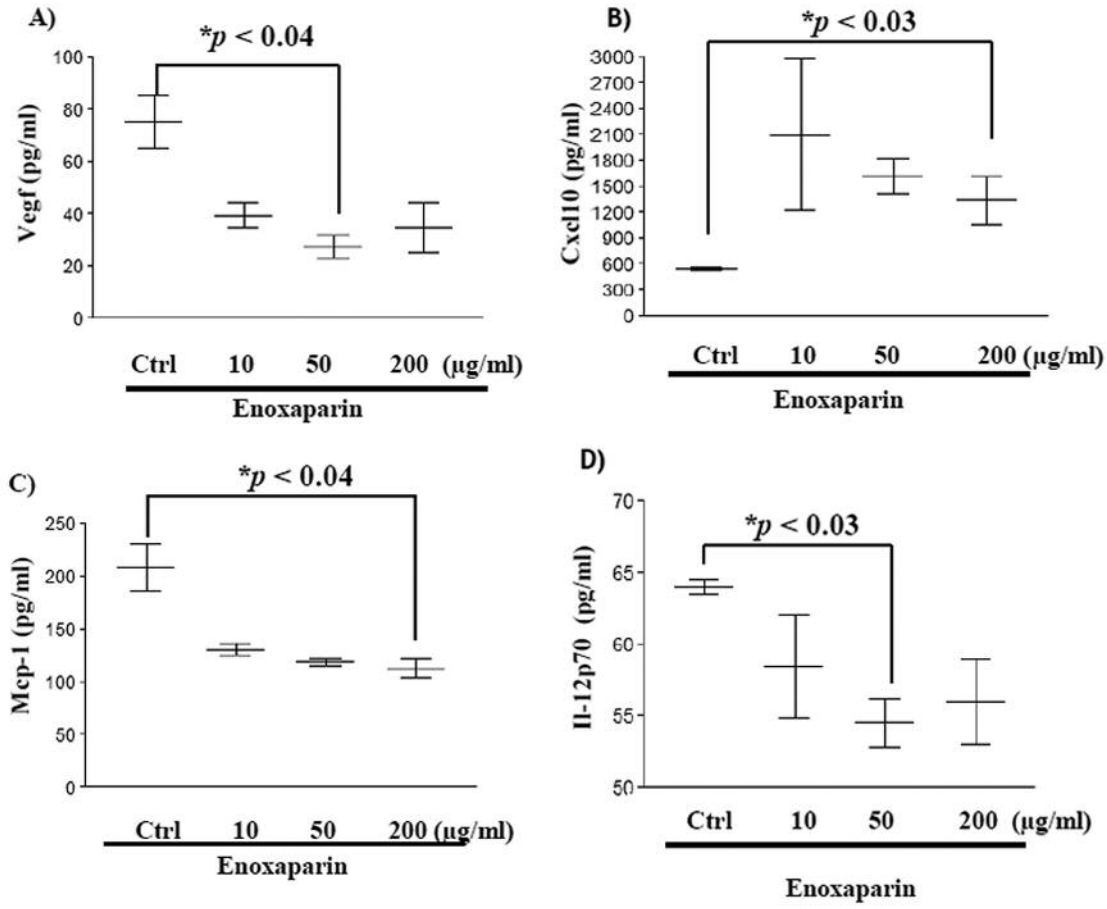

Figure 5. *Effects of enoxaparin on MCA38 cell lines. MCA38 were treated with increasing enoxaparin doses. Supernatants were collected and analyzed for Vegf, Cxcl10, MCP1, Il-12p70 and Il-1β using 20-BioPlex assays. Enoxaparin treatment decreases Vegf at a higher dose (50 µg/ml) vs. (Ctrl) (n=3, *p<0.04). (b) Enoxaparin increases the production of Cxcl10 at a higher dose (50 µg/ml) vs. (Ctrl) (n=3, *p<0.03). (c-e) Inflammatory cytokines' production was affected by enoxaparin treatment: Mcp-1 (n=3, *p<0.04), Il-12p70 (n=3,*p<0.03). Each analysis was repeated 3 times and the mean±SEM is indicated in the Results section. The values (picogram per ml) were plotted with box-and-whisker graphs and the line in the middle is the median. Groups were compared using the Student's t-test.*

the level of chemokines, with significantly lower Cxcl9 and Cxcl10 levels in treated mice (44.81 *vs.* 107.08 (Ctrl), $p<0.04$, n=8, and 28.11 *vs.* 58.45 (Ctrl), $p<0.02$, n=8) (Figure 4A (h, i)). mRNA expression levels of cytokines and chemokines were subsequently analyzed from same livers. Enoxaparin decreased the mRNA expression of *Ifn-γ* (0.661, n=8, *vs.* 0.276 (Ctrl), n=8, $p=0.09$) (Figure 4b (a)), *Cxcl9* (0.290 *vs.* 2.25 (Ctrl), $p<0.02$, n=8) and *Cxcl10* (0.391 *vs.* 0.749 (Ctrl), $p<0.03$, n=9) (Figure 4b (b, c)).

Cytokine and chemokine profile of MCA38 cells in response to enoxaparin treatment. To understand and characterize the direct effects of enoxaparin on murine colon carcinoma cells, the supernatant of MCA38 cells treated with increasing doses of enoxaparin was analyzed for the levels of Vegf, Mcp1, Il-12p70, using a Bioplex Assay. Supernatants from enoxaparin-treated cells *versus* non-treated cells showed decreased levels of Vegf (27.44±4.440 (50 µg/ml) *vs.* 75.38±10.00 (Ctrl), $p<0.04$, n=3) (Figure 5a), Mcp1 (113.6±8.875 (200 µg/ml) *vs.* 208.7±21.34 (Ctrl), $p<0.04$, n=3) (Figure 5c) and Il-12p70 (54.54±1.66 (50 µg/ml) *vs.* 64.04±54.1.66 (Ctrl), $p<0.03$, n=3) (Figure 5d). In contrast, the level of Cxcl10 was increased in supernatants from enoxaparin-treated cells (1621±200.4 (50 µg/ml) *vs.* 546.9±10.03 (Ctrl), $p<0.03$, n=3) (Figure 5b).

Study of heparanase-overexpressing MCA38HPA cell line vs. control cell lines. In an effort to further define the link between heparanase, enoxaparin treatment and metastasis, a cell line overexpressing heparanase was established (further referred to as MCA38HPA). MCA38 cells and empty vector-

transfected cells (MCA38pWPI) were used as negative controls. RT-PCR revealed a 2.7-fold increase in relative expression of heparanase mRNA in MCA38HPA cells compared to MCA38, as well as MCA38pWPI cells (3.625±0.3301 (MCA38HPA) *vs*. 1.375±0.3706 (MCA38) and 1.384±0.2353 (MCA38pWPI) n=4, $p<0.008$) (Figure 6a). Western blotting analysis revealed increased heparanase protein expression in MCA38HPA cells when compared to negative controls, MCA38 and MCA38pWPI cells (1.384±0.2353 *vs*. 0.2625±0.09945 (MCA38) and 0.1575±0.03092 (MCA38pWPI), n=4, $p<0.01$) (Figure 6b). Heparanase expression in MCA38HPA was comparable to the melanoma cell line B16F16, that expresses high levels of heparanase (Figure 6b). To investigate the influence of enoxaparin on heparanase expression, MCA38HPA and MCA38 cells were treated with increasing enoxaparin doses. The results showed that a 24-h treatment with enoxaparin at 200 μg/ml decreased heparanase expression significantly in MCA38 cells (0.5700±0.05859 (Ctrl) *vs*. 0.1303±0.07545 (200 μg/ml) n=3, $p<0.01$) (Figure 6c). Significant enoxaparin effect was observed in the MCA38HPA cell line between different doses (Figure 6c). To further characterize the MCA38HPA cells overexpressing heparanase, we studied their capacity to proliferate and migrate in dose-dependent manner of enoxaparin treatment. A higher percentage of EdU uptake was measured in MCA38HPA, when compared to negative control cell line (35.25±3.473 (MCA38HPA) *vs*. 20.25±2.394 (MCA38), n=4, $p<0.012$) (Figure 6d). Higher dose of enoxaparin (200 μg/ml) treatment affected only MCA38 cell line, when compared to non-treated conditions (20.25±2.394 (Ctrl) *vs*. 13.00±2.198 (200 μg/ml), n=4, $p<0.06$) (Figure 6d), while the enoxaparin treatment did not affect neither MCA38HPA nor B16F10 proliferation (Figure 5d). The migration capacity of MCA38 cells was assessed using a transwell system and PDGF-BB, a growth factor known to induce migration. As a positive control, migration of MCA38 cells was increased by PDGF-BB compared to non-treated MCA38 cells (8933±806.1 (PDGF-BB) *vs*. 828.3±229.6 (MCA38), n=4) (Figure 6e). Heparanase-overexpressing MCA38HPA cells showed a significantly increased migration compared to MCA38 and MCA38pWPI cells (3900±689.2 (MCA38HPA) *vs*. 828.3±229.6 (MCA38), n=4, $^{**}p<005$ and 434.0 ± 147.8 (MCA38pWPI), n=4, $^{**}p<0.002$) (Figure 6e).

Taking into account the effect of enoxaparin on MCA38 cells *in vitro*, we investigated the effect of enoxaparin treatment *in vivo* using our mouse model. Analysis of mice livers, retrieved 15 days after injection, showed that mice injected with MCA38HPA cells developed significantly larger tumors than animals that had received MCA38 and MCA38pWPI cells (751020±102280 (MCA38HPA), n=9 *vs*. 268316±51635 (MCA38), n=8, $^{**}p<001$ and 321008±90813 (MCA38pWPI), n=8, $^{**}p<0.007$) (Figure 7a and b). However, treatment with enoxaparin of mice injected with MCA38HPA did not show reduced tumor size (790526±124238 (MCA38 HPA+Enoxaparin) *vs*. 751020±102280 (MCA38HPA), n=8) (Figure 7 a and b). Collectively, these results suggested that increased heparanase expression in colorectal cancer cells promote migration and tumor growth. However, an inhibitory effect of enoxaparin on tumor growth of heparanase-overexpressing MCA38HPA cells could not be observed.

Discussion

Currently, the major obstacle in treating cancer patients is linked to the progression of the disease to a metastatic stage. Heparin and LMWHs, in addition to their traditional anticoagulant properties, have shown substantial anti-metastatic activity in recent studies (36-38), especially in experimental models (30, 5). However, the molecular mechanisms and the levels of LMWH action on HS bioactive binding molecules at early stages of colorectal cancer development remain unknown. In the present work, we tested the effect of enoxaparin as a LMWH in a murine model of hepatic metastatic cancer developing after injection of MCA38 cells, a mouse colorectal carcinoma cell line. We first showed that the MCA38 cell line constitutively expresses heparanase. Furthermore, we observed that addition of enoxaparin inhibits significantly the expression of heparanase in MCA38 cells and has an apoptotic effect on MCA38 cells *in vitro*. In our *in vivo* model, enoxaparin treatment inhibited significantly the development of liver metastases and conferred survival advantage in treated mice compared to non-treated mice. We also showed that enoxaparin-treated mice exhibited a significant decrease in heparanase expression at mRNA and protein level in liver. As mentioned, heparanase expression is up-regulated in many tumors, where it significantly increases the angiogenic and the metastatic potential of tumor cells (39, 40). The inhibition of the heparanase gene expression or enzyme activity suppresses metastasis formation, thus indicating that heparanase is involved in the extravasation and invasion process of cancer (15, 41). Our results showed that inhibition of liver heparanase gene expression by enoxaparin was significant and reached 50%.

In our study, we provide evidence that heparanase expression may contribute to the facilitation of hematogenous metastasis at early stages of colorectal murine cancer. These results were consistent with previous studies that demonstrated a direct effect of heparanase on tumor cell invasion and metastasis *in vivo* (42). To further investigate the potential mechanisms of enoxaparin on the invasion and metastasis of MCA38 colorectal cells, we studied the change in expression of various invasion and metastasis molecules, such as the *Ccnd1* and chemokines, as examined by RT-PCR, western blotting and Bioplex analysis. Cyclin D1 proto-oncogene is an

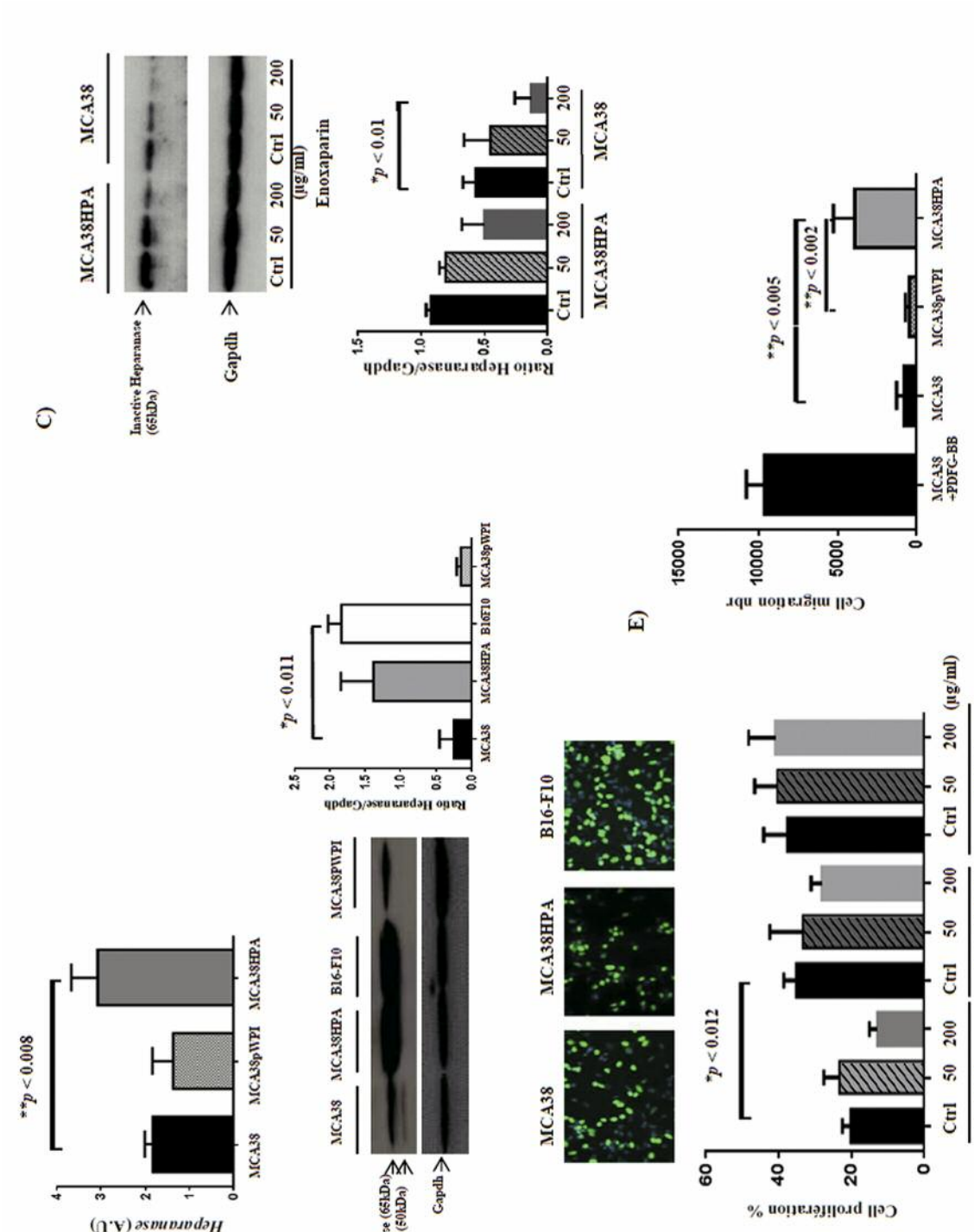

Figure 6. *Overexpression of heparanase promoted tumor growth and migration in vitro. (a) Increased heparanase expression was observed in MCA38HPA compared to MCA38 and B16F10 cell lines (n=4, **p<0.008, MCA38HPA vs. MCA38). (b) An over-production of heparanase in MCA38HPA was assessed compared to negative control cell lines (n=4, *p<0.01, MCA38HP vs. MCA38 and MCA38pWPI). (c) Treatment with enoxaparin attenuated heparanase expression in MCA38HPA (n=3, *p<0.01, Ctrl vs. 200 µg/ml). (d) MCA38HPA cells showed increased levels of cell proliferation compared to MCA38 cells (n=4, *p<0.012, MCA38HPA vs. MCA38). (e) Increased migration of MCA38HPA compared to MCA38 and MCA38pWPI negative controls (n=4, **p<005, MCA38HPA vs. MCA38; and n=4, **p<0.002, MCA38HPA vs. MCA38pWPI). Each analysis was repeated 3 times and the mean±SEM is indicated in the Results section. Groups were compared using the Student's t-test.*

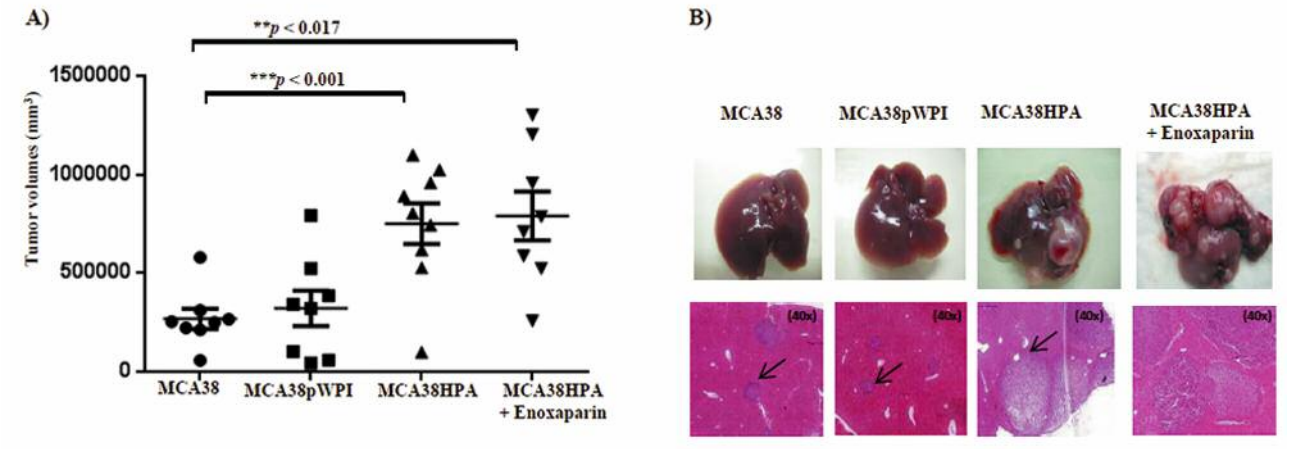

Figure 7. *Study of heparanase-overexpressing MCA38HPA cell line vs. control cell lines. (a) Tumor volumes in MCA38HPA-injected mice were significantly larger than those in control groups (**p<0.001, n=9 (MCA38HPA) vs. n=8 (MCA38); **p<0.007, n=9 (MCA38HPA) vs. n=8 (MCA38pWPI). Mice receiving MCA38HPA and treated with enoxaparin did not show a decrease in tumor volume when compared to non-treated mice (n=8 (MCA38HPA+Enoxaparin) vs. n=8 (MCA38HPA)). (b) Representative photographs (40× magnification) were taken from HE-stained liver sections at day fifteen. Arrows point to the metastatic nodules inside livers. Groups were compared using the Student's t-test.*

important regulator of cell cycle progression. It regulates the progression of G_1 to S phase in many different cell types (43). *Ccnd1* (cyclin D1) is a highly up-regulated gene in many malignancies (44, 45). It has been shown that cyclin D1 overexpression is associated with tumor cell metastasis (46, 47). We demonstrated that cyclin D1 expression is decreased in mouse livers treated daily with enoxaparin and that a high enoxaparin dose (200 μg /ml) decreases its expression *in vitro*, in MCA38 colorectal cells. Since cyclin D1 regulates the G1 to S phase cell cycle transition, the decreased cyclin D1 expression is, probably in part, responsible for the delay and decrease in tumor cell and metastasis development in enoxaparin-treated mice. In addition, we demonstrated a deleterious effect on tumor cells *in vivo* in enoxaparin-treated mice and *in vitro* on MCA38 cell lines. The mechanisms underlying these results are still unclear and require further investigation. In vitro, enoxaparin inhibits heparanase expression at mRNA and protein levels in a dose-dependent manner. Enoxaparin seems to decrease heparanase production in MCA38 cells, in contrast to the B16-F10 cells. Furthermore, LMWH and heparin have been found to have different effects on angiogenesis in previous studies and some of them suppress Vegf production in solid tumors (48, 49, 42). In our *in vivo* experiments, we observed a non-significant decrease of Vegf serum levels *in vivo*. However, we showed *in vitro* that enoxaparin inhibits Vegf production in a dose-dependent manner in MCA38 cell lines. These *in vitro* results corroborate the *in vivo* findings and suggest an anti-angiogenic effect of enoxaparin in our tumor growth model.

In primary and metastatic tumors, monocytes/macrophages represent a major component of infiltrating cells. A number of cytokines and chemokines ensure the recruitment of the infiltrating cells (50-52). Consistent with previous studies, we demonstrated that enoxaparin treatment, in our model, decreases significantly the production and the expression of pro-inflammatory cytokines, such as Ifnγ and Il-12p40. We suggest that the decreased levels of these inflammatory cytokines decrease recruitment of inflammatory cells to tumor tissues but also the inhibition of the recruitment of lymphocytes and macrophages by enoxaparin could cause a decreased production of interferon-γ-. Further studies are needed to explore both possibilities. The interferon-γ-inducible molecules Cxcl9 and Cxcl10 are ligands of the CXCR3 receptor, playing an important role in the recruitment of lymphocytes and macrophages into the tumor tissues (53, 24). In the present study, we observed a significantly decreased level of Cxcl9 and Cxcl10 in serum and in liver mRNA of enoxaparin-treated mice. Interferon-γ stimulates the production of Cxcl10 and Cxcl9, thus causing a continuous recruitment of lymphocytes at the tumor site (54, 53, 24, 55). In our experiment, the decreased level of such chemokines may be explained, in part, by decreased levels of interferon-γ in tumor microenvironment and, on the other hand, are probably due to lack of interferon-γ signaling. To further investigate this hypothesis, we additionally examined the effect of enoxaparin on chemokines' expression by MCA38 cells. Enoxaparin up-regulated the expression and production of Cxcl10 *in vitro*, while it decreased Vegf production. Such decrease can be explained by the significantly elevated levels of Cxcl10 in MCA38 supernatant. Furthermore, Cxcl10 is known as an angiostatic factor capable of inhibiting Vegf production (56, 57). This suggests that LMWHs not only

decrease interferon-γ -inducible chemokines but also increase chemokine expression that attenuates tumor growth through inhibiting vascularization of tumors.

To gain further insight into the specific function of heparanase in colorectal cancer, we developed overexpression approaches to study its effects on biological function(s) in colorectal cell lines. The overexpression of heparanase clearly increases tumor cell proliferation, migration and tumor growth *in vivo*. Our results revealed that the overexpression of heparanase led to notable increase in cell function. This changes observed in colorectal cell lines is consistent with biological functions of heparanase itself.

Once we showed that enoxaparin affects heparanase expression in constitutively- and in up-regulated heparanase-expressing cell lines, we further examined the enoxaparin effect *in vivo*. Interestingly, MCA38HPA cell lines overexpressing heparanase exhibited larger tumor volumes compared to MCA38 cells in our mouse model, demonstrating that heparanase expression is correlated with tumor size. The enoxaparin treatment reduced metastatic growth from MCA38 cells moderately expressing heparanase but failed to inhibit significantly the development of tumor metastatic development when heparanase was up-regulated in MCA38 cells. We evaluated the heparanase expression in our MCAHPA38 cells to be increased by 10-fold. It is, thus, possible that, at such increased levels, enoxaparin is not able to inhibit heparanase activity sufficiently to observe an effect on tumor growth. Further, *in vivo* studies are required to understand why the MCA38HPA cells are resistant to enoxaparin treatment.

In summary, in our model, treatment with enoxaparin significantly attenuated colon carcinoma metastatic tumors in the mouse liver at early stages of development. We suggest that the observed effects are due to inhibition of heparanase. We additionally observed that enoxaparin regulated chemokine expression involved in inflammatory events. Understanding the mechanisms of the anti-metastatic effect of LMWH may allow developing analogs with low antithrombotic action that can be helpful to treat cancer independently of thrombotic prophylaxis.

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