Heparin regulates colon cancer cell growth through p38 mitogen-activated protein kinase signalling

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Abstract

Objectives: Heparin acts as an extracellular stimulus capable of activating major cell signalling pathways. Thus, we examined the putative mechanisms utilized by heparin to stimulate HT29, SW1116 and HCT116 colon cancer cell growth.

Materials and methods: Possible participation of the mitogen-activated protein kinase (MAPK) cascade on heparin-induced HT29, SW1116 and HCT116 colon cancer cell growth was evaluated using specific MAPK cascade inhibitors, Western blot analysis, real-time quantitative PCR and FACS apoptosis analysis.

Results: Treatment with a highly specific p38 kinase inhibitor, SB203580, significantly (50–70%) inhibited heparin-induced colon cancer cell growth, demonstrating that p38 MAPK signalling is involved in their heparin-induced proliferative response. This was shown to be correlated with increased (up to 3-fold) phosphorylation of 181/182 threonine/tyrosine residues on p38 MAP kinase. Furthermore, heparin inhibited cyclin-dependent kinase inhibitor p21^{WAF1/CIP1} and p53 tumour suppressor gene and protein expression up to 2-fold or 1.8-fold, respectively, and stimulated cyclin D1 expression up to 1.8-fold, in these cell lines through a p38-mediated mechanism. On the other hand, treatment with heparin did not appear to affect HT29, SW1116 and HCT116 cell levels of apoptosis.

Conclusions: This study demonstrates that an extracellular glycosaminoglycan, heparin, finely modulates expression of genes crucial to cell cycle regulation through specific activation of p38 MAP kinase to stimulate colon cancer cell growth.

Introduction

Colon cancer is one of the most commonly diagnosed types of cancer among men and women worldwide (1,2). Cancer progression requires a continually evolving network of interactions between neoplastic cells and the surrounding microenvironment (3,4). Different organs have unique extracellular matrix (ECM) properties, which by regulating tumour cell proliferation and migration, can either attenuate or stimulate their ability to metastasize. Liver is the major victim of colon cancer metastasis, and importantly, hepatocyte-derived ECM has been reported to stimulate proliferation of colon cancer cell lines (5). Specifically, the heparan sulphate (HS) component of hepatocyte ECM, structurally similar to highly sulphated heparin (6), has been suggested to enhance colon cancer cell population growth (5). Furthermore, the key role of heparan sulphate-derived metabolites in tumorigenesis of colon neoplasia is supported by reports correlating heparanase expression with colon cancer progression (7–9).

Inhibitory effects of heparin on *in vitro* proliferation of many cell types, including normal osteoblasts and human osteosarcoma cells (10,11), vascular smooth muscle cells (12), normal fibroblasts (13,14) and human melanoma cells (15) have been reported previously. On the other hand, some investigations show that heparin can stimulate cell growth (16–18). In our recent study, heparin was demonstrated to stimulate colon cancer cell proliferation (19), whereas previously liver HS-derived disaccharides have been shown to both inhibit and to enhance colon cancer

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cell proliferation in a sulphation pattern-dependent manner (20).

Numerous mechanisms of heparin action have been postulated, including regulation of growth factors/growth factor receptors (21,22), cytokines, mitogen-activated protein kinase (MAPK) (23) activities, or modulation of cell cycle progression (24). Thus, heparin and structurally related HS act as extracellular stimuli capable of activating key cell signalling pathways and consequently key cell functions (25).

Transduction of signals from the cell surface to the nucleus is mediated by several protein kinases including the family of MAPKs (26–29), serine/threonine kinases with three major classes: extracellular-regulating kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38 MAPKs, that differ in their substrate specificity and responses to extracellular signals. MAPK signalling pathways have been implicated in a wide range of cell functions such as proliferation, apoptosis and migration (30–34). Importantly, the ERK1/2 cascade is frequently dysregulated in cancer and implicated in colon cancer tumorigenesis (35), whereas c-Jun NH2-terminal kinase 1 plays a critical role in colon cancer cell proliferation (36), but although p38 has been proposed primarily as a MAP kinase participating in apoptosis and the cell response to stress, there is evidence that p38 activation is required for colon cancer cell proliferation and survival (37–39).

In this study, we have investigated possible mechanisms that the extracellular glycosaminoglycan, heparin, utilizes to regulate HT29, SW1116 and HCT116 colon cancer cell growth.

Materials and methods

Materials

RPMI-1640 medium, foetal bovine serum (FBS), penicillin, streptomycin and gentamicin were obtained from Gibco-Invitrogen (Paisley, UK). Heparin (H3933), p38 (SB202190) and JNK (SP600125) inhibitors were obtained from Sigma (St Louis, MO, USA). p38 inhibitor SB203580 was purchased from Calbiochem (La Jolla, CA, USA). MEK1/2 (U0126) inhibitor, anti-caspase 3, anti-cleaved caspase 3, as well as polyclonal antibodies specific for p38 and phosphorylated p38 MAPK were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-p-EGFR (pY1068) was obtained from Biosource International (Camarillo, CA, USA). Polyclonal antibody against the protein core of actin, anti-cyclin D1, anti-p53, anti-p21, as well as anti-rabbit, anti-mouse and anti-goat HRP-conjugated secondary antibodies, were purchased from Santa Cruz Biochemicals (Santa Cruz, CA, USA). Fluorometric CyQUANT cell proliferation

Assay Kit was obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Epidermal growth factor (EGF) was purchased from R&D Systems (Minneapolis, MN, USA). MAPK inhibitors were dissolved in dimethyl sulphoxide (DMSO) according to the manufacturer's instructions. All other chemicals used were of the best available grade.

Cell culture

HT29, SW1116 and HCT116 human colon adenocarcinoma cell lines were obtained from ATCC. HT29 and SW1116 cells were grown in a humidified atmosphere at 37 °C, 5% (v/v) CO₂, in RPMI-1640 medium supplemented with 10% FBS and 0.5% gentamicin. HCT116 cell line was grown in RPMI-1640 supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin.

Real-time PCR

Total RNA was isolated using the TRIzol method (Gibco, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Five micrograms of total RNA was used for cDNA synthesis, which was carried out using the DyNAmo cDNA synthesis Kit (Finnzymes, Keilaranta, Finland) according to the manufacturer's instructions. Primers were mRNA specific to avoid misleading results from traces of DNA contamination: p21^{WAF1/CIP1} (F: CTGCCCCAAGCTCTACCTTCC, R: CAGGTCCACATGGTCTTCCT), p53 (F: CGTCTGGGCTTCTTGCATTC, R: AAGGCCTGCCCTGTGCAGC), cyclin D1 (F: GCCCTCGGTGTCCTACTTCAA, R: TCCTCCTCGCACTTCTGTTCC) and GAPDH (F: GGAAGGTGAAGGTCGAGTCA, R: GTCATTGATGGCAACAATATCCAC T). For real-time PCR, the QuantiTech SYBR Green master mix (Qiagen, Valencia, CA, USA) with a total volume of 20 µL was used. Standard curves were run in each optimized assay which produced a linear plot of threshold cycle (C_t) against log (dilution). Real-time PCR was carried out using a Mx300P cycler. Amounts of each target were quantified, based on concentration of the standard curve and were presented as arbitrary units. Quantity of each target was normalized against quantity of GAPDH.

SDS-PAGE and Western blotting

Proliferative cells from non-confluent cultures were harvested and seeded in 24-well culture plates and allowed to rest overnight in RPMI supplemented with 10% FBS. Cells were serum-starved for 24 h and then treated with heparin (50 and 100 µg/mL) or/and specific MAPK inhibitors or gefitinib for 30 min and 1 h respectively. After treatment, cells were lysed using 50 mmol/L of

Tris-HCl, 0.5 mol/L of EDTA, 1% Triton X-100, 0.1% NaCl and protease/phosphatase inhibitors (1 mmol/L phenylmethylsulphonyl fluoride, 5 mmol/L NEM, 5 mmol/L benzamidine, 1% sodium chlorate and 1 mmol/L orthovanadate). Cell extracts were electrophoresed on 8% polyacrylamide Tris/glycine gels and transferred to nitrocellulose membranes. Membranes were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and 5% (w/v) low-fat milk powder, overnight at 4 °C. Incubation with primary antibodies, rabbit anti-p38 (1:1000), rabbit anti-phospho-p38 (1:1000), rabbit anti-p-EGFR (1:1000), rabbit anti-cleaved caspase 3 (1:1000), mouse anti-p53 (1:200), rabbit anti-cyclin D1 (1:200), mouse anti-p21 (1:100) and goat anti-actin (1:200) in PBS-T and 1% (w/v) low-fat milk powder was carried out for 1 h at room temperature. Immune complexes were detected after incubation with peroxidase-conjugated anti-rabbit (1:2000 or 1:4000), anti-mouse (1:2000) or anti-goat (1:4000) antibodies, diluted in PBS-T + 1% low-fat milk, using the Super Signal West Pico Chemiluminescent substrate (Pierce, Holmdel, NJ, USA) according to the manufacturer's instructions.

Cell proliferation assay

Exponentially growing cells from non-confluent cultures were harvested and seeded into a flat-bottom 96-well black plate (Corning, NY, USA) at a density of 3×10^3 cells (HT29 and HCT116) or 7×10^3 cells (SW1116) per well, in the presence of 10% FBS, and were incubated for 24 h. Cells were serum-deprived for 24 h prior to treatment with heparin (100 µg/mL) in serum-free culture medium, this heparin preparation utilized has been finely biochemically characterized previously (10). In experiments using SP600125 (5 µM), U0126 (30 µM), SB202190 (5 µM) and SB203580 (0.4 or 5 µM) inhibitors, cells were pre-incubated with the respective inhibitors 1 h prior to addition of heparin. As the MAPK inhibitors were dissolved in DMSO according to the manufacturers' instructions, equivalent DMSO vehicle controls were used to rule out any potential non-specific DMSO effects. After 48 h of treatment, cells were lysed and their numbers were determined using the fluorometric CyQUANT cell proliferation Assay Kit, which measures DNA content using fluorescent dye binding. A separate standard curve for each cell line was used to convert fluorescence units to cell numbers. All measurements were performed in triplicate.

Assay of cell cycle regulator gene expression

Colon cancer cells (HT29, SW1116 and HCT116) were plated on flat-bottom 24-well plates at a density of 2×10^5 (HT29 and HCT116) or 4×10^5 (SW1116) cells

per well as described earlier. After 0, 48 and 96 h of treatment with heparin (100 µg/mL) and/or SB203580 inhibitor (5 µM), total RNA was isolated by the TRIZOL method (Gibco, Invitrogen, Carlsbad, CA, USA) and p21^{WAF1/CIP1}, p53 and cyclin D1 expression was determined using real-time PCR analysis.

Apoptosis and cell cycle analysis assay

HT29, SW1116 and HCT116 cells were seeded in 12-well plates in the presence of 10% FBS for 24 h. The cells were serum starved for 24 h whereupon the utilized medium was replaced with fresh medium supplemented with heparin at a concentration of 100 µg/mL. In experiments using the SB203580 (5 µM) inhibitor, cells were pre-incubated with the respective inhibitor for 1 h before addition of heparin. Apoptosis was quantified using annexin V-FITC and PI (BD Pharmingen, San Diego, CA, USA, Cat. no. 556547) according to the manufacturer's instructions. Briefly, cells were washed twice with cold PBS and then harvested in binding buffer (BB, provided in the kit) at a concentration of 10^6 cells/mL. Annexin V-FITC (5 µL) and PI (5 µL) were added at 100 µL/ 10^5 cells and incubated for 15 min at room temperature in the dark. After incubation, 400 µL of BB was added and cells were analysed using Beckton-Dickinson FACSArray apparatus (Beckton-Dickinson, Franklin Lakes, NJ, USA); data were analysed using CELLQuest software (Beckton-Dickinson); DNaseI (3 U/mL)-treated cells were used as positive control. Flow cytometry was also used to analyse cell cycle phase distribution. After 48 h treatment, cells were washed and harvested in PBS. After centrifugation (5 min, 160 g), the pellet was resuspended in 1 mL of cold 1:1 mixture of PBS and McIlvaine's buffer (0.2 M Na₂HPO₄, 0.1 M citric acid, pH 7.5) followed with drop-by-drop addition of two volumes of cold ethanol, whereupon they were incubated for 1 h at 4 °C. Cells (10^6 cells/mL) were then centrifuged (10 min, 440 g), washed in distilled water and incubated in staining solution (PBS, 100 µg/mL RNase A, 10 µg/mL propidium iodide for 1 h at 37 °C. DNA content was measured using the Beckton-Dickinson FACSArray apparatus. The sum of all cells obtained during cell cycle analysis was set to 100% and distribution into G0/G1, S and G2/M was determined.

Statistical analysis

Statistical significance was evaluated using *t*-test and one-way completely randomized variance analysis (ANOVA) using Microcal Origin (version 5.0) software. Independence/dependence between characteristics were tested at a significance level of $P < 0.01$.

Results

Effect of specific MAPK inhibitors on heparin-stimulated colon cancer cell proliferation

MAPK intracellular pathways are crucial in regulation of cell proliferation (29,40,41) and are implicated in colon cancer progression (35,37). We have examined possible participation of the MAPK cascade on heparin-induced colon cancer cell population growth using specific MAPK cascade inhibitors. SP600125, a specific inhibitor of JNK and U0126, a specific inhibitor of MEK1/2 (which is an upstream activator of ERK1/2) as well as two independently acting specific inhibitors of p38 (SB203580 and SB202190) were utilized in concentrations that did not affect cells' morphologically as verified by light microscopy (data not shown). Administration of 5 μ M of SP600125 or 30 μ M of U0126 had no effect on proliferation of heparin-induced HT29, SW1116 and HCT116 cells (Fig. 1a–c). On the other hand, treatment with the highly specific p38 kinase inhibitors, SB203580 and SB202190 at concentration of 5 μ M, and SB203580 at low concentration of 0.4 μ M (42), significantly inhibited their heparin-induced division (Fig. 1a–c) but did not have any statistically significant effects on their basal cell proliferation (data not shown). In order to establish the net heparin-induced population growth effect, values of cells in media alone growth rates were subtracted from heparin treated cells; SP600125 (5 μ M) were subtracted from heparin-SP600125 (5 μ M) co-treatment; U0126 (30 μ M) were subtracted from heparin-U0126 (30 μ M) co-treatment; SB202190 (5 μ M) were subtracted from heparin-SB202190 (5 μ M) co-treatment; and SB203580 (0.4 or 5 μ M) were subtracted from heparin-SB203580 (0.4 or 5 μ M) co-treatments; resulting data expressed as percentages of with heparin-induced population growth. DMSO vehicle controls demonstrated that this solvent, under conditions used, did not affect proliferation of HT29, SW1116 and HCT116 cells (Fig. 1a–c). These results indicate that p38 MAPK signalling is involved in the heparin-induced colon cancer cell proliferative response.

Heparin modulates phospho-p38 activation

To elucidate the effect of heparin on p38 signalling in HT29, SW1116 and HCT116 human colon cancer cell lines, antibodies against p38 and phospho-p38 were used, to determine total p38 protein content (anti-p38) and phosphorylation levels (anti-pp38). Anti-pp38 antibody, which specifically detects the phosphorylated serine/tyrosine 181/182 residues, demonstrated that heparin stimulated p38 activation in all three cell types in a dose-dependent manner (Fig. 2a,b). Increase in p38 MAPK

Figure 1. Effect of MAPK inhibitor treatment on heparin-induced colon cancer cell proliferation. HT29 (a), SW1116 (b) and HCT116 (c) human colon cancer cell lines were treated with SP600125 (5 μ M), U0126 (30 μ M), SB202190 (5 μ M) and SB203580 (0.4 or 5 μ M) (JNK, MEK1/2 and p38 MAPK inhibitors, respectively) or MAPK inhibitors in combination with heparin (100 μ g/mL). In order to establish net heparin-induced proliferation effect, values of media growth levels were subtracted from the heparin treated levels, alone; SP600125 (5 μ M) were subtracted from heparin-SP600125 (5 μ M) co-treatment; U0126 (30 μ M) were subtracted from heparin-U0126 (30 μ M) co-treatment; SB202190 (5 μ M) were subtracted from heparin-SB202190 (5 μ M) co-treatment; and SB203580 (0.4 or 5 μ M) were subtracted from heparin-SB203580 (0.4 or 5 μ M) co-treatments and resulting data expressed as percentage of heparin alone induced proliferation. Also presented are DMSO vehicle controls. Results represent average of three separate experiments in triplicate. Mean $\pm$ SEM plotted; statistical significance: *** P < 0.001 compared to control and to DMSO vehicle.

phosphorylation was not related to changes in total p38 protein content as p38 protein expression was not affected (Fig. 2a,b). These data demonstrate that heparin induces activation of p38 in these colon cancer cells. In control experiments, the effect of heparin on p38 phosphorylation was examined in the presence of the highly specific p38 kinase inhibitor, SB203580 (5 μ M) (Fig. 2c,d), which abolished heparin-induced p38 activation.

Heparin regulates colon cancer cell cycle through a p38-dependent mechanism

To characterize molecular mechanisms involving the p38 pathway on heparin-induced colon cancer cell

Figure 2. Effect of heparin on human colon cancer cell p38 activation. (a) Western blot analysis of pp38 activation in HT29, SW1116 and HCT116 cells treated with 50 and 100 µg/mL heparin. (b) Bands, from three separate experiments, were quantified using image analysis software (IMAGEJ 1.4.3.67 Launcher Symmetry Software, Watkinsville, CA, USA) and used to calculate ratio of phosphorylated p38/total p38 (pp38/p38). (c) Activation of pp38 in colon cancer cells treated with heparin (100 µg/mL) in combination with SB203580 (5 µM) and (d) densitometric analysis of the resulting bands. Representative blots are presented. Mean $\pm$ SEM plotted.

proliferation, expression of three cell cycle-related genes was analysed. Initially, HT29, SW1116 and HCT116 cells were grown for 96 h (at which point they reached growth arrest) and p21^{WAF1/CIP1}, p53 and cyclin D1 expression were evaluated at 0, 48 and 96 h points (Fig. 3). Real-time PCR analysis demonstrated that p21^{WAF1/CIP1} and p53 transcript expression was significantly higher at 48 and 96 h time points ($P < 0.001$), whereas levels of cyclin D1 transcripts were significantly lower ($P < 0.001$) compared to those at 0 h time point, in all three cell lines. These results demonstrate that expression of examined cell cycle-related genes correlates well with the growth curves of HT29, SW1116 and HCT116 cells. To investigate possible effects of heparin on p21^{WAF1/CIP1}, p53 and cyclin D1 expression, HT29, SW1116 and HCT116 cells were

Figure 3. Analysis of p21^{WAF1/CIP1}, p53 and cyclin D1 mRNA expression over 96 h. p21^{WAF1/CIP1}, p53 and cyclin D1 mRNA expression in human colon cancer (HT29, SW1116 and HCT116) cells after 0, 48 and 96 h of serum starvation, as determined using real-time PCR. Mean $\pm$ SE plotted; $n = 3$. Statistical significance: ** $P < 0.01$, *** $P < 0.001$ compared to 0 h control.

treated with heparin, SB203580 (5 µM), or combinations for 48 h, and p21^{WAF1/CIP1}, p53 and cyclin D1 mRNA and protein expression were analysed. Heparin modulated p21^{WAF1/CIP1}, p53 and cyclin D1 expression in a p38-dependent manner. Specifically, p21^{WAF1/CIP1}, and p53 transcripts and protein levels were strongly reduced in all the three cell lines ($P < 0.001$), whereas cyclin D1 expression both at mRNA and protein level was significantly higher in the presence of heparin ($P < 0.001$) (Fig. 4a–c). Furthermore, cell cycle distribution of HT29 and HCT116 cells under heparin treatment (100 µg/mL) and/or SB203580 (5 µM) treatment was analysed by flow cytometry. Heparin effects caused a significant rise in percentage of HT29 and HCT116 cells during S phase of the cell cycle ($P < 0.001$), an effect which was inhibited when they were co-treated with the p38 inhibitor SB203580 (Fig. 4d). Cell cycle analysis of SW1116 cells could not be reproducibly determined using FACS analysis due to their tendency to form clusters. Collectively, these results

Figure 4. Effect of heparin on human colon cancer cell cycle. p21^{WAF1/CIP1}, p53 and cyclin D1 mRNA and protein expression in HT29, SW1116 and HCT116 colon cancer cells treated with heparin (H) (100 µg/mL), SB203580 (SB) (5 µM) or their combinations (SB + H) for 48 h, as determined using real-time PCR (a) and Western blot (b) analysis. (c) Bands were densitometrically analysed and ratios of p53, p21 and cyclin D1/actin are presented. (d) Cell cycle distribution of HT29 and HCT116 cells after 48-h treatment with heparin (100 µg/mL) and/or SB203580 (5 µM), as determined by propidium iodide staining and subsequent FACS analysis. Percentage of cells in G0/G1, S and G2/M phases is indicated. Mean ± SE plotted; *n* = 3. Statistical significance: ****P* < 0.001 compared with respective control (c) and SB203580 treatment: ++*P* < 0.01, +++*P* < 0.001 heparin as compared to heparin/SB203580.

suggest that heparin is involved in transcriptional control of cell cycle genes through a p38-dependent mechanism.

Effect of heparin on colon cancer cell apoptosis

p38 MAPK is defined as a key cellular check-point in the cascade leading to colon cancer cell apoptosis (37). A specific apoptosis assay was used to assess levels of apoptosis in our cells treated with a p38 inhibitor (SB203580, 5 µM), heparin (100 µg/mL), or combinations (Fig. 5a). FACS analysis demonstrates that at the 48-h time point

the apoptotic levels of HT29 and HCT116 colon cancer cells were not affected by heparin treatment. As apoptosis of SW1116 cells could not be reproducibly determined using FACS analysis due to their tendency to form clusters when harvested mechanically, Western blot analysis was used to examine cleavage of caspase 3, a key mediator of apoptosis of mammalian cells. As shown in Fig. 5b, SB203580 and heparin/SB203580 treatment resulted in increased cleaved caspase 3 protein expression in SW1116 cells, which correlated well with the FACS analysis results for HT29 and HCT116 cells.

Figure 5. Effect of heparin on colon cancer cell apoptosis. (a) Apoptotic effect of heparin (100 μ g/mL), SB203580 inhibitor (5 μ M) or their combinations on HT29, SW1116 and HCT116 cells after 48 h treatment, as determined by annexin V/propidium iodide staining of apoptotic cells and subsequent FACS analysis. DNaseI (3 U/mL)-treated cells were used as positive control. Levels of apoptosis were calculated and expressed as percentage of cells stained with annexin V. (b) Expression of cleaved caspase 3 protein after 48 h heparin and/or SB203580 treatment in SW1116 cells, as determined using Western blotting, and ratio of cleaved caspase 3/actin measured after digital image analysis. Mean $\pm$ SEM plotted.

Effect of EGFR-mediated signalling on heparin-stimulated colon cancer cell proliferation

Previously, heparan sulphate has been specifically implicated in stimulation of colon cancer cell proliferation via EGFR signalling pathways (20). Thus, gefitinib, a specific inhibitor of PTK activity of EGFR, was used to address the possibility that heparin-stimulated colon cancer cell

division might be dependent on EGFR signalling. Pilot experiments showed that gefitinib treatment dose-dependently inhibited basal EGFR phosphorylation in HT29 and SW1116 cells (Fig. 6a,b). Addition of gefitinib at concentration of 10 μ M, abolished EGF-stimulated HT29 and SW1116 cell proliferation (Fig. 6c), whereas heparin in the presence of gefitinib stimulated proliferation of HT29 and SW1116 cells to the same level as that of heparin alone-treated control (Fig. 6c). As gefitinib had a toxic effect on HCT116 cells, as indicated by their low basal

Figure 6. Effect of EGFR-mediated signalling on heparin-stimulated colon cancer cell proliferation. (a) Serum-starved HT29 and SW1116 cells treated with 0, 0.1, 1 and 10 μ M gefitinib were lysed and equal amounts of protein/lysate were analysed for phosphorylated EGFR using Western blotting and (b) ratio of phosphorylated EGFR/actin measured after digital image analysis. (c) Effects of EGF (50 ng/mL), heparin (100 μ g/mL), gefitinib (10 μ M) treatment or their combinations on proliferation of HT29, SW1116 and HCT116 colon cancer cells. Results represent average of three separate experiments in triplicate. Mean $\pm$ SEM plotted; statistical significance: *** P < 0.001.

proliferation level and the semiquantitative method of trypan blue dye exclusion assay (% cytotoxicity: 45.8% gefitinib 0.1 μM , 58% gefitinib 1 μM , 71.4% gefitinib 10 μM), the role of EGFR signalling on their heparin-induced cell proliferation could not be estimated (Fig. 6c). Basal proliferation levels of HT29 and SW1116 cells were not affected by gefitinib treatment as demonstrated by the cell proliferation assay (Fig. 6c) and verified using the trypan blue dye exclusion assay (data not shown). These data indicate that the heparin-induced effect on human colon cancer cell proliferation is not dependent on the EGFR signalling pathway.

Discussion

Site-specific metastasis is strongly dependent on local environment prevalent in the organ to be colonized by metastatic tumour cells, in particular the ECM of these organs (3,43,44). Organ-specific ECM may have either a stimulatory or inhibitory effect on tumour cell proliferation (5). Proteoglycans/glycosaminoglycans are key ECM molecules, which have been shown to affect survival and proliferation of cancer cells (45,46). Previously, hepatocyte-derived HS has been found to stimulate colon cancer cell proliferation *in vitro* (5). In a recent study, we have shown that heparin dose-dependently stimulates the proliferation of HT29, SW1116 and HCT116 cells (47). Furthermore, in liver-colonizing colon cell lines, HS disaccharides were found to stimulate the division of weakly metastatic cells and to inhibit the proliferation of strongly metastatic colon cancer cell lines, grown on fibronectin substrate, depending on their structure and sulphation patterns (20). These data highlight the importance of HS-dependent pathways on colon cancer cell growth and metastasis.

In view of the fact that involvement of MAPK signalling in colon cancer cell division is well established (35–37,42), three major MAPK pathways, JNK, ERK1/2 and p38, were examined for their participation on the mitogenic effect of heparin. Heparin-stimulated HT29, SW1116 and HCT116 colon cancer cell proliferation was inhibited by p38 inhibition, while it was not modulated by MEK1/2 or JNK inhibition, thus demonstrating that the mitogenic effect of heparin is perpetrated through p38 MAPK signalling. Previously, the p38 MAPK pathway has been established as a central mediator of colorectal cancer cell homeostasis involved in processes such as proliferation, differentiation and cell death (37,39,48,49). Thus, treatment with the SB202190 inhibitor specific for p38 α/β kinases causes cell cycle arrest; autophagy and cell death of colon cancer cells (37). In another study, olive oil polyphenols were found to inhibit p38 phosphorylation initiating anti-proliferative effects in human colon

adenocarcinoma cells (49). In contrast, PEITC induces G1 cell cycle arrest through activation of the p38 pathway, which appears to involve modulation of major colon cancer cell cycle regulator expression (48). These data therefore indicate that the relationship of the p38 MAPK pathway with cell cycle arrest, proliferation and apoptosis is complicated and may be dependent on the exact cellular context, cell type and stimuli.

In the present study, the effect of heparin on cyclin D1, p21^{WAF1/CIP1} and p53 cell cycle-related proteins/genes, important in colon cancer progression was examined. Cyclin D1 is a protein coded for by an oncogene that drives G1/S cell cycle progression in a manner dependent on extracellular stimuli (50). Expression of cyclin D1 has been correlated with colon cancer progenitor cell malignant transformation, and to resistance to chemotherapy (51). Cyclin-dependent kinase inhibitor p21^{WAF1/CIP1} involved in control of G1/S and G2/M transitions, is suggested to have a key role in regulation of colon cancer cell proliferation (52), whereas p53 tumour suppressor protein exerts its population growth inhibitory activity by activating and interacting with diverse signalling pathways; deregulation of its activity supports tumour progression (53). Our results demonstrate that heparin, through a p38-dependent mechanism, modulates mRNA and protein expression of these key cell cycle regulators in a manner supportive of colon cancer cell proliferation. Furthermore, heparin treatment caused a significant increase in percentage of HT29 and HCT116 cells in S phase of the cell cycle, an effect which was inhibited when the cells were co-treated with the p38 inhibitor SB203580. The results indicated that heparin promoted cell cycle progression in cells with increased S-phase entry.

Dependence of colon cancer cell growth and metastasis on the glycosaminoglycan content of the surrounding ECM is well established. Therefore, this study proposes a novel mechanism directly linking extracellular glycosaminoglycan, heparin, with regulation of the colon cancer cell cycle.

Cancer results from an imbalance between cell proliferation, differentiation and apoptosis (54). Previously, p38 MAPK-dependent pathways have been determined to support colon cancer cell proliferation and survival, and to confer these cells with protection against autophagy and apoptosis (37,47). In this study, treatment with heparin did not appear to affect colon cancer cell apoptosis levels. Thus, the observed effect of heparin on colon cancer cell growth is most likely perpetrated through the demonstrated modulation of cell cycle regulator expression.

Previous studies have shown that HS chains secreted by liver cells, as well as exogenous HS disaccharides, stimulated proliferation of colon cancer cells through a

mechanism involving increased expression of tyrosine kinase receptors of the erbB family (20,55,56). The possibility that EGFR signalling affects heparin-induced colon cancer cell proliferation was examined using an EGFR tyrosine kinase inhibitor, gefitinib. Combined treatment of HT29 and SW1116 human colon cancer cell lines with gefitinib and heparin demonstrated that the heparin-induced effect on proliferation of these cells is not dependent on EGFR signalling. On the other hand, gefitinib had a toxic effect on HCT116 cells prohibiting us from evaluation of the role of EGFR signalling on heparin-induced proliferation of these cells. Previously, EGFR activation has been shown to be dependent on HS structure (20). Lack of EGFR involvement in the heparin-induced effect demonstrated in this study could well be correlated with disaccharide sequence and fine sulphation pattern of the utilized heparin preparation, as shown previously in other model systems (46). Furthermore, the authors cannot exclude the possibility that specific organ-derived HS chains and/or those utilized in this study might influence the p38 MAP kinase-dependent pathway to regulate colon cancer cell division.

In conclusion, these novel results demonstrate that an extracellular glycosaminoglycan, heparin, may modulate expression of genes crucial to colon cancer cell cycle regulation, promoting increased S-phase entry through specific activation of the p38 MAP kinase pathway. Furthermore, discovery of the effect of heparin-mediated p38 MAPK pathway on colon cancer cell growth may provide a target for future effective therapy.

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