Heparin Increases Human Gastric Carcinoma Cell Growth

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Abstract. *Background: Heparin has been widely used to prevent cancer-associated thromboembolism in cancer patients. Recent evidence reveals that heparin could modulate cell proliferation in the stomach. The effect of heparin on gastric cancer growth, however, is unknown. The effect of heparin on the proliferation of a human gastric adenocarcinoma cell line, BGC-823, was investigated. Materials and Methods: Cell proliferation was assessed by [^{3}H]-thymidine incorporation. The expressions of several growth-related genes were determined by RT-PCR and Western blot. Results: Heparin significantly increased cell proliferation in BGC-823 cancer cells by 15.5% at the dose of 0.2 μg/ml. Heparin also up-regulated c-Myc protein expression by 14.4%. In contrast, mRNA and protein levels of epidermal growth factor receptor (EGFR) were, respectively, down-regulated by 12.7% and 8.2% with no effect on cyclooxygenase-2 mRNA or protein expression. Conclusion: Our results suggest that heparin can promote the proliferation and up-regulation of c-Myc protein expression in gastric cancer cells.*

Heparin is a heterogeneous group of straight-chain anionic mucopolysaccharides, called glycosaminoglycans, which is normally present in mast cell secretory granules with anticoagulant properties. It is primarily used as an antithrombotic for therapeutic and thromboprophylactic indications, including the management of unstable angina, the treatment of hemodynamic disorders and the surface coating of biomedical devices. Heparin exerts its anticoagulant effect by interacting with antithrombin III and potentiating the inhibition of thrombin and activated factor X (1, 2). In the context of cancer chemotherapy, heparin is used as an adjunct to reduce the risk of cancer-associated thromboembolism. Heparin, in this respect, has been validated in terms of its efficacy and safety in the prevention of venous thromboembolic disease in cancer patients (3).

Independent of its antithrombotic properties, heparin has been reported to play a role in the modulation of cell proliferation (4-7). With regard to tissue repair in the stomach, for instance, it was found that heparin could dose-dependently elicit rat gastric epithelium regeneration, accompanied by increased levels of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and constitutive nitric oxide synthase (cNOS) activity following acute injury (8). Heparin could also increase prostaglandin E_2 (PGE_2) levels and angiogenesis in gastric mucosal cells (9). Transient and self-limiting expression of these growth-related genes is conducive to wound healing, but sustained or uncontrolled expression might drive the transformed cells toward the pathway of carcinogenesis.

In the gastric mucosa, it is known that overexpression of several growth-related genes, in particular, *c-Myc*, EGF receptor (*EGFR*) and cyclooxygenase-2 (*COX-2*), are closely related to tumorigenesis (10-12). *c-Myc* is an example of an immediate early gene related to cell proliferation. Mitogenic stimulation increases the expression of *c-Myc*, which drives the cell cycle from G_0 to G_1. It was discovered that *c-Myc* expression was higher in gastric cancers than in noncancerous tissues. The mechanism by which *c-Myc* regulates cell cycle progression is not fully elucidated, but it has been hypothesized to involve the inhibition of the cyclin-dependent kinase inhibitors p27 and p21 (13). *COX-2* also plays a pivotal role in gastric cancer tumorigenesis. *COX-2*, in this regard, was found to be overexpressed in gastric cancer and its expression correlated well with tumor invasiveness and proliferative activity (14, 15). The use of nonsteroid anti-inflammatory drugs (NSAIDs), which inhibit both *COX-1* and *COX-2*, was associated with a reduced risk of gastric cancer (16). Regarding *EGFR*, previous studies demonstrated that it was overexpressed in 30% of gastric carcinomas (17). EGFR tyrosine kinase inhibitor could also effectively suppress gastric tumor growth and angiogenesis in nude mice model (18), implicating that *EGFR* was involved in the carcinogenesis of gastric cancer.

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Based on the facts that heparin could modulate cell proliferation in gastric glands and that it is widely used as an adjuvant therapeutic in cancer patients, it is likely that heparin might modulate cancer growth in gastric cancer patients. The aim of the present study, was to investigate the effect of heparin on the growth of the gastric carcinoma cell line BGC-823. The expressions of the above-mentioned tumor-related genes, namely, *c-Myc, COX-2* and *EGFR*, were analyzed in relation to cell proliferation and heparin treatment.

Materials and Methods

All the chemicals and reagents used were purchased from Sigma (Sigma Chemical Company, St. Louis, USA) unless otherwise specified.

Cell culture. The Human gastric cancer cell line BGC-823 was obtained from the Institute of Cell Biology (Shanghai, China). The cells were maintained in 100-mm culture dishes in RPMI 1640 (GibcoBRL, Grand Island, USA) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) (GibcoBRL) in an incubator at 37˚C, with 95% humidity and 5% carbon dioxide. When confluent, the cells were washed twice with Hank's balanced salt solution (GibcoBRL) and trypsinized with 2 ml of 0.25% trypsin/EDTA (GibcoBRL). The cells were then centrifuged and resuspended in RPMI 1640 medium with 10% FBS for culture. Cell viability was assayed using the 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method, as described previously (19).

Cell proliferation assay. Cell proliferation was assessed as DNA synthesis. To evaluate DNA synthesis in cells, the incorporation of [^{3}H]-thymidine (Amersham, USA) into DNA was determined. Briefly, cells ($5x10^4$/well) were seeded into a 24-well plate and cultured for 24 h for attachment. They were then washed twice with 0.01M phosphate-buffered saline, followed by incubation with 1 ml/well of the medium containing various concentrations of unfractionated heparin for 5 h. In the next step, 0.5 μCi of [^{3}H]-thymidine was added to each well, and the cells were further incubated for 5 h. The incorporation of [^{3}H]-thymidine into the cells was measured with a liquid scintillation counter (LS-6500; Beckman Instruments, Inc.)

RT-PCR analysis of COX-2 and EGFR mRNA levels. Total RNA was isolated from BGC-823 gastric cancer cells using TRIZOL Reagent (Gibco BRL, Gaithersburg, MD, USA) and 5 μg of the total RNA was used to generate the first-strand cDNA by reverse transcription (Gibco BRL), in accordance with the manufacturer's instructions. PCR for EGFR, COX-2 and GAPDH was performed using the THERMOSCRIPT™ RT-PCR system (GibcoBRL). The amplification was initiated by 2 min at 94˚C for denaturing followed by 30 cycles at 94˚C for 45 sec, 55˚C for 45 sec, and 74˚C for 45 sec. After the last cycle of amplification, the samples were incubated at 74˚C for 5 min for extension. The specific primer sets used for human EGFR were: 5'-CGCTGCTGGCTGCGCTCTG-3' (forward) and 5'-AGCCACCTCCTGGATGGTC-3' (reverse); for human COX-2: 5'-ACT GCGCCTTTTCAAGGATG-3' (forward) and 5'-CACCCCATTCAGGATGC-3' (reverse); for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-ACGGAT TTGGTCGTATTGGG-3' (forward) and 5'- TGATTTGGAGGGA TCTCGC-3' (reverse). The PCR products were electrophoresed on 1.5% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide. Gel photographs were then analyzed semi-quantitatively in a multianalyzer (Bio-Rad, Hercules, CA, USA).

Western blot analysis: cellular levels of c-Myc, COX-2 and EGFR. After incubating the BGC-823 cells with or without unfractionated heparin for 5 h, the cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.5% α-cholate acid, 0.1% SDS, 2 mM EDTA, 1% Triton X-100 and 10% glycerol), containing 1.0 mM phenylmethylsulfonyl fluoride and 1 mg/ml aprotinin, and the mixture was subjected to 30 sec-sonication on ice and centrifuged at 12,000 xg for 20 min at 4˚C. The protein concentration in the supernatant was determined by the protein assay kit (Bio-Rad) using bovine serum albumin as the standard. The same amount of protein (50 μg/each lane) was separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to Hybond C nitrocellulose membranes (Amersham Corporation, Arlington Heights, IL, USA). The membrane was probed with c-Myc, COX-2 or EGFR antibodies (Santa Cruz, USA) overnight at 4˚C and incubated for 1 h with secondary antibodies conjugated with peroxidase. The membranes were developed by enhanced chemiluminescence (Amersham Corporation) and exposed on X-ray film (FUJI Photo Film Co., Ltd., Tokyo, Japan). The quantification of the protein bands was carried out by video densitometry (Scan Marker III, Microtek, USA).

Statistical analysis. The results are expressed as the mean±SE of at least triplicate determinations, and statistical comparisons are based on Student's *t*-test or ANOVA. $P<0.05$ was considered to be significant.

Results

Enhancement of BGC-823 cell proliferation by heparin treatment. The BGC-823 cells were treated without or with 0.02-0.5 μg/ml unfractionated heparin for 5 h, and the [^{3}H]-thymidine incorporated into the cells was counted (Figure 1). Heparin, at all concentrations examined, increased [^{3}H]-thymidine incorporation, with the maximum 15.5% increase occurring at 0.2 μg/ml, compared with the non-treated cells. Hereafter, we chose 0.2 μg/ml unfractionated heparin as the working concentration for the subsequent gene expression analysis in this study. The MTT assay also showed that unfractionated heparin, only at the dose of 50 μg/ml, compromised BGC-823 cell viability by 10%, whereas unfractionated heparin at 0.02, 0.05, 0.2, or 0.5 μg/ml had no effect.

Increase in c-Myc protein level by heparin. Since c-Myc is a transcription factor important in modulating cell cycle progression (10), we examined whether heparin treatment could increase the cellular level of c-Myc protein (Figure 2). The 5h-treatment of BGC-823 cells with 0.2 μg/ml unfractionated heparin significantly raised the level of c-Myc protein by 14.4%, compared with nontreated cells.

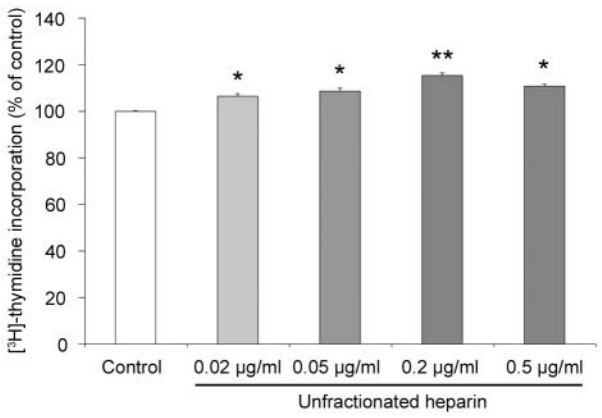

Figure 1. *Increased proliferation of BGC-823 cells by heparin, as measured by [^{3}H]-thymidine. Cells were treated without or with the indicated concentrations of unfractionated heparin for 5 h. *p<0.05; **p<0.005 vs. control group.*

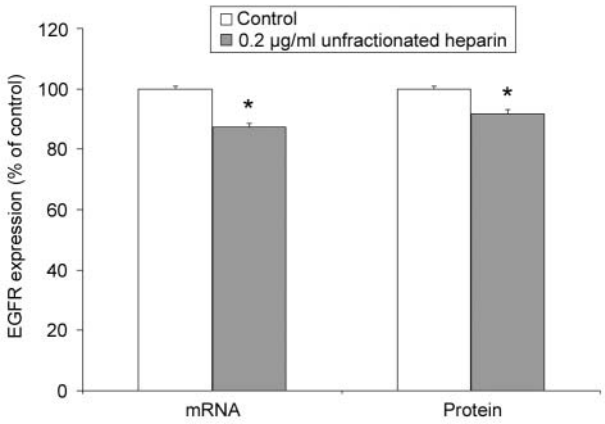

Figure 3. *Down-regulation of EGFR mRNA and protein expression in heparin-treated BGC-823 cells, as determined by RT-OCR and Western blot, respectively. Five-h-treatment of BGC-823 cells with 0.2 μg/ml unfractionated heparin significantly reduced EGFR expression. *p<0.05 vs. control group.*

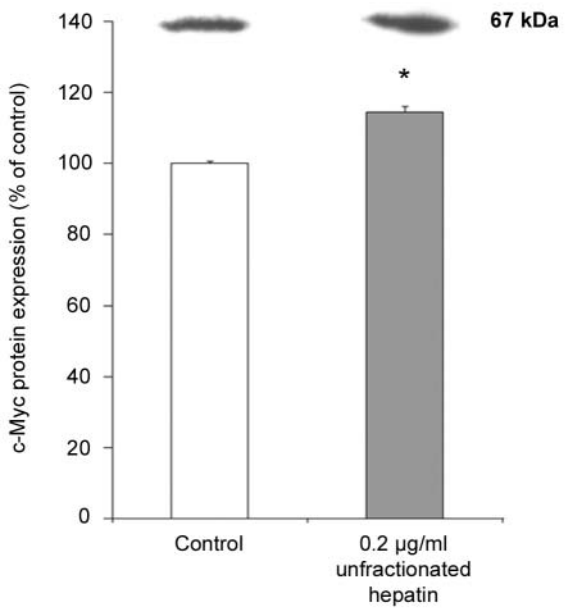

Figure 2. *Elevated protein expression of c-Myc in BGC-823 cells by heparin, as determined by Western blot. Cells were treated without or with 0.2 μg/ml unfractionated heparin for 5 h. *p<0.05 vs. control group.*

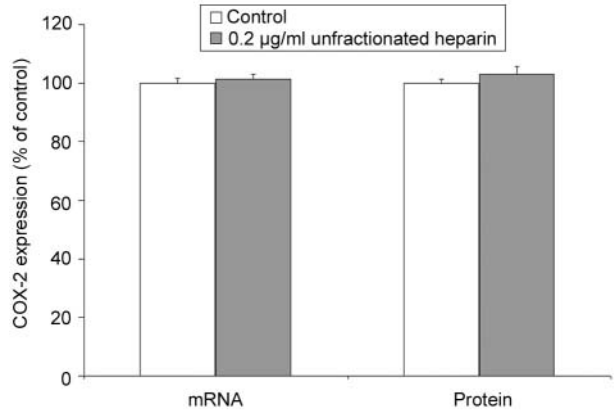

Figure 4. *No effect of heparin treatment on COX-2 mRNA and protein expression in BGC-823 cells, as determined by RT-PCR and Western blot, respectively. Five-h-treatment of BGC-823 cells with 0.2 μg/ml unfractionated heparin failed to alter EGFR expression.*

Decrease in EGFR mRNA and protein levels by heparin treatment. As shown in Figure 3, the 5 h-treatment of BGC-823 cells with unfractionated heparin decreased the EGFR mRNA level by 12.7% and the EGFR protein level by 8.2% at 0.2 μg/ml, a concentration at which heparin elevated the [^{3}H]-thymidine incorporation (Figure 1) and the c-Myc protein level (Figure 2) in BGC-823 cells.

No effect of heparin treatment on COX-2 mRNA and protein levels. Since COX-2 protein levels have been shown to correlate with proliferative activity of human gastric cancer cells (15), and since heparin stimulated BGC-823 cell proliferation (Figure 1), we investigated whether heparin could raise COX-2 mRNA and protein levels in BGC-823 cells (Figure 4). The 5-h-treatment with 0.2 μg/ml unfractionated heparin failed to alter COX-2 mRNA and protein levels, in contrast to the modulatory effects of heparin on [^{3}H]-thymidine incorporation, c-Myc protein level, and EGFR mRNA and protein levels.

Discussion

Heparin is widely used in the prophylaxis of thromboembolism in cancer patients, but its direct effect on cancer growth is poorly understood. In the present study, we demonstrated that heparin could significantly increase BGC-823 gastric carcinoma cell proliferation, accompanied with an elevation of c-Myc protein level, suggesting that the use of heparin might pose a risk of increased tumor growth in gastric cancer patients. Pathogenically, the accumulation of mast cells around gastric tumors was known to increase vascularization, tumor growth and metastasis and was associated with poor prognosis (20, 21). Our results, to this end, suggest that one of the mast cell secretory products, heparin, might contribute directly to the proliferation of gastric cancer cells.

The mechanism by which heparin promotes cell proliferation was unknown, but might possibly involve the stabilization or enhanced release of growth factors. For instance, it has been reported that heparin could modify the interaction between the extracellular matrix and growth factors (*e.g.* bFGF and HGF) and thereby exert its mitogenic action (22, 23). Moreover, heparin has been shown to increase the levels of EGF in the gastric mucosa (8). It is therefore speculated that the mitogenic action of heparin on BGC-823 gastric cancer cells might be mediated by these growth factors. The current study also reveals that heparin treatment elevates c-Myc expression in gastric cancer cells. It has been reported that the transcription factor nuclear factor-κb (NF-κB) is involved in the regulation of c-Myc expression (24-26). Heparin, in this regard, was known to enhance the stability of bFGF, that was shown to activate NF-κB in breast cancer cells and in healing soft tissue (20, 27, 28). It is, therefore, possible that heparin up-regulates c-Myc expression through a bFGF-dependent NF-κB-mediated pathway.

Our results indicate that heparin could down-regulate EGFR expression, but has no effect on COX-2 expression in gastric cancer cells, thus implying that the mitogenic action of heparin is independent of COX-2. Regarding EGFR down-regulation, the transcription factor activator protein-1 (AP-1) was known to increase the gene transcription of EGFR (29). It has been reported that heparin or heparan sulfate proteoglycan could suppress AP-1 activation (30). In addition, the low molecular weight fraction of heparin could also prevent the enhanced binding of nuclear proteins to the regulatory AP-1 site (31). It is therefore likely that the down-regulation of EGFR by heparin may be mediated through the suppression of AP-1 activation. Our finding is also consistent with the *in vivo* observation that EGFR expression is down-regulated in chemically-induced squamous cell carcinoma of the stomach, in which abundant mast cell infiltration was observed (32).

In conclusion, our results suggest that heparin can promote proliferation and elevate c-Myc expression in gastric cancer cells. Although there is no epidemiological evidence to date to support the notion that the use of heparin would aggravate gastric cancer, the present findings however, may pose a safety concern to current heparinized gastric cancer patients. We, therefore, suggest that an appropriate surveillance on this subset of patients be instituted to monitor the effect of heparin on the progression of gastric cancer or that an alternative antithrombotic agent be used in these patients.

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