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Effect of heparin on proliferation and signalling in BC3H-1 muscle cells

Evidence for specific binding sites

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We studied binding and growth inhibitory properties of different glycosaminoglycans in growing and differentiated BC3H-1 muscle cells. Heparin (10 μg/ml) and heparan sulfate (10 μg/ml) significantly inhibited DNA synthesis in growing and differentiated cells, as monitored by [^{3}H]thymidine incorporation. Binding of heparin to BC3H-1 cells was specific and time-dependent. Heparan sulfate was the only glycosaminoglycan able to displace [^{3}H]heparin (IC_{50}, 3.2×10^{-7} M), although it was 10-fold less effective than heparin itself (IC_{50}, 3.6×10^{-8} M). Scatchard analysis revealed the existence of high-affinity heparin binding sites (K_d, 5×10^{-8} M). Furthermore, heparin inhibited serum-induced stimulation of inositol lipid turnover. Taken together, these results indicate that heparin inhibits BC3H-1 cell growth by interacting with the cell surface, possibly disrupting the flow of growth factor-related mitogenic signalling.

Heparin; Cell growth; Inositol lipid; Cell signalling

1. INTRODUCTION

Several studies in vivo and in vitro showed that heparin inhibits proliferation of vascular smooth muscle cells [1–3]. The molecular mechanism(s) involved in this effect are not known as yet; some studies do not support the possibility of direct interaction between heparin and growth factors [1]. Recent evidence, on the other hand, suggests that the effects of heparin on vascular smooth muscle cells are receptor-mediated [4]. A heparin-binding protein of 78 K_d from bovine uteri, thought to be involved in the inhibition of vascular smooth muscle cell proliferation, has been described [5]. Interestingly, the antiproliferative effect of heparin on vascular smooth muscle cells is reversed by EGF, but not by PDGF, IGF-1 or thrombin [6]. In this case, heparin, acting intracellularly, seems to cause a decrease of EGF receptors [6]. In BC3H-1 cells EGF, acting in synergism with FGF, is reported to induce cell growth [7].

The effect of heparin on cell growth differs greatly in varying experimental conditions. Endothelial cells provide a good example of this; heparin stimulates endothelial cell proliferation by interacting with, and potentiating the effect of ECGF and a-FGF [8,9]. In human umbilical vein endothelial (HUVE) cells, heparin fails to stimulate cell growth in the absence of growth factors [10]. In human omental microvascular endothelial (HOME) cells, heparin inhibits the proliferation of exponentially growing cells, but not that of serum-deprived cultures [11].

Given such a complexity, we decided to study the effects of heparin in BC3H-1 cells, i.e. a cell line that exhibits different traits according to the stage of differentiation [12]. The clonal cell line BC3H-1 was obtained by Shubert et al. from a nitrosoethylurea-induced tumor in the C3H mouse [13]. These cells grow logarithmically in culture and are able to differentiate toward a more mature muscle phenotype after being serum-starved and growth-arrested. The process of differentiation does not involve cell fusion. Ultrastructural features typical of smooth muscle cells have been observed by electron microscopy. Among other traits indicative of smooth muscle origin are expression of the muscle isoform of creatine phosphokinase, myokinase, and smooth muscle α-actin [13–16]. A recent study, however, points out that differentiated BC3H-1 cells express sarcomeric muscle-specific contractile protein genes, thus resembling skeletal myoblasts that are defective for commitment to terminal differentiation [12].

Therefore, we decided to characterize the response of this clonal cell line to heparin, and related GAGs, taking into consideration growing and differentiated cells. Here we demonstrate that heparin and heparan sulfate,

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Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor-1; ECGF, endothelial cell growth factor; a-FGF, acidic fibroblast growth factor; GAGs, glycosaminoglycans; VLMW-heparin, very low molecular weight heparin; BSA, bovine serum albumin; PBS, phosphate-buffered saline

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but not other GAGs, bind to, and inhibit the growth of BC3H-1 cells in culture, independently of the stage of differentiation. Furthermore, we describe the inhibitory effect of heparin on serum-induced inositol lipid turnover, thus suggesting that heparin might disrupt the normal flow of mitogenic signalling at the cell surface.

2. MATERIALS AND METHODS

[^{3}H]Heparin (400 μCi/mg, average molecular mass 15 kDa), and [^{3}H]thymidine (82.2 Ci/mmol) were purchased from New England Nuclear. Heparin EP 756 from bovine intestinal mucosa (average molecular mass 12.9 kDa; 150 U/mg), very low molecular weight heparin obtained from peroxidative cleavage of bovine heparin (VLMW-heparin 1027/45 OP; average molecular mass 2.1 kDa), and dermatan sulfate OP 435, were kindly provided by Opocrin Research Laboratories, Modena, Italy. Heparan sulfate (from bovine lung) was donated by Professor Cifonelli (Department of Pediatrics, University of Chicago, USA). Hyaluronic acid (from human umbilical cord), chondroitin sulfate C (from shark cartilage), and chondroitin sulfate A were from Sigma Chemical. Sephacryl S-200 was from Pharmacia.

The BC3H-1 cell line was obtained from American Type Cell Cultures (Rockville, MD, USA). The cells for each experiment were plated at an initial cell density of 4000–5000 cells/cm^2, and cultured at 37°C in DMEM containing 10% fetal calf serum for the length of time indicated in each experiment. The medium was not changed during the course of the experiment. Binding experiments were performed on fully differentiated cells, i.e. 5 days after confluence.

[^{3}H]Thymidine incorporation was studied in cells two days before confluence, at confluence, and for the next 6 days. Briefly, [^{3}H]thymidine (4 μCi/ml) was added to the cultures for 1 h. At the end of the incubation period, the medium was removed and the radioactivity incorporated into TCA-precipitable material was counted. The antiproliferative effect of differential GAGs on growing BC3H-1 cells was determined by adding 10 μg/ml of each compound 24 h before pulse labelling. Each experiment was performed in quadruplicate. The protein concentration was determined by the Bradford method, using BSA as standard [17].

Binding experiments were performed on BC3H-1 cells cultured in 17-mm plates. Five days after confluence, cultures were washed with cold PBS and precooled at 4°C for 30 min. Binding experiments were initiated by adding PBS (1 ml) containing 0.1% human serum albumin, increasing concentrations of [^{3}H]heparin (7.5–30 nM) without unlabelled heparin, and [^{3}H]heparin (30 nM) with increasing concentrations of unlabelled ligand (10–100 nM). Non-specific binding was determined in the presence of 100-fold excess of unlabelled ligand. Cells were incubated at 4°C for 2 h. The reaction was stopped by removing the incubation medium; cells were collected from the dishes onto GF/C filters (Whatman) and washed 5 times with cold PBS (5 ml). Filters were dried and counted for ^{3}H-radioactivity in a liquid scintillation counter. The unspecific binding of [^{3}H]heparin to filters was negligible.

The effect of heparin on serum-induced inositol lipid turnover was monitored as accumulation of [^{3}H]inositol phosphates in cells prelabelled with [^{3}H]*myo*-inositol (10 μCi/ml) for 48 h. Heparin was added either during labelling (24 or 1 h before the experiment), or concomitantly with serum. At the end of incubation, cultures were washed extensively to remove unincorporated [^{3}H]*myo*-inositol and, when present, heparin added 24 or 1 h earlier. Cultures were stimulated in the presence of lithium (10 mM) in order to favor the accumulation of water-soluble inositol phosphates. The reaction was terminated by adding ice-cold methanol, and inositol phosphates were extracted and separated as described [18]. Total inositol phosphates were eluted from Dowex AG 1 × 8 columns (Bio-Rad) with 1.2 M ammonium formate/0.1 M formic acid.

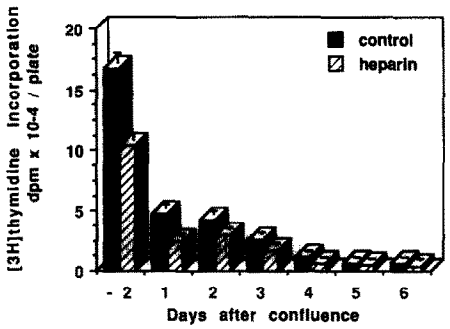

Fig. 1. Inhibitory effect of heparin on [^{3}H]thymidine incorporation in BC3H-1 cells. BC3H-1 cells were grown in DMEM supplemented with 10% fetal calf serum. 24 h before the pulse (1 h) labelling with [^{3}H]thymidine, cells were incubated with regular DMEM (100 μl; solid bars), or with heparin-containing DMEM (10 μg/ml per plate; hatched bars). Measurements were performed on cell cultures 2 days before confluence (−2), and at the indicated days after confluence. Results are expressed as [^{3}H]thymidine incorporation and are the means ± SE of 4 determinations for each point.

3. RESULTS

The effect of heparin on BC3H-1 cell growth at different stages of differentiation is shown in Fig. 1. Incorporation of [^{3}H]thymidine was drastically reduced when cells reached confluence. Complete inhibition of thymidine incorporation, however, occurred 4 days after confluence. Heparin (10 μg/ml) caused 30–50% inhibition of [^{3}H]thymidine incorporation (Fig. 1). The antiproliferative effect was independent of the stage of differentiation, since it was evident in growing as well as in differentiating cells. Since BC3H-1 cells exhibit smooth or skeletal muscle cell characteristics according to the stage of differentiation [12], it can be concluded that the inhibitory effect of heparin is unaffected by the process of differentiation, or by the phenotypic traits of the cells.

In order to ascertain whether the effect of heparin was specific, we compared the effect of heparin to that of other GAGs on BC3H-1 cell growth (Table I). Heparin and heparan sulfate were the only GAGs able

Table I

Effect of different glycosaminoglycans on [^{3}H]thymidine incorporation in BC3H-1 cells

Addition	% inhibition
None	0
Heparin EP 756	30 −50
Heparan sulfate	25 −40
Hyaluronic acid	0 − 1
Chondroitin sulfate C	−1.1− 0
Dermatan sulfate OP 435	1 − 2.5
VLMW-heparin 1027/45	0 − 2

% inhibition was calculated according to this formula: (1 − dpm of [^{3}H]thymidine in treated cells/dpm of [^{3}H]thymidine in control cells) × 100. Values represent the range of inhibition observed in 3 experiments, each performed in quadruplicate

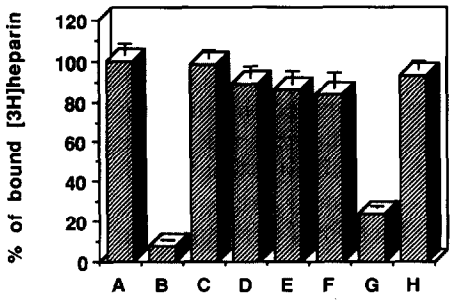

Fig. 2. Specificity of [^{3}H]heparin binding to BC3H-1 cells. Fully differentiated BC3H-1 cells were incubated for 2 h at 4°C with [^{3}H]heparin (4.0×10^5 dpm/ml per plate) in the absence (A), or in the presence of: 50 μg/ml per plate of heparin EP 756 (B); chondroitin sulfate A (C); hyaluronic acid (D); chondroitin sulfate C (E); dermatan sulfate OP 435 (F); heparan sulfate (G); very low molecular weight heparin OP 1027/45 (H). Results are expressed as % of [^{3}H]heparin bound to cells, and are means ± SE of triplicate samples from an experiment representative of 3 others which gave identical qualitative results.

to inhibit cell proliferation. Identical concentrations of VLMW-heparin (1027/45 OP) and heparin-unrelated GAGs were completely ineffective.

The time course of [^{3}H]heparin binding to BC3H-1 cells at 4°C was essentially the same in growing, confluent, and postconfluent cultures (not shown). Specificity of [^{3}H]heparin (30 nM) binding to BC3H-1 cells was determined by incubating the cultures with 100-fold excess of the following unlabelled compounds: heparin EP 756; VLMW-heparin 1027/45 OP; hyaluronic acid; heparan sulfate; dermatan sulfate OP 435; chondroitin sulfate A and C (Fig. 2). Heparin binding was inhibited by 100-fold excess of unlabelled heparin EP 756, but not by 100-fold excess of heparin-unrelated GAGs. Heparan sulfate was the only GAG able to compete for the major portion of specific [^{3}H]heparin binding. Fig. 3A shows that the calculated IC_{50} for heparin was 3.6×10^{-8} M, whereas the IC_{50} for heparan sulfate was 3.2×10^{-7} M. Scatchard analysis of data (Fig. 3B) indicated a single class of high-affinity binding sites with a K_d value of 5.0×10^{-8} M.

A major question about the antiproliferative effect of heparin refers to whether heparin acts extra- or intracellularly. In order to help clarify this point, we studied the effect of heparin on serum-induced inositol lipid metabolism. Serum-starved (4 days after confluence) BC3H-1 cells were prelabelled with [^{3}H]*myo*-inositol and stimulated with serum. Phosphoinositide metabolism was monitored as the accumulation of inositol phosphates. In order to eliminate errors due to variability in cell number or labelling efficiency as differentiation proceeded, we took the ratio of counts in inositol phosphates to the total counts in all labelled phospholipids. Previous studies demonstrated that this procedure of normalization is accurate and reliable in a variety of experimental conditions [19–21]. We found that heparin did not affect the basal rate of inositol lipid turnover, either when administered during the incubation period with [^{3}H]*myo*-inositol, or just before the assay (not shown). It seems therefore that heparin does not interact per se with the inositol lipid signalling machinery. Heparin, however, significantly inhibited serum-induced inositol phosphate formation when administered concomitantly with serum (Fig. 4). This effect was strictly related to the binding properties of heparin, since VLMW-heparin and chondroitin sulfate (that did not displace heparin from the cell surface, see Fig. 2) did not affect serum-stimulated inositol lipid metabolism (not shown).

4. DISCUSSION

The results presented in this paper indicate that heparin and heparin-like GAGs specifically bind to BC3H-1 cells and inhibit proliferation independently of the stage of cell differentiation. VLMW-heparin and other GAGs unrelated to heparin failed to show these effects. These results suggest that the GAG molecular weight and sulfatation degree are important in deter-

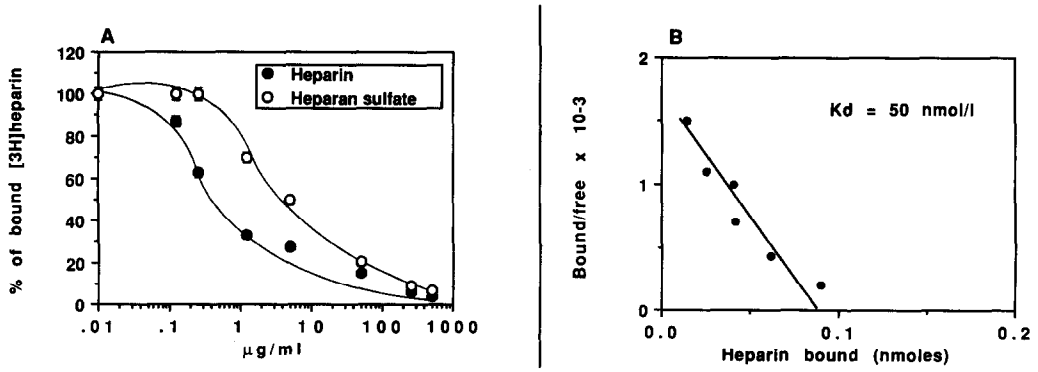

Fig. 3. Displacement of [^{3}H]heparin and Scatchard analysis of [^{3}H]heparin bound to BC3H-1 cells. (A) BC3H-1 cells were incubated for 2 h at 4°C with [^{3}H]heparin alone, or in the presence of increasing concentrations of unlabelled compounds. Results are expressed as % of bound [^{3}H]heparin and are means of triplicate samples from an experiment representative of 3 others which gave identical qualitative results. (B) Scatchard analysis. Each point is the mean of quadruplicate samples.

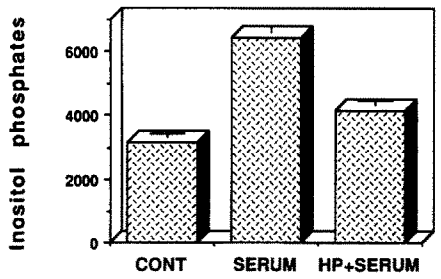

Fig. 4. Effect of heparin on serum-induced inositol lipid metabolism. Serum-starved differentiating BC3H-1 cells (4 days after confluence at the time of the experiment), were preincubated for 48 h with [^{3}H]*myo*-inositol (10 μCi/ml). Cells were then stimulated with serum (10%), in the presence or in the absence of heparin (10 μg/ml per plate; HP). [^{3}H]Inositol phosphates were separated by anion exchange chromatography. Results, expressed as the radioactivity recovered in the total inositol phosphate fraction (dpm/10^5 dpm in total inositol phospholipids) are means ± SE of 3 experiments.

mining the ability to bind to the cell surface. Consistently, heparan sulfate, although active, was less efficient than heparin, whereas VLMW-heparin was unable to compete for heparin binding.

In order to assess the mechanism(s) underlying the antiproliferative effect of heparin, we studied the turnover of inositol lipids, a major mitogenic signalling pathway [20,21]. Heparin, administered either during labelling or just before the assay, did not affect the basal rate of inositol lipid metabolism, thus suggesting that it does not interfere with intracellular GTP-binding protein(s) and/or enzymes involved in phosphoinositide synthesis and degradation. Heparin, however, significantly inhibited serum-induced inositol phosphate formation. This effect, just as the inhibition of growth, was strictly related to heparin binding to the cell surface; consistently, VLMW-heparin and chondroitin sulfate failed to show any effect on inositol lipid turnover and cell proliferation. From the data discussed here, it is proposable that heparin, by selectively binding in the proximity of certain areas of the cell surface, might interfere with growth factor/receptor coupling, thus disrupting the flow of mitogenic signalling from the surface to the nucleus. An interaction between heparin and growth factors has been proposed to explain some effects of heparin on endothelial cell growth [22,23]. The matter, however, is far from being clear, since heparin enhances the effect of ECGF and a-FGF [9], but inhibits proliferation of exponentially growing HOME cells [11].

Additionally, heparin might act intracellularly after being taken up by vascular smooth muscle cells [24,25]. A putative target of the intracellular action of heparin is the phosphoinositide-related calcium signalling. In vascular smooth muscle cells, heparin inhibits inositol trisphosphate-dependent, but not guanine nucleotide-dependent intracellular calcium mobilization [26]. These findings were confirmed by a recent report demonstrating that heparin inhibits inositol trisphosphate-dependent calcium release in permeabilized smooth muscle cells [27]. The extra- and intracellular action of heparin might be interconnected, as it has been proposed that heparin-induced decrease of EGF receptor number is a consequence of heparin acting inside the cell [6]. Most likely, binding to the cell surface is a necessary prerequisite for heparin internalization. Therefore, heparin might exert a dual action: at the cell surface, interacting with growth factors and/or their receptors; inside the cell, affecting mitogenic signalling pathways. Another mechanism might be related to the heparin effect on synthesis and processing of extracellular matrix proteins secreted by vascular smooth muscle cells [28–30]. Further studies are in progress to characterize in detail the molecular mechanism(s) underlying the antiproliferative effect of heparin.

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