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INHIBITION OF BC3H-1 CELL GROWTH BY HEPARIN IS RELATED TO DECREASED MITOGENIC SIGNALLING

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SUMMARY. We examined the effect of heparin and heparin fragments on BC3H-1 muscle cell proliferation. Heparin significantly inhibited BC3H-1 cell growth and this inhibitory effect was related to the ability of heparin to bind to cell surface; low molecular weight heparins were poorly efficient in binding and inhibiting proliferation. Analysis by gel filtration of heparin bound to cell surface showed selective binding of the high molecular weight fraction. Heparin inhibited serum-stimulated incorporation of [^{3}H]thymidine; this effect, however, was only evident when heparin was administered concomitantly with serum. Similarly, heparin inhibited serum-induced inositol lipid turnover only when present with serum. Heparin fragments unable to inhibit cell growth did not affect the metabolism of inositol lipids. Taken together these data suggest that heparin inhibits cell growth by interfering with growth factor-mediated mitogenic signalling.

INTRODUCTION. Heparin, although can be considered neither a growth factor nor a growth inhibitor, is known to affect the proliferation of several cell lines. Its effect, however, varies depending on cell lineage (1,2), growth factor requirement (3-5), and stage of cell differentiation (6); heparin has been reported either to stimulate or inhibit cell proliferation (1-6). Interaction of heparin with growth factors (7-8), exposure of growth factor receptors (9), inhibition of intracellular signalling (10,11), and effects on extracellular matrix protein synthesis (12-14) have been reported.

Heparin is widely distributed among tissues, both as endogenous heparin (15) and following therapeutic administration for antithrombotic purposes. Work from our laboratory showed that endogenous heparin is released from human platelets (16), and that is present in human plasma

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assembled with phosphatidylcholine in a supramolecular complex (17). We also described the metabolism of endogenous heparin in endothelial cells in culture (18). In our effort to clarify physiological roles of heparin, we decided to study the interference of heparin with the growth of BC3H-1 cells, a clonal muscle cell line able to differentiate toward a more mature muscle phenotype upon serum starvation (19).

EXPERIMENTAL. [^{3}H]heparin, [^{3}H]thymidine and [^{3}H]*myo*-inositol were purchased from New England Nuclear. Different heparins and heparin fragments obtained from peroxidative cleavage were provided by Opocrin Research Laboratories (Modena, Italy) (20). BC3H-1 cell line was obtained from American Type Cell Cultures (Rockville, MD, USA). Cells for each experiment were plated at initial cell density of 4000-5000 cell/cm^2, and cultured at 37 ^{o}C in DMEM containing 10% fetal calf serum. Experiments were performed on fully differentiated cells, *i.e.* five days after confluence.

Studies on thymidine incorporation were performed by adding [^{3}H]thymidine (0.5 µCi/ml) to the cultures for 1 h. At the end of the incubation period, medium was removed and radioactivity incorporated into TCA-precipitable material was counted. The antiproliferative effect of different heparins on BC3H-1 cells was determined by adding each compound 24 h before pulse labelling.

For binding experiments, cultures were washed with cold PBS and pre-cooled at 4 ^{o}C for 30 m. [^{3}H]heparin (27 pmol/plate) was added and cells were incubated at 4 ^{o}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. Unspecific binding was negligible. Binding to BC3H-1 cells was determined by displacement experiments (20) in the presence or in the absence of unlabelled compounds (50 µg/ml).

For the analysis of [^{3}H]heparin bound to BC3H-1 cells, cultures were incubated at 4 ^{o}C for 2 h with unfractionated [^{3}H]heparin. Incubation medium was then removed, cells were washed 4 times with ice-cold PBS, and bound [^{3}H]heparin was displaced by adding 100 fold excess of unlabelled heparin for 2 h at 4 ^{o}C. Labelled heparin removed from cell surface was analyzed on Sephacryl S-200 column (1x90 cm), eluted with 4M guanidinium hydrochloride (4 ml/h). 2 ml fractions were collected and counted. Unfractionated [^{3}H]heparin mixed with 2 mg of unlabelled heparin was also analyzed on Sephacryl S-200.

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, heparins added 24 or 1 h earlier. Inositol phosphates were extracted and separated as described (21).

RESULTS and DISCUSSION. Fig. 1 shows that heparin significantly inhibited [^{3}H]thymidine incorporation; low molecular weight heparin was only partially effective whereas very low molecular weight heparin was ineffective (A). Thus, heparin molecular weight and sulfatation degree seemed important in determining growth inhibitory effects. Next we measured the binding of heparin and its fragments to BC3H-1 cell surface, and we compared capability to bind to growth inhibitory property. Fig. 1 shows that binding capability and DNA synthesis inhibition were closely related; heparins HP 756 and sm 1026 (*i.e.* two high molecular weight heparins, see 20), efficiently displaced bound [^{3}H]heparin (B) and significantly inhibited [^{3}H]thymidine incorporation (A). Low molecular weight heparin had intermediate efficacy in displacing bound labelled heparin and inhibiting cell growth. Very low molecular weight heparin had no binding capability and did

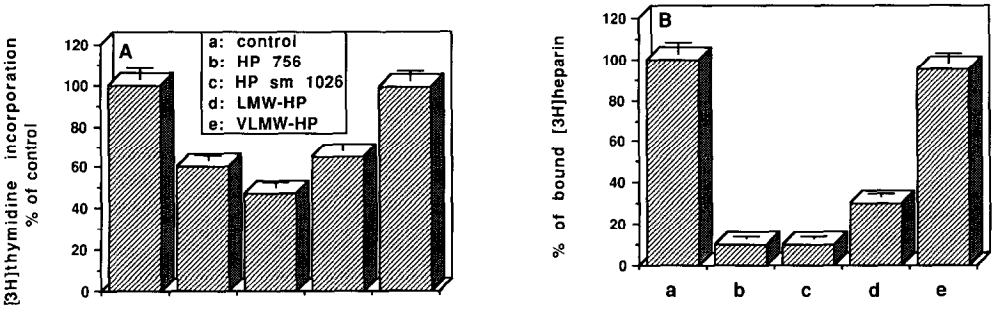

Figure 1. Effect of heparins on BC3H-1 cell growth: relationship with their ability to bind to the cell surface. Cell growth was assessed as [^{3}H]thymidine incorporation, in the presence of indicated compounds (10 µg/ml), in post-confluent BC3H-1 cells (A). Results are expressed as % of control (untreated cells), and are means±S.E. of three determinations for each point. Heparin binding (B) was measured in cells incubated for 2 h at 4 ^{o}C with [^{3}H]heparin ($4x10^5$ dpm/ml/plate) in the absence (a) or in the presence of 50 µg/ml/plate of: heparin HP 756 (b); heparin sm 1026 (c); low molecular weight heparin LMW-HP 2123/850 (d); very low molecular weight heparin VLMW-HP 1027/45 (e) (20). Results are expressed as % of [^{3}H]heparin bound to cells and are means±S.E. of triplicate samples from an experiment representative of three others which gave identical qualitative results.

not show any effect on cell proliferation. Similar results were obtained by measuring the effect of heparin on cell number (not shown). These data indicate that binding of heparin to cell surface was necessary prerequisite for growth inhibitory effect. Binding capability seemed to be influenced by heparin molecular weight. Sephacryl S-200 gel chromatography confirmed that the high molecular weight heparin fraction was the one selectively bound (Fig. 2).

The next step was measuring the effect of heparin on serum-stimulated cell growth. Serum (2% FCS) stimulated by approximately two fold [^{3}H]thymidine incorporation (control, 9875±312; FCS, 22176±489, dpm±S.E.). Heparin (HP 756, 1 µg/ml) significantly inhibited serum-stimulated [^{3}H]thymidine incorporation (10556±541, dpm±S.E.). This inhibitory effect was observed only when heparin was administered concomitantly with serum. Pretreatment of cells with saturing amount of heparin for 24 or 1 h, followed by extensive washing, did not modify responsiveness to serum (not shown). From these results it seems that heparin had to be present during serum stimulation in order to inhibit mitogenic signalling.

The turnover of inositol lipids is considered a major signalling pathway involved in growth factor-stimulated cell proliferation (22). We measured the effect of heparin on basal and serum-stimulated inositol lipid metabolism. Fig. 3

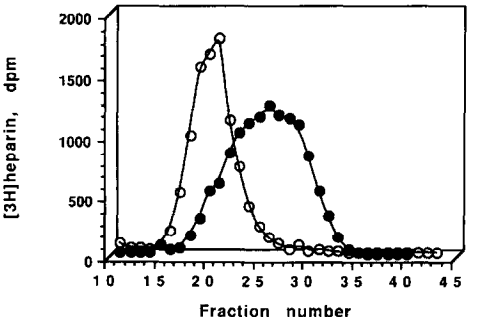

Figure 2. Analysis on Sephacryl S-200 of [^{3}H]heparin bound to BC3H-1 cells. (-●-): [^{3}H]heparin used in the experiment; (-o-): [^{3}H]heparin bound to cell surface. [^{3}H]heparin bound to cell surface was displaced by addition of 100 fold excess of unlabelled heparin HP 756 for 2 h at 4 °C. Results are expressed as ^{3}H-radioactivity associated with each fraction. This figure reports data from an experiment representative of three others which gave identical qualitative results.

shows that pretreatment of cells with heparin for 24 or 1 h (hatched bars), followed by extensive washing, did not alter the pattern of inositol lipid metabolism neither in resting nor in serum-stimulated cells. This finding indicates that heparin did not interact *per se* with either degradative or synthetic pathways within the phosphoinositide cycle. Since all the enzymes (and G-proteins) involved in inositol lipid degradation and synthesis are located intracellularly, it appears that heparin did not interfere with phosphoinositide signalling from inside the cell. Heparin, however, administered concomitantly with serum, significantly inhibited serum-induced inositol phosphate accumulation. Very low molecular weight heparin was ineffective, thus paralleling the results observed with [^{3}H]thymidine incorporation (Fig. 1). These data indicate that heparin inhibited serum-induced phosphoinositide turnover by acting from outside the cell, probably interfering with growth factor/receptor coupling.

Although we demonstrate here that heparin exerted its inhibitory effect mainly from outside the cell, this observation does not rule out the possibility that heparin, once uptaken, could affect intracellular mitogenic pathways. In particular, heparin has been proposed to interfere with

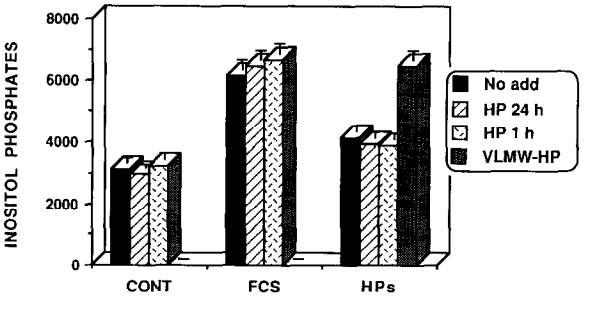

Figure 3. Inhibition by heparin of serum-induced inositol lipid turnover. Serum-starved, post-confluent BC3H-1 cells were preincubated for 48 h with [^{3}H]*myo*inositol (10 μCi/ml). As indicated, cells were preincubated with heparin for 24 or 1 h, extensively washed, and then stimulated with 10% serum in the presence or in the absence of heparin (10 μg/ml). Stimulation with serum was performed in the presence of 10 mM lithium. [^{3}H]inositol phosphates were extracted and 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±S.E of three experiments.

inositol polyphosphate-regulated calcium mobilization (10,11). Therefore, although most of heparin bound was of the high molecular weight, a less abundant, perhaps lower molecular weight intracellular component generated by metabolism of high molecular weight heparin (18,20), could be active at a "high affinity" site. Elucidation of the mechanism by which heparin inhibits the growth of cultured cells will prove useful in understanding other roles of heparin beside its effect on blood coagulation.

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