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Heparin inhibits A431 cell growth independently of serum and EGF mitogenic signalling

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In several cell types heparin exerts an antiproliferative action; here we report that heparin inhibited the growth of human epidermoid carcinoma A431 cells. Heparin binding to the cell surface was necessary for growth inhibition; binding was influenced by the molecular weight of heparin. Inhibition of A431 cell proliferation was evident in the presence and in the absence of serum, thus indicating that heparin did not act by binding and 'subtracting' nutrients or other serum factors. In confluent A431 cells, EGF induced DNA synthesis, but heparin did not inhibit this effect; consistently, it did not affect inositol lipid turnover triggered by EGF or bradykinin.

Heparin; Epidermal growth factor; Inositol lipid; A431 carcinoma cells; Cell growth

1. INTRODUCTION

Proliferation of cultured cells is controlled by proteins able to stimulate or inhibit cell growth. A number of growth factors and growth inhibitors has been described, and several mitogenic signalling pathways have been elucidated [1]. Heparin is known to interfere with the control of cell proliferation [2–4]; its effects, however, range from stimulation to inhibition, depending on cell lineage, growth factor requirement and experimental conditions [5–7]. It has been proposed that stimulation of endothelial cell growth by heparin is due to interaction with specific growth factors, thus stabilizing their binding to receptors [8]. Inhibition of smooth muscle cell proliferation has been ascribed to interaction with growth factors, receptors, or intracellular signalling [9–12]. We showed that inhibition of serum-induced DNA synthesis in BC3H-1 cells was accompanied by inhibition of stimulated inositol lipid turnover [13]; this observation indicated that heparin could interfere with the mitogenic signalling cascade activated by growth factors.

However, the effect of heparin on cell proliferation has been mainly studied in normal cells which were dependent on exogenous growth factors for proliferation and survival. Searching for a mechanism of action that could elucidate the role of heparin as growth inhibitor, we decided to study its effect on A431 human epidermoid carcinoma cells which grow in the absence of serum and overexpress the EGF receptor [14]. Another rationale for choosing A431 cells lay in the observation that heparin does not bind EGF or its receptor [9], thus ruling out the possibility that heparin might inhibit cell growth simply by 'subtracting' growth factors from the medium.

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Abbreviations: EGF, Epidermal growth factor; APTT, activated partial thromboplastin time; AXa, anti-activated coagulation Factor X activity; TCA, trichloroacetic acid; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]heparin, [^{3}H]thymidine and [^{3}H]*myo*-inositol were purchased from New England Nuclear. Different heparins, heparin fragments, and dermatan sulfate OP 435 were provided by Opocrin Research Laboratories (Modena, Italy). Heparins HP 756 and sm 1026 were extracted from bovine intestinal mucosa. Low molecular weight heparin (LMW) (2123/850) was obtained by peroxidative cleavage of bovine heparin. Very low molecular weight heparin (VLMW) (1027/45) was obtained by fractionation on Sephacryl S-200 of a low molecular weight heparin (LMW 886-87/12 OP - av. M.W. 5.0 kDa). Molecular weights (kDa) were determined by polyacrylamide gel electrophoresis [15]. The degree of sulfatation was evaluated by potentiometric analysis, and expressed as molar ratio of sulfate (SO_3^-) and carboxyl (COO^-) groups determined for each compound [16]. Activated partial thromboplastin time (APTT) was measured according to Basu et al. and expressed as U/mg [17]. Anti-activated coagulation Factor X (AXa) activity was tested in a chromogenic assay as described by Teien et al., and expressed as U/mg [18]. A summary of the properties of these compounds is given in Table I. Hyaluronic acid (from human umbilical cord), chondroitin sulfate B, and C (from shark cartilage) were from Sigma. Tissue culture media were from Gibco. Bradykinin was from Calbiochem. EGF (purified, receptor grade) was from Collaborative Res. A431 human epidermoid carcinoma cells were kindly donated by Dr Bruni, Istituto di Chimica Biologica, Florence, Italy.

Table I

Properties of opocrin compounds used in the experiments

	M_w	SO_3^-/COO^-	APTT	AXa
HP 756	12.9	2.15	164	114
sm 1026	36.9	"	295	119
LMW 2123/850	4.5	2.19	37	86
VLMW 1027/45	2.1	2.27	2	38
DS OP435	30.0	1.11	1	0

Glycosaminoglycan molecular weight (M_w) is expressed in kDa Degree of sulfatation (SO_3^-/COO^-) is expressed as molar ratio of sulfate and carboxyl groups determined for each compound [16]. Activated partial thromboplastin time (APTT) is expressed as U/mg [17]. Anti-activated coagulation Factor X (AXa) activity was tested in a chromogenic assay and is expressed as U/mg [18]. Abbreviations used: HP, heparin; sm, slow moving; LMW, low molecular weight; VLMW, very low molecular weight; DS, dermatan sulfate.

Determination of cell growth

Cell growth was assessed as [^{3}H]thymidine incorporation. 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, the medium was removed and radioactivity incorporated into TCA-precipitable material was counted. The anti-proliferative effect of different glycosaminoglycans was determined by adding each compound 24 h before pulse labelling. The effect of EGF and heparin on DNA synthesis cells was studied in confluent and semi-confluent cultures, routinely grown in 17-mm dishes with 10% FCS. After 24 h of serum starvation, the cultures were repeatedly washed with fresh medium (DMEM) and incubated for 24 h with EGF and/or heparin as indicated. At the end of incubation, [^{3}H]thymidine (0.5 μCi/ml) was added for 1 h, and incorporation was assessed.

Measurement of heparin binding

Binding experiments were performed essentially as previously described [2]. Briefly, to evaluate the K_d, confluent cultures were washed with cold PBS and precooled at 4°C for 30 min. Increasing concentrations of [^{3}H]heparin (7.5–30 nM) without unlabelled heparin, and [^{3}H]heparin (30 nM) with increasing concentrations of unlabelled heparin (HP 756, 10–100 nM), was added and 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. Unspecific binding was negligible. Binding specificity of [^{3}H]heparin was studied incubating confluent cultures with [^{3}H]heparin for 2 h at 4°C, with or without adding 100-fold excess of unlabelled glycosaminoglycans.

Analysis of inositol lipid turnover

The effect of heparin on basal and stimulated 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 stimuli (EGF or bradykinin). 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 with chloroform/methanol and separated by ion-exchange chromatography [2].

3. RESULTS AND DISCUSSION

The effect of heparin and other glycosaminoglycans on cell proliferation was assessed by measuring [^{3}H]thymidine incorporation in cells cultured for 24 h in the presence of these compounds. Fig. 1 shows that A431 cell growth was dramatically inhibited by high molecular weight heparins (HP 756, sm 1026); low molecular weight heparin (LMW 2123/850) had intermediate efficacy, and very low molecular weight heparin (VLMW 1027/45) was ineffective. Qualitatively identical results were obtained by assessing growth inhibition as the decrease of cell numbers. These results indicate that the molecular weight of heparin was directly related to growth inhibition. Since the molecular weight of heparin is known to influence its binding to the cell surface [2], we measured binding as monitored by displacement of [^{3}H]heparin (Fig. 1). The calculated K_d value for high molecular weight heparin (HP 756) was 1×10^{-7} mol/l. Low molecular weight heparin bound less efficiently, and very low molecular weight heparin did not bind to the cell surface. In order to determine specificity of binding, we measured [^{3}H]heparin displacement by other glycosaminoglycans which did not inhibit cell growth (i.e. chondroitin sulfate B and C, hyaluronic acid, and dermatan sulfate OP 435); none of these compounds was able to displace bound [^{3}H]heparin, thus demonstrating that heparin binding to the cell surface was specific for growth inhibitory heparins.

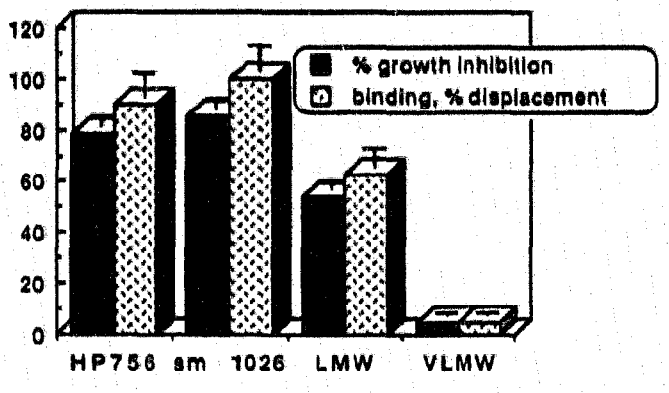

Fig. 1. Binding and antiproliferative effect of heparins. Cell growth was assessed as thymidine incorporation in the presence of 10 μg/ml of indicated compounds which were added for 24 h before pulse labelling (1 h) with [^{3}H]thymidine. Data are expressed as % inhibition of [^{3}H]thymidine incorporation compared with controls (no addition, i.e. no inhibition = 0%; full inhibition = 100%). Results are means of at least 3 experiments, each performed in triplicate, ± SE. Heparin binding was measured in cells incubated for 2 h at 4°C with [^{3}H]heparin (4×10^5 dpm/ml/plate) in the absence or in the presence of 50 μg/ml/plate of indicated compounds [2]. Results are expressed as % of [^{3}H]heparin displacement, and are means of triplicate samples ± SE.

Experiments reported in Fig. 1 were performed in cells grown in the presence of serum. However, since transformed cells are able to survive in the absence of serum, we decided to study the effect of heparin in serum-starved A431 cells in order to determine whether growth inhibition was due to the interaction of heparin with nutrients or other factors present in serum. Fig. 2 shows that heparin significantly inhibited [^{3}H]thymidine incorporation in serum-starved A431 cells for

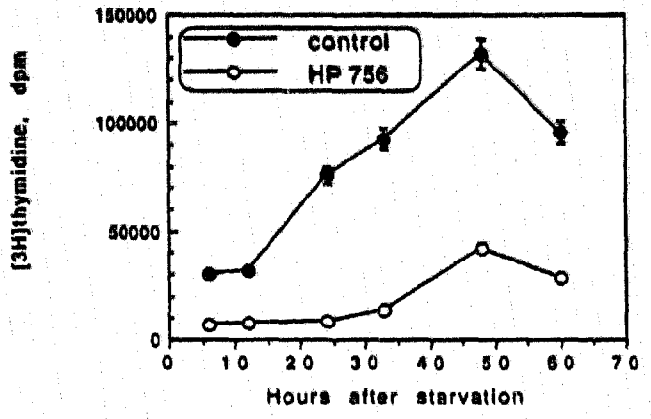

Fig. 2. Antiproliferative effect of heparin on serum-starved A431 cells. Confluent A431 cells were serum-starved for the indicated length of time. High molecular weight heparin HP 756 (10 μg/ml) was added 24 h before pulse (1 h) labelling. Medium was not changed for the length of the experiment and cell proliferation was assessed as [^{3}H]thymidine incorporation. Results are expressed as dpm of incorporated [^{3}H]thymidine, and are means ± SE ($n=4$) of an experiment representative of 3 others which gave qualitatively identical results.

up to 60 h after starvation. Similar results were obtained by determining growth inhibition as the decrease of cell numbers (not shown). These data indicate that growth inhibition could not be ascribed to 'removal' of essential factors present in the serum.

Although the role of EGF as a 'bona fide' mitogen in A431 cells is rather questionable [14], evidence has accumulated that EGF stimulates DNA synthesis in confluent cultures after 24 h of incubation (14,19). Fig. 3A shows that EGF induced significant DNA synthesis after 24 h of incubation in confluent cultures, while inhibiting [^{3}H]thymidine incorporation in semi-confluent dishes. Consistent with the results shown above, heparin inhibited basal (unstimulated) A431 cell proliferation both in confluent and semi-confluent cultures. EGF-induced DNA synthesis in confluent cultures was unaffected by heparin: % increase of EGF-induced DNA synthesis over control (no EGF addition) was about the same in untreated and heparin-treated cells as if the glycosaminoglycan inhibited components distinct from the EGF mitogenic pathway (Fig. 3B).

To substantiate this observation, we decided to study the effect of heparin on basal and stimulated inositol lipid turnover. A431 cells over-express the EGF receptor, and addition of EGF results in the hydrolysis of phosphoinositides [20]. Heparin did not affect basal inositol lipid turnover (Table II), thus indicating that it did not interfere per se with components of the inositide pathway. EGF and bradykinin stimulated inositol lipid turnover in A431 cells with the formation of water-soluble inositol phosphates (Table II). Heparin (100 μg/ml) did not cause any change in EGF- (50 ng/ml), or bradykinin- (1 μM) induced inositol phosphate formation in A431 cells, whereas it inhibited serum-stimulated phosphoinositide hydrolysis in BC3H-1 cells [13]. Pretreatment of cells with heparin for 24 or 1 h, followed by extensive washing, did not modify the pattern of EGF-, or bradykinin-induced inositol lipid turnover. Please note that, in order to detect any possible effect of heparin on agonist-stimulated inositol lipid metabolism, we used a concentration of heparin 10-fold higher than that used to inhibit cell growth (Fig. 1), or EGF-induced thymidine incorporation (Fig. 3). Inhibition of serum-induced inositol phosphate formation, however, was observed with 10 μg/ml of heparin [2].

Heparin is known to have other effects beside inhibition of blood coagulation. Interference with cell growth has been described in several systems, but the overall picture is confused as heparin has been reported to stimulate and/or inhibit proliferation in different cell types and under different experimental conditions [5–7]. Also it is not clear which component(s) of the mitogenic signalling cascade are affected by heparin; in-

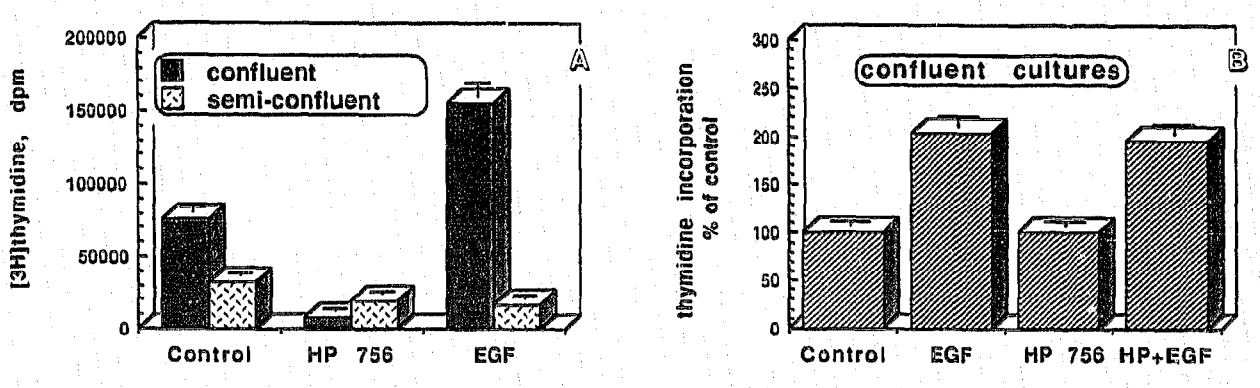

Fig. 3. Effect of EGF and heparin on [^{3}H]thymidine incorporation in A431 cells. Serum-starved confluent and semi-confluent A431 cells were stimulated with EGF (50 ng/ml), with or without heparin (HP 756, 10 μg/ml), for 24 h. [^{3}H]thymidine was then added for 1 h. A. Results are expressed as dpm of incorporated [^{3}H]thymidine, and are means ± SE ($n=4$) of an experiment representative of three others which gave qualitatively identical results. B. Results are expressed as % increase of EGF-induced [^{3}H]thymidine incorporation in confluent A431 cells. Basal values (i.e. no EGF addition; indicated in the Figure as 100%) of [^{3}H]thymidine incorporation were: control, 75560 ± 3245; HP 756, 9154 ± 423, dpm, means ± SE ($n=4$).

Table II

Effect of heparin on inositol lipid metabolism

Treatment	Inositol phosphates
None	3385 ± 121
HP 756	3021 ± 89
EGF	8876 ± 239
HP + EGF	8659 ± 342
Bradykinin	7863 ± 199
HP + Bradykinin	7984 ± 233

Cell monolayers in 35-mm wells were labelled with [^{3}H]inositol for 48 h as described [2]. Stimulation with different inositol lipid turnover agonists was carried out in the presence of 10 mM lithium. Heparin (HP 756, 100 μg/ml), when present, was added 5 min before the agonists. Inositol phosphates and inositol phospholipids were extracted and separated as described [2–24]. Results, expressed as the radioactivity recovered in the total inositol phosphate fraction (dpm/10^5 dpm in total inositol phospholipids) [2], are means ± SE of 3 experiments.

teraction with growth factors, receptors, intracellular signalling mechanisms, and extracellular protein synthesis has been described [9–12, 21–23]. Our results demonstrate that heparin binding to the A431 cell surface did not interfere with mitogenic signalling elicited either by EGF or the nonapeptide bradykinin (Table II, Fig. 3); therefore, the antiproliferative effect of heparin can be ascribed neither to 'removal' of growth factors, nor to the formation of a 'coat' on the cell surface.

Given these data,it is tempting to speculate on the opposite effects of heparin in different cell lines postulating that heparin might act either on the external cellular surface or inside the cell. An external action could be envisaged in BC3H-1 muscle cells, where heparin inhibits serum-stimulated proliferation and inositol lipid turnover [13]; in this case it could either bind and subtract serum factors or prevent their interaction with receptors. A similar inhibitory mechanism, however, cannot be postulated for A431 cells, since heparin does not bind EGF, and consistently does not inhibit its mitogenic signalling pathway. Heparin might, however, bind and 'neutralize' autocrine growth factors, other than EGF, secreted by A431 cells. Although our experiments do not rule out this possibility, the observation that heparin does not alter basal inositol lipid signalling suggests that these putative growth factors would act through pathways distinct from that of the activated EGF receptor. Alternatively, heparin could first be bound and internalized, and then interfere with mitogenic passages located downstream of the activated receptor and its immediate signalling machinery. By whatever mechanism heparin perturbs cell proliferation, our studies provide evidence linking its antiproliferative effect to multiple targets within the mitogenic signalling cascade.

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REFERENCES

[1] Heldin, C.H., Betsholtz, C., Claesson-Welsh, L. and Westermark, B. (1987) Biochim. Biophys. Acta 707, 219–244.
[2] Vannucchi, S., Pasquali, F., Fiorelli, G., Bianchini, P. and Ruggiero, M. (1990) FEBS Lett. 263, 137–141.
[3] Wright, T.C., Pukac, L.A., Castellot, J.J., Karnovsky, M.J., Levine, R.A., Kim-Park, H.J. and Campisi, J. (1989) Proc. Natl. Acad. Sci. USA 86, 3199–3203.
[4] Zaragoza, R., Battle-Tracy, K.M. and Owen, N.E. (1990) Am. J. Physiol. 258, C46–C53.
[5] Hoover, R.L., Rosenberg, R., Haering, W. and Karnovsky, M.J. (1980) Circ. Res. 47, 578–583.
[6] Tiozzo, R., Cingi, M.R., Pietrangelo, A., Albertazzi, L., Calandra, S. and Milani, M.R. (1989) Arzneim.-Forsch./Drug Res. 39, 15–20.
[7] Bikfalvi, A., Dupuy, E., Ruan, C., Tobelem, G., Leseche, G. and Caen, J. (1988) Cell. Biol. Int. Rep. 12, 931–942.
[8] Schreiber, A.B., Kenney, J., Kowalski, W.J., Friesel, R., Mehlman, J. and Maciag, T. (1985) Proc. Natl. Acad. Sci. USA 82, 6138–6145.
[9] Reilly, C.F., Fritze, L.M.S. and Rosenberg, R.D. (1987) 131, 149–157.
[10] Kobayashi, S., Somlyo, A.V. and Somlyo, A.P. (1988) Biochem. Biophys. Res. Commun. 153, 625–631.
[11] Chopra, L.C., Twort, C.H.C., Ward, J.P.T. and Cameron, I.R. (1989) Biochem. Biophys. Res. Commun. 163, 262–268.
[12] Ghosh, T.K., Eis, O.S., Mullaney, J.M., Elbert, C.L. and Gill, D.L. (1988) J. Biol. Chem. 263, 11075–11079.
[13] Vannucchi, S., Pasquali, F., Chiarugi, V.P. and Ruggiero, M. (1990) Biochem. Biophys. Res. Commun. 170, 89–95.
[14] Gill, G.N. and Lazar, C.S. (1981) Nature 293, 305–307.
[15] Hilborn, J.C. and Anastassiadis, P.A. (1971) Anal. Biochem. 38, 88–92.
[16] Vannucchi, S., Pasquali, F., Porciatti, F., Chiarugi, V., Magnelli, L. and Bianchini, P. (1988) Thromb. Res. 49, 373–383.
[17] Basu, D., Gallus, A., Hirsh, J. and Cade, J. (1972) N. Engl. J. Med. 287, 324–327.
[18] Teien, A.M., Lie, M., Abilgaard, U. (1976) Thromb. Res. 8, 413–416.
[19] Polet, H. (1990) Exp. Cell Res. 186, 390–393.
[20] Hepler, J.R., Nakahata, N., Lovenberg, T.W., DiGuiseppi, J., Herman, B., Earp, H.S. and Harden, T.K. (1987) J. Biol. Chem. 262, 2951–2955.
[21] Cochran, D.L., Castellot, J.J., Robinson, J.M. and Karnovsky, M.J. (1988) Biochim. Biophys. Acta 967, 289–295.
[22] Majack, R. and Bornstein, P.J. (1985) Cell Biol. 100, 613–619.
[23] Majack, R., Cook, S. and Bornstein, P.J. (1985) Cell Biol. 101, 1059–1070.
[24] Lapetina, E.G., Silio, J. and Ruggiero, M. (1985) J. Biol. Chem. 260, 7078–7083.