

Short Communications

Heparin inhibits the growth of astrocytes in vitro

Patricia L. Robertson and Gary W. Goldstein

Department of Pediatrics and Neurology, University of Michigan, Ann Arbor, MI 48109 (U.S.A.)

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Heparin at a concentration of 100 $\mu\text{g/ml}$, inhibits neonatal rat astrocyte proliferation by 45%, and [^3H]thymidine incorporation into DNA by 55% when they are stimulated with 2% fetal bovine serum (FBS) in culture. Higher serum concentrations up to 10% FBS decreased this inhibition to 20% and 29% respectively. Inhibition of [^3H]thymidine incorporation by heparin is dose-dependent with maximal inhibition at 100 $\mu\text{g/ml}$, but 39% inhibition is still seen at 1 $\mu\text{g/ml}$ on stimulation by 5% FBS. Heparin or heparin-like molecules in the extracellular matrix of brain capillary walls may be important in the regulation of astrocyte growth in vivo.

Heparin and heparin-like glycosaminoglycans alter cellular behavior in many systems including blood vessel walls. Heparin stimulates the migration of endothelial cells in vitro, can induce neovascularization in some experimental settings, and is known to augment the in vitro biologic activity of some endothelial cell growth factors^{1,25,26}. In contrast, heparin inhibits the proliferation of blood vessel smooth muscle cells in vitro, and after arterial injury in vivo^{7,10}. A role for cell-extracellular matrix (ECM) interactions in the control of cell growth and differentiation is well recognized, and heparin and heparin-related glycosaminoglycans are components of the ECM in many cell types including those of blood vessels^{2,12}. Such molecules are thus available in vivo to mediate physiologic growth responses. Further, heparin-like molecules have been identified in the conditioned medium of some endothelial cells⁹.

In brain, the microvasculature is functionally and structurally different from capillaries of other organs²². Capillary endothelial cells are intimately associated with astrocytes, which is a cell type unique to brain. Astrocytic end feet are in contact with the antiluminal surfaces of endothelial cells forming a glial sheath, and they share a common basement

membrane³. Although the exact composition of this shared ECM is unknown in vivo, it is likely to contain heparin-like molecules, since both astrocytes and a number of endothelial cells in culture produce a pericellular matrix containing heparin-related glycosaminoglycans^{6,14}. The close anatomic relationship between the astrocyte and brain capillary endothelial cell suggests that trophic interactions between them may play a role in the regulation of their growth and differentiation. Components of their common matrix, such as heparan sulfate, may be important in mediating or modulating such cellular interactions. It was therefore of interest to investigate the effect of heparin on the growth responses of brain astrocytes.

Primary cultures of astrocytes were prepared from rat brains by a modification of the method of Franzakis¹³. Briefly, cerebral hemispheres were removed from 12 1–2 day old Sprague–Dawley rat pups, cleared of meninges and choroid plexus, chopped into small pieces and serially digested with 3 mg/ml neutral dispase (Boehringer-Mannheim) in Dulbecco's Minimal Essential Medium (DMEM). Dissociated cells were centrifuged and the pellet resuspended and plated in 10% fetal bovine serum (FBS) in DMEM with 100 U/ml penicillin and 100

$\mu\text{g/ml}$ streptomycin (P/S) (Gibco), in five 75 cm^2 plastic tissue culture flasks. The cultures were grown in 5% $\text{CO}_2/95\%$ air at 37 °C, fed on alternate days and became confluent 10–14 days after plating. Oligodendrocytes sometimes grew above the astrocytes in a stratified manner and these were removed from the cultures by the orbital shaking method of McCarthy and DeVellis¹⁸. Of the remaining cells, 90–95% in these primary cultures resembled polygonal astrocytes and were positive on immunocytochemical staining for glial fibrillary acidic protein (GFAP), a specific marker for astrocytes⁵. For GFAP staining, after being fixed with 1:1 acetone/ethanol, the cells were rinsed 3 times with phosphate buffered saline (PBS) and treated with normal goat serum (1:20) for 15 min at 37 °C. They were then exposed to rabbit anti-GFAP at a 1:10 dilution (Accurate Chemical and Scientific) for 30 min, rinsed $\times 3$ with PBS, and then treated with goat antiserum against rabbit immunoglobulin conjugated with fluorescein, at a 1:20 dilution (Cappel Labs.). The cells were rinsed a final time and viewed with epifluorescence optics for GFAP staining. In the growth studies, the cells were used at first passage which were also 90–95% GFAP positive. For measurement of cell proliferation, primary cultures were passed at 7000 cells/ cm^2 , to 24 well plastic tissue culture plates (Costar) in 10% FBS/P/S. After 24 h, the cells were placed on serum-free DMEM for another 24 h, and then changed to DMEM/P/S containing various concentrations of FBS with and without heparin (Sigma) at 100 $\mu\text{g/ml}$, in quadruplicate wells. The cells were fed once more after two days and counted after 5 days, using a Coulter Counter. For the [³H]thymidine incorporation assays, the astrocytes were passed from the primary cultures at 30,000 cells/ cm^2 , into 24 well plastic tissue culture plates in 10% FBS/P/S. The cells were fed every 2–3 days until they were confluent (usually 5–7 days), and then placed on serum free DMEM growth arrest medium for 72 h. This medium was then replaced with DMEM/P/S containing various concentrations of FBS, human platelet-free plasma, (PFP), or with platelet-derived growth factor (PDGF-Collaborative Research) with or without heparin at various concentrations for 20 h. After removal of the test media, the cells were rinsed with serum-free DMEM and pulsed with [³H]thymidine at 1 $\mu\text{Ci/ml}$ DMEM (New England Nuclear SA 80

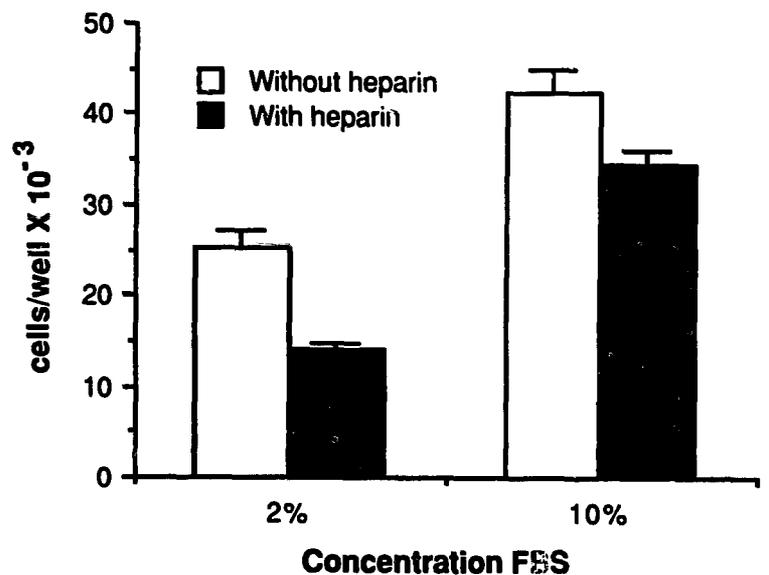


Fig. 1. First passage neonatal rat astrocytes were plated at 7000 cells/ cm^2 in 10% FBS/DMEM. After 24 h, the medium was replaced with serum-free DMEM for 48 h. Cells were then stimulated with 2% and 10% FBS/DMEM with or without heparin at 100 $\mu\text{g/ml}$ and counted 5 days later. Results are the mean of 4 determinations expressed as cells/well \pm S.E.M.

Ci/mM) for 2 h, after which they were processed for scintillation counting of tritium incorporated into DNA. The cells were fixed with 2.5% glutaraldehyde for 15 min, rinsed 3 times with 70% ethanol for 10 min each and then rinsed in tap water 3 times for 5 min each. After the wells were drained and air-dried, 0.5 ml of 1 M HCl was added to each well and the plates incubated at 60 °C for 2 h. The HCl extracts were then added to 5 ml of Safety Solve (Research Products International) and counted in a liquid scintillation counter.

Serum was a potent mitogen for the rat brain astrocytes in culture. After low density plating, 2% serum stimulated a 2.6-fold increase in cell number after 5 days in culture. This rose to 4.2-fold in 10% serum (Fig. 1). The mitogenicity of serum for astrocytes was also reflected in the stimulation of [³H]thymidine incorporation into confluent cultures of growth arrested cultures of astrocytes. Serum at 0.5% concentration in DMEM produced a 3.2-fold stimulation above control in these cultures. This increased to 4.5-fold at 10% serum concentration (Fig. 2). We found that heparin inhibited both the serum stimulated cell proliferation and [³H]thymidine incorporation in the astrocyte cultures. The stimulation of cell proliferation produced by 2% serum, decreased 45% in the presence of heparin at 100 $\mu\text{g/ml}$. This concentration of heparin also inhibited the stimulation of astrocytes by 10% serum, but the effect was somewhat dimin-

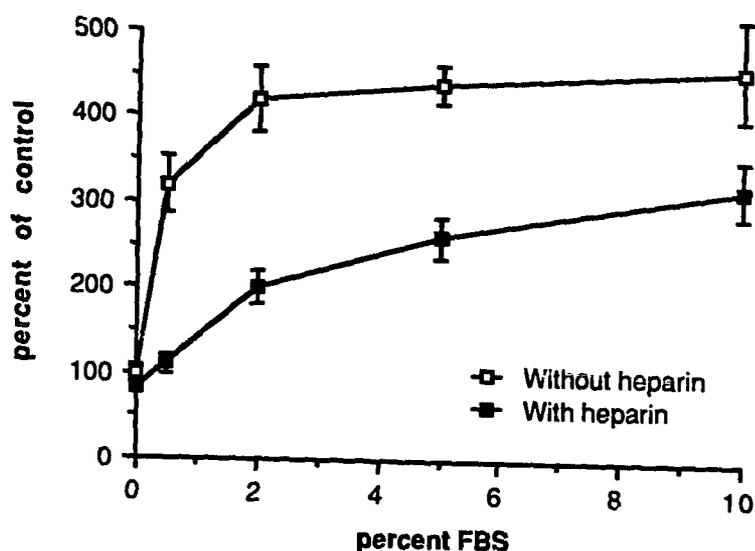


Fig. 2. Incorporation of [^3H]thymidine into confluent growth arrested cultures of first passage rat astrocytes, on stimulation by various concentrations of FBS with or without heparin at 100 $\mu\text{g}/\text{ml}$. Results are the mean counts per well from 4 determinations \pm S.E.M. expressed as percent of control.

ished (Fig. 1). Maximum inhibition of [^3H]thymidine incorporation in the confluent growth arrested astrocyte cultures, was 65% on stimulation by 0.5% serum in the presence of heparin at 100 $\mu\text{g}/\text{ml}$. As was noted in the cell proliferation assays, the inhibition also decreased but did not disappear as the serum concentration was increased to 10% (Fig. 2). This was the concentration of serum that produced maximum stimulation of the astrocytes, and there was also no further diminution of the heparin-mediated inhibition at higher serum concentrations up to 20% (data not shown). When stimulation of [^3H]thymidine incorporation by 5% serum was tested with varying concentrations of heparin, inhibition occurred in a dose-dependent manner with maximum inhibition at 100 $\mu\text{g}/\text{ml}$, but there was significant inhibition also at as low as 1 $\mu\text{g}/\text{ml}$ of heparin (Fig. 3). Human platelet-free plasma stimulated [^3H]thymidine incorporation into astrocytes just as well as did FBS, and this stimulation was also inhibited by heparin to an equal degree (Table I). PDGF stimulation of astrocytes in the absence of serum, however, was not affected by the presence of heparin (Table I).

Inhibition of growth by heparin is known to occur in several other types of cells^{7,17,23,27}. This phenomenon has been studied best in smooth muscle cells (SMC). Heparin inhibits proliferation and migration of SMC in vitro, as well as in vivo after arterial injury^{7,10}. In these cells, heparin appears to exert its inhibitory effect during the $G_0 \rightarrow S$ transition in the cell cycle⁷. Heparin binds specifically to receptors on

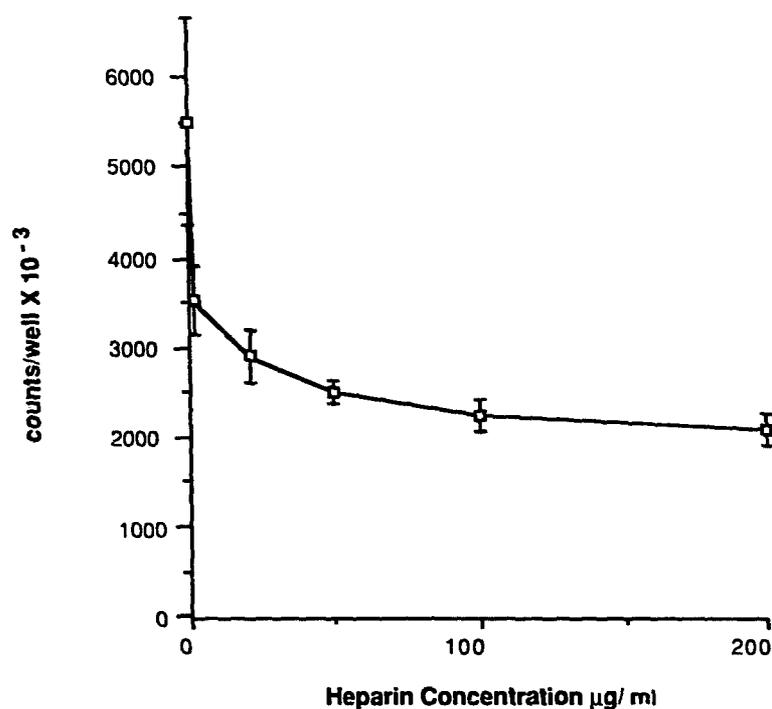


Fig. 3 [^3H]Thymidine incorporation was measured in confluent growth arrested first passage neonatal rat astrocyte cultures, on stimulation with 5% FBS/DMEM in the presence of varying concentrations of heparin. Results are the mean of 4 determinations expressed as counts/well \pm S.E.M.

the cell surface, is partially internalized by receptor-mediated endocytosis, and induces the expression of several new cellular proteins^{8,11,16}. Astrocyte growth inhibition by heparin may occur in a similar manner, but this will require further exploration.

The mitogens in serum that are responsible for heparin inhibitable growth in SMC are unknown. There is conflicting evidence as to whether PDGF, a known SMC mitogen and a factor present in serum in high concentration, is among them. Heparin does not bind PDGF or block the PDGF receptor on SMC²¹. Heparin retains its antiproliferative activity when added to SMC cultures up to 20 h after their release from G_0 by serum, whereas PDGF exerts its growth stimulating effect within 60 min after exposure to cells^{7,19,24}. However, in an assay in which PDGF was the sole mitogen for the SMC, an inhibitory effect of heparin was demonstrated, though not as great as when the cells were stimulated with serum²⁰. From our experiments, two pieces of data suggest that PDGF is not a mitogen inhibited by heparin in astrocyte cultures. PDGF is a known mitogen for astrocytes⁴, but PDGF stimulated [^3H]thymidine incorporation into astrocyte cultures in the absence of serum, is not inhibited by heparin (Table I). Moreover, stimulation of [^3H]thymidine incorporation in the astrocytes by human platelet-free plasma, which contains

TABLE I

Heparin inhibition of [³H]thymidine incorporation in rat astrocyte cultures

Percent inhibition of [³H]thymidine incorporation by heparin at 100 µg/ml, on stimulation by various concentrations of fetal bovine serum (FBS), human platelet-free plasma (PFP), or platelet-derived growth factor (PDGF). Percent inhibition = 1 - (stimulation without heparin/stimulation with heparin) × 100.

Mitogen	Percent inhibition by heparin
2% FBS	52
10% FBS	28
2% PFP	65
10% PFP	30
5 U/ml PDGF	4

little or no PDGF, is just as potently inhibited by heparin as when they are stimulated with FBS (Table I).

Heparin inhibits serum-stimulated [³H]thymidine incorporation and cell proliferation in astrocytes, but we also found that increasing serum concentration has a somewhat mitigating effect on this inhibition. At 100 µg/ml, heparin inhibits 0.5% serum stimulation 65%, but as the concentration of serum increases, inhibition decreased linearly to a low of 29% with 10% serum stimulation. This might be explained in several possible ways. Increasing the concentration of serum mitogens responsible for the heparin inhibitable stimulation, if they are competitive with

heparin at cell surface receptors or at some subsequent step intracellularly, could decrease the inhibition. It is also possible that increased concentration of serum proteins that can bind heparin, such as antithrombin III or platelet factor 4, could compete with cellular receptors for heparin, thus decreasing its cellular antiproliferative effects. This latter mechanism appears to be operative in the reversal by serum of heparin-inhibited mitogenesis in human umbilical vein endothelial cells (HUVE), although the potency of heparin for these cells is less than for the astrocytes or smooth muscle cells, and the heparin-mediated inhibition of HUVE is completely reversed by 10% serum²³.

The heparin-mediated inhibition of serum stimulated astrocyte mitogenesis in vitro may be important in the regulation of their growth in vivo. Under normal adult physiologic conditions, astrocytes and endothelial cells are not actively proliferating¹⁵. Given the sensitivity of astrocytes to stimulation by mitogens in serum, and the possibility of exposure to these factors under physiologic conditions, heparin-like molecules in the basement membrane could play a role in maintaining astrocytes in their normal quiescent growth state. Such growth inhibition may also be linked to maintaining the cells in a differentiated state necessary for their normal physiologic interactions.

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