

The Effect of Heparin on Growth of Mammalian Cells *in Vitro*<sup>1</sup> (40290)

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Heparin has been used primarily as a therapeutic anticoagulant agent (1), and clinically used to treat inflammatory and allergic diseases (2). It has also been shown to accelerate recovery of burn patients and promote wound healing in humans and animals (3-5). The mechanism and process of burn and wound healing from these observations is still not well elucidated. It is, therefore, of interest to investigate whether heparin can stimulate proliferation of human skin diploid cells *in vitro* which might be related to the mode of action of the healing process.

The effect of heparin and other acid mucopolysaccharides on the growth of various cell types, mainly malignant cells, has been studied by a number of investigators. The results obtained often have been controversial. Some investigators find inhibitory effects on cell growth (6-8), some stimulatory (8-10), and some report morphological changes (11). Therefore, this study was carried out in an attempt to provide more information about the nature of the effect of heparin on the growth of cultured mammalian cells.

*Materials and methods. Chemicals.* Amino acids, vitamins and newborn calf serum were purchased either from International Scientific Industries, Inc., Cary, IL, or Grand Island Biological Co., Grand Island, NY; prednisolone-21-sodium-succinate (PSS) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES) from Sigma Chemical Co., St. Louis, MO; S-210 medium from Grand Island Biological Co.; Waymouth 752/1 dry powder medium from Schwarz/Mann Inc., Orangeburg, NY; fatty acid-free bovine serum albumin (FAF-BSA) from Miles Laboratories, Inc., Elkhart, IN; oleic acid from Nu-Chek Prep, Inc., Elysian, MN; various forms of heparin were kindly supplied by Riker Co., Division of 3M Co., St. Paul, MN;

Calbiochem, La Jolla, CA, Upjohn Co., Kalamazoo, MI; and highly purified heparin was a gift from Dr. J. A. Cifonelli, University of Chicago, Chicago, IL.

*Cell cultures.* The sources of the cells and the methods used for cultivation were the same as described previously. Monkey kidney (MK-2) cells were cultivated as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% newborn calf serum (MEM<sub>5</sub>) (12). Novikoff hepatoma cells were grown in shaker culture in S-210 medium (13). Human prepuce cells were grown as monolayers in Eagle's MEM medium (14) supplemented with 10% newborn calf serum (MEM<sub>10</sub>) and baby hamster kidney cells (BHK-21) were grown in shaker culture using a modified Waymouth 752/1 medium (15).

*Growth of cells in the presence of heparin.* Prepuce cells were grown in Eagle's MEM supplemented with 4% newborn calf serum (MEM<sub>4</sub>) or MEM<sub>10</sub> in the presence of a wide range of heparin. Hanks' balanced salt solution (BSS) was used as a base (16). The cells were used at an initial density of  $2.0-3.0 \times 10^5$  cells/flask in a volume of 4 ml and were placed in 25 cm<sup>2</sup> cell culture flasks (Corning Glass Works, Corning, NY). The cells were incubated at 37° for 6-10 days and were enumerated at varying intervals of time after trypsinization with the aid of a Coulter counter.

Thirty ml of BHK-21 cells were suspended in modified Waymouth 752/1 medium (15) containing 2.5% newborn calf serum and different amounts of heparin. The initial cell population contained  $3.5 \times 10^5$  cells/ml and were incubated at 37° in a New Brunswick gyratory shaker. At 0, 24, 48, 72 and 96 hr the cells were enumerated with a Coulter counter.

An initial population of  $3.0 \times 10^5$  MK-2 cells in 4 ml of modified Waymouth 752/1 medium (15) was added to 25 cm<sup>2</sup> flasks. In order to establish monolayers of cells, the medium was supplemented with 1% newborn

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calf serum. After 24-hr incubation at 37°, the medium was discarded and the cells rinsed with BSS. Fresh modified Waymouth medium without the serum supplement containing different amounts of heparin were added to the cells. The cells were incubated at 37° again and were enumerated at varying intervals of time.

Novikoff hepatoma cells were grown in shaker culture in S-210 medium in the presence of different amounts of heparin. A starting density of  $2 \times 10^5$  cells/ml was incubated at 37° for 4 days. Cell numbers were determined every 24 hr.

*Combined effect of heparin and PSS on the growth of BHK-21 and prepuce cells.* Varying amounts of PSS and heparin were added in various combinations to the growth medium to cultivate BHK-21 cells in shaker cultures and prepuce cells in monolayers. The cell growth was measured at varying intervals of time. The procedures were the same as described above for testing the effect of heparin alone.

A minimum of two independent experiments were performed for all studies. Each treatment was carried out in triplicate, and triplicate counts were made on each sample. The results were analyzed for significant differences by using a student's *t* test.

*Results.* The effect of Riker's hog mucosal heparin on the growth of prepuce cells cultivated in MEM<sub>10</sub> is shown in Table I. There were no immediate differences observed in growth between control and heparin-treated cells during the first 3 days after incubation. On day 5, the cells cultivated in the medium containing 5 and 10 µg/ml of heparin had an increase of 30% and 23%, respectively, in cell numbers over that of the control cells. There was a modest increase in the number of cells

grown in medium containing 15 and 20 µg/ml of heparin, whereas the cells treated with 80 µg/ml of heparin had a 21% decrease in cell population when compared to control cells. The population of cells treated with 0, 5, 10 and 20 µg/ml of heparin began to decline after day 5, whereas cells treated with 15 and 80 µg/ml of heparin continued to increase in cell number.

When prepuce cells were cultivated in MEM<sub>4</sub> after initially incubating the cells in MEM<sub>10</sub> for 24 hr, no differences in the growth between heparin-treated and untreated cells were observed until 8 days after incubation (Table II). On day 8, cells treated with 5 µg/ml of heparin showed a 90% increase in cell numbers over that of the untreated cells. Cells treated with higher concentrations of heparin which were less stimulatory than the cells treated with 5 µg/ml of heparin showed an increase of about 35% in population. On day 10, the cells treated with 5, 10 and 15 µg/ml of heparin all showed about a 30% increase in cell number over the untreated cells. Cells treated with 80 µg/ml of heparin had about the same growth rate as that of the untreated cells.

Various heparins with different anticoagulant activity obtained from Upjohn Co. and Wilson Labs and further purified by J. A. Cifonelli showed similar stimulatory effects on the growth of prepuce cells (Table III). Each of the three heparins at a concentration of 5 µg/ml increased the number of cells about 30–50% from day 5 to 8 after incubation.

Heparins from different sources at a concentration of 5 µg/ml showed similar stimulatory effect on the growth of prepuce cells (Table IV), except there was slightly higher cell population when the cells were grown in

TABLE I. EFFECT OF RIKER'S HOG MUCOSAL HEPARIN ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM SUPPLEMENTED WITH 10% NEWBORN CALF SERUM.

Day	Heparin (µg/ml)					
	0	5	10	15	20	80
1	1.33 ± 0.03 <sup>a</sup>	1.27 ± 0.03	1.30 ± 0.00	1.27 ± 0.07	1.17 ± 0.03	1.30 ± 0.12
3	2.97 ± 0.09	3.40 ± 0.21	3.20 ± 0.10	2.60 ± 0.17	2.70 ± 0.27	2.63 ± 0.18
5	4.70 ± 0.10	6.10 ± 0.35 <sup>b</sup>	5.77 ± 0.22 <sup>b</sup>	5.03 ± 0.07	5.30 ± 0.20	3.73 ± 0.70
7	4.20 ± 0.29	5.67 ± 0.35 <sup>b</sup>	5.30 ± 0.47	6.20 ± 0.31 <sup>b</sup>	4.53 ± 0.29	4.83 ± 0.46

<sup>a</sup> Average cell number  $\times 10^5$ /flask (25 cm<sup>2</sup>)  $\pm$  SEM from three flasks each counted in triplicate.

<sup>b</sup> Significantly different from control (*P* < 0.05). These data are typical results from a minimum of three independent experiments.

TABLE II. EFFECT OF RIKER'S HOG MUCOSAL HEPARIN ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 4% NEWBORN CALF SERUM.

Day	Heparin ( $\mu\text{g}/\text{ml}$ )					
	0	5	10	15	20	80
1	1.50 $\pm$ 0.07 <sup>a</sup>					
2	2.00 $\pm$ 0.06	1.93 $\pm$ 0.09	1.67 $\pm$ 0.07	1.93 $\pm$ 0.15	1.93 $\pm$ 0.07	2.23 $\pm$ 0.07
4	2.77 $\pm$ 0.03	2.90 $\pm$ 0.10	2.73 $\pm$ 0.07	2.73 $\pm$ 0.22	2.53 $\pm$ 0.07	2.37 $\pm$ 0.03
6	2.83 $\pm$ 0.03	3.17 $\pm$ 0.35	3.17 $\pm$ 0.19	3.03 $\pm$ 0.18	3.37 $\pm$ 0.34	2.60 $\pm$ 0.06
8	2.83 $\pm$ 0.03	5.40 $\pm$ 0.32 <sup>b</sup>	3.60 $\pm$ 0.36	4.10 $\pm$ 0.72	3.83 $\pm$ 0.52	3.27 $\pm$ 0.43
10	4.87 $\pm$ 0.09	6.43 $\pm$ 0.23 <sup>c</sup>	6.40 $\pm$ 0.46 <sup>c</sup>	6.30 $\pm$ 0.15 <sup>b</sup>	5.77 $\pm$ 0.09 <sup>b</sup>	4.83 $\pm$ 0.19

<sup>a</sup> Average cell number  $\times 10^5/\text{flask}$  ( $25 \text{ cm}^2$ )  $\pm$  SEM from three flasks each counted in triplicate.

<sup>b</sup> Significantly different from control ( $P < 0.01$ ).

<sup>c</sup> Significantly different from control ( $P < 0.05$ ). These data are typical of results from three independent experiments.

TABLE III. EFFECT OF HEPARINS (5  $\mu\text{g}/\text{ml}$ ) WITH DIFFERENT SPECIFIC ACTIVITIES ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 4% NEWBORN CALF SERUM.

Day	Heparins			
	Control	A <sup>a</sup>	B <sup>b</sup>	C <sup>c</sup>
1	1.37 $\pm$ 0.09 <sup>d</sup>			
3	3.07 $\pm$ 0.15	3.27 $\pm$ 0.20	3.17 $\pm$ 0.13	3.47 $\pm$ 0.29
5	3.17 $\pm$ 0.09	4.57 $\pm$ 0.23 <sup>e</sup>	4.33 $\pm$ 0.19 <sup>e</sup>	4.27 $\pm$ 0.22 <sup>e</sup>
6	4.03 $\pm$ 0.12	6.00 $\pm$ 0.40 <sup>e</sup>	5.37 $\pm$ 0.02 <sup>e</sup>	5.63 $\pm$ 0.30 <sup>e</sup>
8	5.20 $\pm$ 0.15	7.10 $\pm$ 0.32 <sup>e</sup>	6.80 $\pm$ 0.46 <sup>e</sup>	6.63 $\pm$ 0.09 <sup>f</sup>

<sup>a</sup> A: Beef lung heparin from Upjohn Co. further purified by gel filtration on Sephadex G-75 by J. A. Cifonelli (specific activity of 144  $\text{I}\mu/\text{mg}$ ).

<sup>b</sup> B: Beef lung heparin from Wilson Labs further purified by J. A. Cifonelli (specific activity of 180  $\text{I}\mu/\text{mg}$ ).

<sup>c</sup> C: Beef lung heparin from Wilson Labs (specific activity of 110  $\text{I}\mu/\text{mg}$ ).

<sup>d</sup> Average cell number  $\times 10^5/\text{flask}$  ( $25 \text{ cm}^2$ )  $\pm$  SEM from three flasks each counted in triplicate.

<sup>e</sup> Significantly different from control ( $P < 0.05$ ).

<sup>f</sup> Significantly different from control ( $P < 0.01$ ). These data are typical from three independent experiments.

TABLE IV. EFFECT OF VARIOUS HEPARINS (5  $\mu\text{g}/\text{ml}$ ) ON GROWTH OF HUMAN PREPUCE CELLS IN EAGLE'S MINIMUM ESSENTIAL MEDIUM CONTAINING 10% NEWBORN CALF SERUM.

Day <sup>a</sup>	Heparins				
	A	B	C	D	E
1	1.17 $\pm$ 0.03 <sup>b</sup>	1.17 $\pm$ 0.03	1.27 $\pm$ 0.03	1.10 $\pm$ 0.06	1.23 $\pm$ 0.03
3	1.90 $\pm$ 0.06	2.30 $\pm$ 0.10 <sup>c</sup>	2.23 $\pm$ 0.09	2.57 $\pm$ 0.20 <sup>c</sup>	2.90 $\pm$ 0.10 <sup>c</sup>
5	2.67 $\pm$ 0.13	3.13 $\pm$ 0.24	3.27 $\pm$ 0.35	3.00 $\pm$ 0.06	3.77 $\pm$ 0.22 <sup>c</sup>
7	3.17 $\pm$ 0.18	3.73 $\pm$ 0.19	5.13 $\pm$ 0.39 <sup>c</sup>	4.23 $\pm$ 0.23 <sup>c</sup>	4.10 $\pm$ 0.25 <sup>c</sup>
9	3.23 $\pm$ 0.09	4.10 $\pm$ 0.17	4.07 $\pm$ 0.03 <sup>d</sup>	4.03 $\pm$ 0.28	4.40 $\pm$ 0.06 <sup>d</sup>

<sup>a</sup> A: Control, B: Riker's hog mucosal heparin, C: Riker's beef lung heparin, D: Riker's crude hog mucosal heparin, E: Calbiochem's hog mucosa heparin.

<sup>b</sup> Average cell number  $\times 10^5/\text{flask}$   $\pm$  SEM from three flasks each counted in triplicate.

<sup>c</sup> Significantly different from control ( $P < 0.05$ ).

<sup>d</sup> Significantly different from control ( $P < 0.01$ ). These data are typical from three independent experiments.

the medium containing heparin from porcine intestinal mucosa from Calbiochem.

Heparin showed little or no effect on the growth of MK-2, BHK-21 and Novikoff rat hepatoma cells, when the cells were cultivated in the medium containing several concentrations of newborn calf serum in the presence

of a wide range of concentrations of different heparins.

PSS at concentrations of 0.5  $\mu\text{g}/\text{ml}$  and 2  $\mu\text{g}/\text{ml}$  inhibited the growth of BHK-21 (50%) and prepuce (25%) cells, respectively (Figs. 1 and 2). In an attempt to reverse this inhibitory effect of PSS on cell growth, hog

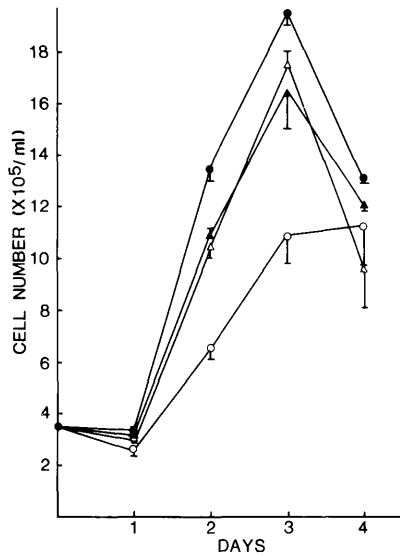


FIG. 1. Combined effect of prednisolone-21-sodium-succinate (PSS) and heparin on growth of BHK-21 cells cultivated in shaker cultures. 30 ml of a cell suspension in modified Waymouth 752/1 medium containing an initial population of  $3.5 \times 10^6$  cells/ml was placed in 125 ml Erlenmeyer screw-cap flasks. LSS alone or a combination of PSS and heparin was added to the medium. The cells were incubated at  $37^\circ$  and enumerated with a Coulter counter at varying intervals of time. ●—●, 0  $\mu\text{g/ml}$  PSS, heparin; ○—○, 0.5  $\mu\text{g/ml}$  PSS; ▲—▲, 0.5  $\mu\text{g/ml}$  PSS + 5  $\mu\text{g/ml}$  heparin; △—△, 0.5  $\mu\text{g/ml}$  PSS + 10  $\mu\text{g/ml}$  heparin. Vertical bars represent standard error of mean.

mucosal heparin was added into the culture medium. It was found that cells grown in the medium containing both PSS and heparin had about the same cell numbers as those in mediums containing no PSS (Figs. 1 and 2), showing reversal of heparin on the inhibitory effect by PSS.

**Discussion.** Previous results about the effect of heparin and other acid mucopolysaccharides on cell growth have been equivocal. Fischer (17) first claimed inhibition of mitosis using heart fibroblasts and concentrations of heparin varying from 20–500  $\mu\text{g/ml}$ . Costachel (7) reported cytotoxic action of heparin at 100–2000  $\mu\text{g/ml}$  using Syrian hamster sarcoma cells. Lippman (8) demonstrated that heparin at 50  $\mu\text{g/ml}$  inhibited growth of mouse "L" cells. King *et al.* (18) found that heparin at 1–1000  $\mu\text{g/ml}$  showed little effect on cell division of mouse "L" cells grown in suspension cultures.

Our data showed that heparin appears to promote the growth of prepuce cells but did not stimulate growth of BHK-21, MK-2 or Novikoff rat hepatoma cells. Takeuchi (10) noted that acid mucopolysaccharides have some promoting activity on tumor growth. Ozzello *et al.* (19) reported the growth promoting activity of acid mucopolysaccharides on a strain of human mammary carcinoma cells. They ascribed this action to the negative electric charge and the viscosity of acid mucopolysaccharides.

The controversy about the action of heparin on the cell growth is presumably due in part to different dosages of heparin and the cell types used. Heparin in high concentration can be inhibitory to the growth of cells cultivated *in vitro*. If the amount of heparin is maintained at a dose that just inhibits coagulation (2  $\mu\text{g/ml}$ ), it seems to be relatively noncytotoxic (11), and perhaps even stimulatory to cell growth. Zakrezewski (20) claimed that the Jensen sarcoma in tissue culture was inhibited by heparin, but empha-

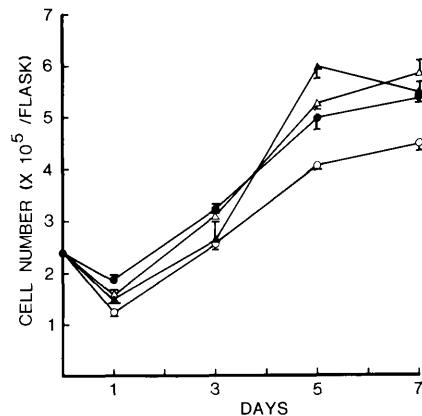


FIG. 2. Combined effect of prednisolone-21-sodium-succinate (PSS) and heparin on growth of prepuce cells cultivated in Eagle's minimum essential medium supplemented with 10% newborn calf serum ( $\text{MEM}_{10}$ ). Prepuce cells at an initial density of  $2.3 \times 10^5$  cells/flask in 4 ml of  $\text{MEM}_{10}$  medium were placed in 25  $\text{cm}^2$  cell culture flasks. PSS alone or a combination of PSS and heparin was added to the medium. The cells were incubated at  $37^\circ$  for 7 days and were enumerated with a Coulter counter at varying intervals of time after trypsinization. ●—●, 0  $\mu\text{g/ml}$  PSS, heparin; ○—○, 2  $\mu\text{g/ml}$  PSS; ▲—▲, 2  $\mu\text{g/ml}$  PSS + 5  $\mu\text{g/ml}$  heparin; △—△, 2  $\mu\text{g/ml}$  PSS + 20  $\mu\text{g/ml}$  heparin. Vertical bars represent standard error of the mean.

sized that this drug was much less effective on normal embryonic tissue.

Medium supplemented with low amounts of serum (4%) was used in one set of experiments to hopefully show a growth stimulatory effect when supplemented with heparin. Growth of prepuce cells might then be magnified when cells were subliminally starved (21). From the results shown in Table II, about 90% increase of cells was observed compared to the control in the presence of 5  $\mu$ g heparin/ml at day 8. Medium containing 2% serum was also tested. No stimulatory effect of heparin was noted when prepuce cells were grown in this medium. Takeuchi hypothesized (10) that acid mucopolysaccharides did not serve as a nutritional component for cell growth but protects the cell surface and promotes the exchange of various metabolites. Our observation indirectly further supports this hypothesis.

Cell populations from monolayer cultures were found to drop in the first 24-hr incubation. This probably is attributed to cell lysis during the trypsinization process. Therefore, the baseline data for all experiments was best interpreted after 24 hr cultivation.

It has been reported that heparin in animal experiments could interact with steroid hormones (2). Our data demonstrated that heparin reversed the inhibitory effect of PSS on the growth of prepuce and BHK-21 cells cultivated *in vitro*. This test system could be used to indirectly show heparin effects on cell growth when little or no activity was noted by heparin directly. This observation confirms the hypothesis of Dougherty and Dolonitz (2).

A question had been raised whether trace metal contaminants or other unknown contaminants of heparin might be responsible for its activity in aiding burn repair. When crude, commercial grade and highly purified heparins from hog mucosa and/or beef lung sources were tested for promoting cell growth, no differences were found in the activity, which seemed to negate the role of heparin contaminants in the cell culture detection systems used.

Since heparin and heparin-like components are normal constituents of the blood and cells of higher animals, it is not surprising to find that heparin at a physiological level is

harmless and even stimulatory to the cell growth *in vitro*.

*Summary.* The effect of heparin on the growth of four cell types cultivated *in vitro* has been investigated. The results suggest that heparin appears to have some growth promoting effects on prepuce cells, while it showed little effect on the growth of Novikoff hepatoma, monkey kidney and baby hamster kidney cells. Heparin reversed the inhibitory effect of prednisolone-21-sodium-succinate on the growth of prepuce and baby hamster kidney cells.

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