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Heparin stimulates the proliferation of intestinal epithelial cells in primary culture

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SUMMARY

Heparin is a sulphated glycosaminoglycan derived from mast cells and has a number of functions including the inhibition of proliferation in several cell types and interactions with a range of heparin-binding growth factors. We report that heparin is a trophic factor in primary cultures of rat small intestinal epithelium. Heparin elicits a dose-dependent increase in epithelial proliferation and inhibits the growth of associated mesenchyme. The trophic effect of this molecule is not reproduced by other glycosaminoglycans including heparan sulphate but is dependent upon extensive molecular sulphation. Highly sulphated polysaccharides that are structurally unrelated to heparin (e.g. dextran sulphate and pentosan polysulphate) also stimulate epithelial proliferation in primary cultures. Heparin may act by the potentiation of mesenchyme-derived heparin-binding growth factors and these data suggest an in vivo role for mast cell-derived heparin in mucosal wound regeneration.

Key words: heparin, epithelial cell, proliferation, small intestine, primary culture, mucosal regeneration

INTRODUCTION

The importance of extracellular matrix (ECM) and stromal components in intestinal epithelial cell proliferation and differentiation is well documented (Kedinger et al., 1988). The development of suitable in vitro models to study growth regulation in intestinal epithelium is therefore important in identifying factors that contribute to the proliferative niche. Using a recently developed primary culture method for suckling rat small intestine epithelium, we have studied the role of stroma and ECM in proliferation of isolated intestinal epithelia. We previously reported that exogenous heparin stimulates proliferation of rat intestine epithelial cells in primary culture (Evans et al., 1992). We now describe more detailed studies into the nature of this mitogenic signal.

There are many polypeptide growth factors, the activities of which are only fully expressed by interactions with ECM components (Ruoslahti and Yamaguchi, 1991; Nathan and Sporn, 1991). The existence of a proliferative niche may therefore be the result of appropriate interactions between regulatory peptides and stromal or ECM constituents. However, heparin is also an effective inhibitor of mesenchymal cell proliferation (Karnovsky et al., 1989), suggesting a bifunctional role for heparin in heterologous primary cultures. Identification of the mechanisms by which heparin acts may be relevant in understanding the controls of proliferation in small intestine epithelia.

In the normal small intestine epithelium, there is a spatial segregation between proliferative (crypt) and differentiated (villus) cellular components. Stem cells in the crypt give rise to immature progeny of limited proliferative potential. These cells migrate up the crypt-villus axis to emerge onto the villus as functionally mature terminally differentiated cells (Potten and Loeffler, 1990). Spatial restriction of proliferative and differentiated cells may require permissive 'niche' environments. A number of reports have suggested roles for a variety of polypeptide growth factors in the regulation of intestinal epithelial proliferation (TGF-β, Kurokowa et al., 1987; IGFs, Laburthe et al., 1988; TGF-α, Malden et al., 1989). Other workers have described the expression of extracellular matrix molecules and their receptors specifically within lower regions of the crypt (Alho and Underhill, 1989; Simo et al., 1991; Beaulieu, 1992) suggesting roles for these molecules in the proliferative niche. However, unlike other tissues, very few reports describe roles for heparin-like molecules in the intestine. Although variations in biochemical properties and tissue distribution of extracellular matrix proteoglycans do occur during intestinal differentiation and development (Hayashi et al., 1987; Bouziges et al., 1991). We now describe the effects of highly sulphated GAGs on the proliferation of epithelial cells in primary cultures of suckling rat small intestine. Observations reported in this paper may have important consequences for understanding growth regulatory roles for heparinoids in the small intestine epithelium.

MATERIALS AND METHODS

Cell culture

Primary intestinal cultures were derived from 5-day post-natal (outbred Wistar) rats, housed under 24-hour light/dark cycles (light: 0700-1900, GMT) and given food and water ad libitum. Animals were sacrificed by cervical dislocation and the entire small intestine removed. Epithelium was isolated as intact organoids by enzymatic dissociation using collagenase (type XI; Sigma Chemical Co., Poole, Dorset) and dispase (Boehringer Mannheim, Lewes, East Sussex, UK) followed by physical dissaggregation (Evans et al., 1992). Organoids were plated in DMEM (single-strength stock, 4.5 g/l glucose; Gibco BRL, Paisley, Scotland) supplemented with 2.5% batch-tested foetal calf serum (Gibco BRL), 20 ng/ml epidermal growth factor (Sigma Chemical Co.), 0.25 i.u./ml insulin (C. P. Pharmaceuticals, Wrexham, UK), 100 i.u./ml benzyl penicillin (Glaxo, Greenford, UK) and 60 μg/ml streptomycin sulphate (Sigma Chemical Co.). Cultures were maintained at 7.5% CO_2 and 37°C on 24-multiwell tissue culture dishes (Costar UK Ltd, High Wycombe, Bucks, UK) that had been previously coated with air-dried bovine dermal collagen (Vitrogen; Imperial Labs, Andover, Hampshire, UK).

Epithelial colonies were identified as described previously (Evans et al., 1992) by ultrastructural and morphological criteria. In addition, functional differentiation of primary epithelial cells was determined by histochemical presence of brush border alkaline phosphatase using Vector red substrate kit (Vector Laboratories, Bretton, Peterborough, UK) in Tris-HCl at pH 9.0. This was applied to primary cultures (previously fixed in 70% ethanol) and paraffin sections of suckling rat small intestine, and the substrate was visualised either by normal or fluorescent microscopy. Fluid transport across epithelial monolayers was also determined by the formation of 'domes' caused by sub-epithelial fluid accumulation.

Mesenchymal cells that expressed smooth muscle α-actin were identified as described previously (Evans et al., 1992) by immunocytochemistry with the 1A4 monoclonal antibody to smooth muscle α-actin (Sigma Chemical Co.).

Cell growth assays

Incremental growth in primary cultures under different conditions was quantified up to 14 days after plating by a crystal violet dye-binding assay (modified from the method of Brasaemle and Attie, 1988). Cultures being assayed were fixed in methanol or 1-2% gluteraldehyde (post-fixation in 70% ethanol), air-dried and stained with 0.1% crystal violet (prepared in citrate buffer; pH 2.5) for 5 minutes at room temperature. Excess stain was removed by washing in acidified (pH 2.5) tap water. Bound dye was solubilised in 10% acetic acid and transferred to 96-well microtitre plates. Optical density of the bound dye was then measured at 540 nm on a TiterTek multiscan (MCC/340). The advantage of this assay above other dye-binding methods is that the dye extraction is non-destructive and therefore cells can be restained for microscopical observation.

In selected experiments, physical cell counts were also performed to analyze effects on epithelial and mesenchymal components of the cultures. The surface area in each well occupied by epithelial or mesenchymal cells was first estimated by using a random point counting method at ×40 magnification. This consisted of a microscope eyepiece graticule of 121 points (formed at the intersections between a grid of lines). The grid intersections (points) lying on epithelial (E_p) or mesenchymal cells (M_p) were scored and expressed as a proportion of total grid points (T_p). These values were converted to area measurements as e.g. area occupied by epithelial cells $E_a=E_p/T_p\times W_a$; where W_a is the area of each well in the plate. At higher magnification (×200), the densities (i.e the number of nuclei) of each cell type was measured within a defined area of this graticule. The number of each cell type per well was then calculated e.g, number of epithelial cells $E_n=E_a/E_d$; where E_d is the mean density of epithelial cells in a defined area of the grid. For point counts and cell density measurements, 5 randomly placed grids were scored within each well, and the means (± standard error) derived from measurements in at least 4 wells per variable. Epithelial and mesenchymal cells were distinguished according to the methods previously described. To account for mesenchymal cells present in multiple layers, the fine focus of the microscope was adjusted during counting.

The extent of proliferation was determined by treating cultures with 1 μCi/ml [methyl-^{3}H]thymidine (6.7 mCi/mmol specific activity, 24.9 GBq/mmol; NEN DuPont, Stevenage, Herts, UK) for 3 hours. Cultures were fixed in 70% ethanol, air-dried and acid-soluble label removed in 5% trichloroacetic acid for 20 minutes. After washing in two further changes of 70% ethanol, acid-insoluble label was solubilized in 0.1 N NaOH for 1 hour. Solubilized samples were mixed with EcoScint® (Mensura Technology Ltd, Wigan, Lancs, UK) and the amount of incorporated label assessed by scintillation counting on a Beckman LS 1801 counter. Alternately, primary cultures were grown in 35 mm dishes (Becton Dickenson UK Ltd, Oxford) in the presence or absence of 50 μg/ml heparin for 7 days before incubation in tritiated thymidine as above. Dishes were fixed in 2% glutaraldehyde for 2 hours, washed extensively in acid ethanol and rinsed in PBS before being dipped in Ilford K5 autoradiography emulsion (Ilford, Mobberley, Cheshire, UK) under darkroom safelight conditions. Autoradiographs were exposed for 10 days and developed by standard procedures. After staining with crystal violet, the percentage of labelled epithelial and non-epithelial cells was scored. Four random graticule areas of epithelial and mesenchymal cells were scored for at least 1000 cells/well of each cell type to determine the labelling indices. The means (± standard error) were derived from measurements in at least 4 wells per variable.

In selected experiments, observable differences in treated and control primary culture growth were analyzed for statistical significance. Two way analysis of variance was used to determine if changes of growth with time or experimental treatment were significantly different from those estimated to be caused by random error. Significance was determined at P=0.05.

Heparin uptake and cellular location

The uptake and intra-cellular location of heparin in stimulated cells was determined by adding tritiated heparin (specific activity 0.016 GBq/mg, 0.44 mCi/mg; NEN Du Pont). Primary cultures were plated in 50 μl of medium onto sterile, serum-coated glass multispot slides (ICN Biomedicals Ltd, High Wycombe, Bucks, UK). Cultures were maintained for 3 days in 2.5% FCS as previously defined after which, growth medium was replaced with 1% FCS containing a mixture of 10 μg/ml [^{3}H]heparin and 10-40 μg/ml cold heparin. At different times after medium change, cultures were fixed in formal saline and the cellular localisation of label determined by autoradiography. The same procedure was repeated using FITC-labelled dextran sulphate (40,000 M_r; Cambridge Bioscience, Cambridge, UK), the location of label being determined by fluorescent microscopy. Unlabelled dextran sulphate (40,000 M_r) was added to the medium as a competitor to assess specificity of uptake. A further control of uptake specificity was incubation with FITC-labelled dextran (40,000 M_r) as a non-specific, unsulphated macromolecule. To localise cells following incubation with FITC-labelled ligands, cultures were pretreated with 0.4 μg/ml Hoescht dye 33258 (Sigma Chemical Co.) for 5 minutes to stain the nuclei and observed under UV illumination.

The role of heparin-binding growth factors

The role of specific growth factor candidates was investigated using proto-typic heparin-binding growth factors acidic and basic FGF (bovine pituitary; British Biotechnology Ltd). These molecules are potent mitogens of certain intestinal epithelium cell lines (Cove and Evans, 1992). Cell growth was assessed in the presence of 10 ng/ml aFGF/bFGF across a range of heparin doses (2-50 μg/ml). This data

was compared with growth rates in the presence of equivalent doses of heparin or FGF in isolation.

Heparin-binding proteins present in serum may contribute to the stimulatory activity of heparin on intestinal epithelia. The efficacy of heparin (0-100 μg/ml) in a range of serum concentrations (1-10%) was therefore compared. In addition, heparin-binding proteins were depleted from serum using a heparin-Sepharose CL-6B column (Pharmacia LKB Biotechnology, Milton Keynes, Bucks, UK). A 5 ml column of heparin-Sepharose was washed in five column volumes of loading buffer (protein-free single-strength DMEM, pH 7.4). FCS was passed through a low protein-binding 0.2 μm Millipore filter, and 1 ml applied to the heparin column. Non-heparin-binding proteins were eluted in four column volumes of DMEM and those bound to the heparin-Sepharose in 3.0 column volumes of DMEM (at 2.0 M NaCl). Protein recovery was monitored by sensitive Coomassie protein assay (Pierce and Warriner, Chester, UK), and high salt fractions were desalted on PD10 columns (Pharmacia LKB Biotechnology). Bound and unbound fractions were diluted to 100 ml in DMEM and appropriate quantities of medium supplements added. After sterile filtration (0.2 μm; Millipore, Watford, Herts, UK) primary cultures were grown in 1% FCS depleted of heparin-binding components, 1% FCS supplemented with additional heparin-binding fractions or control 1% FCS. The effect of porcine heparin (50 μg/ml) in these various media was analyzed and growth rates compared.

Studies into the effect of heparin and related molecules

A range of molecules were screened in primary culture to identify structural characteristics important for the stimulatory activity of heparin. All species of heparin, modified heparins, heparan sulphates, chondroitin sulphates, dermatan sulphate, hyaluronic acid, dextran sulphate, pentosan polysulphate and protamine sulphate were obtained from Sigma Chemical Company except porcine heparan sulphate that was a gift from Dr J. Turnbull (Dept of Tumour Biochemistry, Christie Hospital, Manchester). Hyaluronic acid was tested either as the commercial (high M_r) product or, following autoclaving to produce a mixture of smaller species (low M_r; Laurent and Tengblad, 1980). Unless otherwise stated, organoids were plated in the presence of the compound, culture medium changed every 3-4 days and growth assessed at various times after plating. All molecules were tested across a range (0-100 μg/ml) of concentrations and experiments repeated several times.

Effect on epithelial and mesenchymal cell lines

The action of heparin on pure populations of epithelial and mesenchymal cells was determined using IEC 6, 17 and 18 epithelial cell lines (Quaroni and May, 1980) and an established mesenchymal cell line (UNCL-1) derived in this department from developing rat intestine (Evans et al., 1993). These lines were grown either serum-free in DMEM supplemented with 10 μg/ml transferrin (Sigma), 20 μg/ml EGF and 0.25 i.u./ml insulin or in DMEM supplemented with 1% FCS (EGF and insulin added for IEC cells). Heparin was added to these cultures (0-100 μg/ml) at plating (sub-confluent densities) and growth between days 1 and 4 determined by crystal violet assay as described above.

RESULTS

Characterisation of primary cultures

Isolated organoids of epithelium enclosing a mesenchymal core were plated on collagen-coated plastic where they attached and spread within 48 hours. These colonies were heterogeneous, consisting mostly of epithelial monolayers with some smooth muscle-like mesenchymal cells (Evans et al., 1992). Mesenchymal cells overgrew the epithelial colonies in 5% and 10% foetal calf serum but at 2.5% FCS, their growth was restricted, resulting in sporadic epithelial colonies of a cobblestone morphology surrounded by mesenchymal cells (Fig. 1a and c). Epithelial cells were shown to express the enzyme alkaline phosphatase (Fig. 2a), an enzyme uniformly expressed throughout the epithelium lining the suckling rat small intestine. The fluid transport capacity of these cells was also indicated by the formation of ‘domes’ in areas of sub-epithelial fluid accumulation (Fig. 2b). Ultrastructural components typical of a polarised epithelium have been previously identified in these cultures by transmission electron microscopy e.g. desmosome junctions and well developed microvillus brush borders (Evans et al., 1992). The expression of alkaline phosphatase, and the formation of fluid-filled domes were also observed in cultures when heparin was added to the medium.

Effects of exogenous heparin

Exogenous heparin (derived from porcine intestinal mucosa) was applied to intestinal primary cultures (10-200 μg/ml) in the presence of 2.5% foetal calf serum. Heparin is a known inhibitor of a variety of cell types including smooth muscle cells (Karnovsky et al., 1989). In addition to the expected reduction in mesenchymal contamination, large homogeneous monolayers of tightly packed epithelial cells were observed after 10 days in culture (Fig. 1b and d). A reproducible, dose-dependent increase in proliferation (Fig. 3a) and epithelial cell number (Fig. 3b) was observed in the presence of heparin that was maximal at 50 μg/ml. Over a 14-day period in culture, this stimulation was continuous and statistically significant at $P<0.05$ (Fig. 4). However, in all experiments, a population of mesenchymal cells was constantly found in close association with epithelia (at the periphery of colonies or as small groups of cells lying underneath epithelial monolayers) even when retained in a quiescent state by the addition of maximal dose heparin (Fig. 2c).

Labelling indices determined by autoradiography (Fig. 1e and f) demonstrated a specific increase in epithelial proliferation (6.5% to 22.8%) in the presence of 50 μg/ml heparin compared to controls, while labelling of the mesenchyme was markedly reduced (11.6% to 3.5%). In addition, the action of heparin was not restricted to freshly plated organoids because the same effect was obtained when the cultures were grown for five days in 2.5 % FCS before the addition of heparin to the cultures.

Are heparin-binding factors in serum required for the effect of heparin on epithelium?

A number of polypeptide growth factors can be potentiated by interactions with heparin-like molecules. The presence of such factors in culture medium may be responsible for the stimulatory activities of heparin. FCS is a source of heparin-binding factors, however, the greatest stimulation of epithelial proliferation by heparin was seen in low serum concentrations (1% FCS). High concentrations of serum reversed the effects of heparin leading to reduced epithelial stimulation and increased mesenchymal growth (Fig. 5). In addition, the action of heparin on epithelia was not diminished by removal of heparin-binding components from serum by affinity chromatography (Table 1). Furthermore, when heparin-binding serum components were added to the medium, the stimulation of epithelial growth by

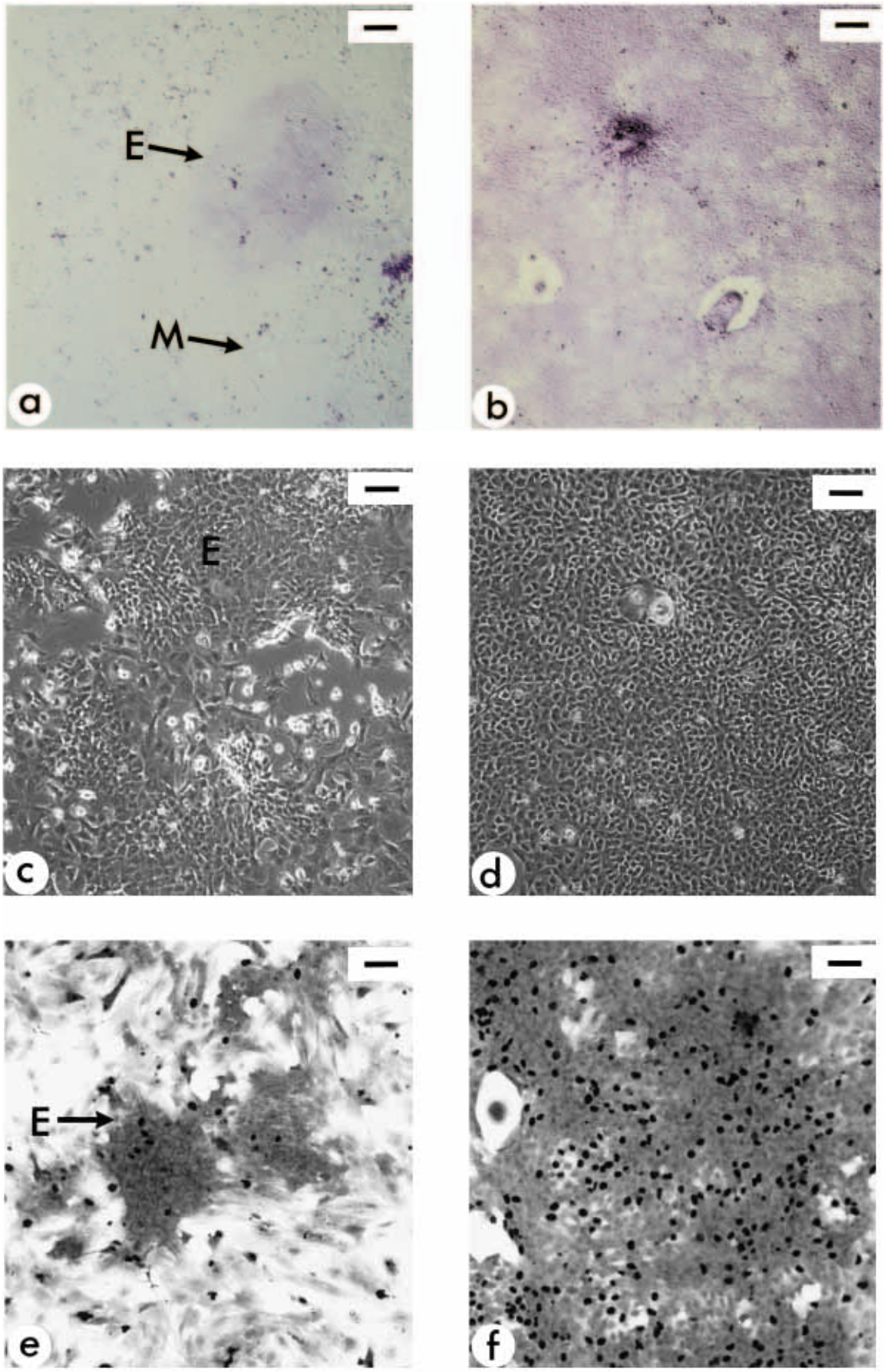

Fig. 1. Primary culture of rat small intestine after 10 days in the absence (a,c,e) or presence (b,d,f) of 50 μg/ml porcine mucosal heparin. Epithelial cells (E) grew as small colonies in the absence of, but as large monolayers in the presence of, heparin. (a and b) Cells stained with crystal violet and visualized under incident light microscopy. At low pH the crystal violet stains epithelial colonies whereas areas of mesenchymal cells (M) are weakly stained. Bars, 170 μm. (c and d) Higher magnification of primary cultures by phase microscopy. Bars, 60 μm. (e and f) Autoradiograph of cultures following incubation in tritiated thymidine. Cells undergoing DNA synthesis are characterised by black nuclei indicating incorporation of labelled thymidine. Bars, 60 μm.

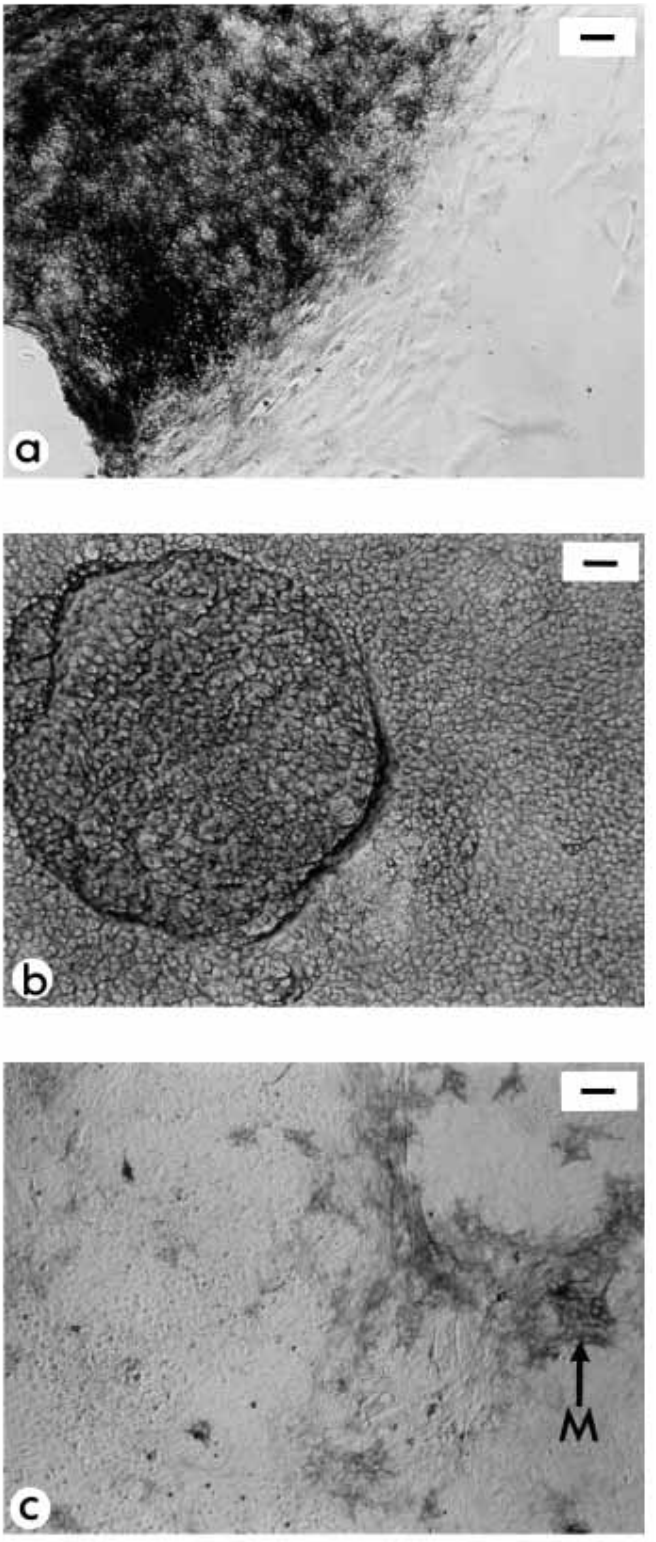

Fig. 2. Characterisation of cells in primary culture seven days after plating in the absence of heparin. (a) Histochemical demonstration of alkaline phosphatase activity in an epithelial colony surrounded by negatively stained mesenchymal cells. Bar, 60 μm. (b) Phase-contrast demonstration of dome formation in an epithelial monolayer. Bar, 50 μm. (c) Smooth muscle α-actin stained cultures demonstrating mesenchyme cells (M) at the edge of an epithelial colony and lying underneath the epithelial monolayer. Bar, 80 μm.

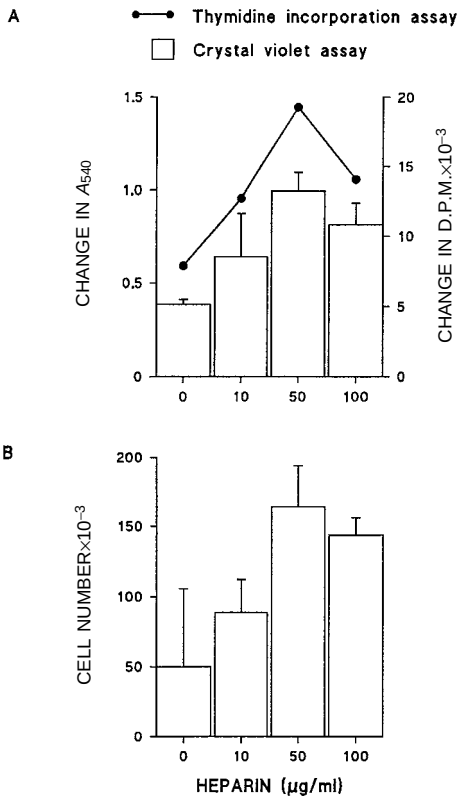

Fig. 3. Dose-responsive increase in epithelial proliferation in the presence of porcine heparin. Maximal stimulation demonstrated at 50 μg/ml. (A) Changes in crystal violet staining intensity and tritiated thymidine incorporation in primary intestinal cultures with increasing doses of porcine heparin. Data represent the change between cultures fixed 2 and 14 days after plating. dpm represents disintegrations per minute (B) Epithelial cell number in primary intestinal cultures with increasing doses of porcine heparin. Data represent direct cell scoring of epithelial cells in cultures 10 days after plating and show a direct, linear relationship to changes measured by crystal violet (correlation coefficient = 0.971) and thymidine incorporation (correlation coefficient = 0.965) assays in (A)

heparin was not significantly increased compared to normal serum. However, the requirement by mesenchyme for serum-derived growth factors was confirmed, since the growth of these cells in FCS depleted of heparin-binding proteins was poor. Under these poor growth conditions, the inhibitory effect of heparin on the mesenchymal cells was also less obvious (Table 1). These findings suggest that serum heparin-binding factors do not account for the growth stimulating effects of heparin on this epithelium, but they can stimulate growth of the mesenchymal cells.

Do mesenchymal cells in primary cultures produce any heparin-binding factors that stimulate selective growth of the epithelial cells?

The effect of heparin on stability, mitogenicity and diffusion of fibroblast growth factors has already been well documented (Vlodavsky et al., 1991). FGFs are produced in vitro by various mesenchymal cells (e.g. smooth muscle cells; Weich et al.,

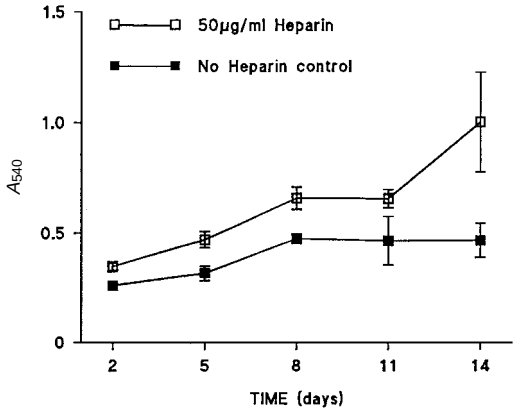

Fig. 4. Incremental growth curves of rat intestinal epithelial primary cultures in the presence or absence of porcine heparin (50 μg/ml). Data represent the intensity of crystal violet stain in cultures fixed at different times up to 14 days after plating. The difference between heparin treated and control cultures was analyzed by two way analysis of variance and was significant at $P<0.05$.

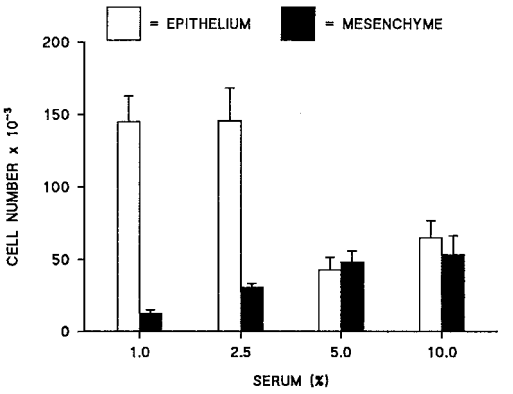

Fig. 5. The effect of heparin (50 μg/ml) on both epithelial stimulation and mesenchymal inhibition was reversed by increasing levels of FCS. Data represent direct cell counting of epithelium and mesenchyme 9 days after plating.

1990) and may therefore have a role in mediating the activity of heparin. However, when added alone, acidic and basic FGF (2-20 ng/ml) had little effect on proliferation of epithelial cells in primary culture. Furthermore, when 10 ng/ml aFGF and bFGF were added in the presence of heparin (10 and 50 μg/ml) the effect of heparin was reversed. Mesenchymal cell growth was enhanced and epithelial growth reduced with respect to control cultures with equivalent dose of heparin alone. An example of the effects for bFGF and heparin is shown in Fig. 6.

Can heparin stimulate the proliferation of established normal intestinal epithelial cell lines that grow in the absence of mesenchyme?

The effect of heparin on established cell lines derived from suckling rat small intestine was also investigated. This provided information on the requirement for epithelial-mesenchymal interactions in the stimulatory activity of heparin. Intestinal epithelial cell lines IEC-6, 17 and 18 were unresponsive to the stimulatory effect of heparin and in fact were slightly inhibited at higher doses (e.g. IEC-18; Fig. 7). However, these cells proliferate well in culture and do not require mesenchyme or extracellular matrix to do this. It would appear therefore that the stimulatory effect of heparin is restricted to freshly isolated epithelia that are dependent on mesenchyme for their proliferation in vitro. In a similar fashion to stromal cells in primary culture, an established smooth muscle-like line derived from suckling rat small intestine was inhibited (to a greater extent when compared to the IEC lines) in a dose-dependent manner by heparin (Fig. 7). This suggests that the effects of heparin on mesenchymal proliferation do not require the presence of, or interactions with, epithelial cells and may occur via different mechanisms.

Is there a cellular uptake of heparin in primary culture?

Cellular distributions of heparinoids using fluorescent dextran sulphate (Fig. 8b) and [^{3}H]heparin (Fig. 8c) were studied. Autoradiography was performed on cells that had been labelled with [^{3}H]heparin for a range of times up to 4 days in culture. Three hours after addition of labelled molecules, heparinoids were taken up into the cytoplasm (Fig. 8c) by most epithelial and mesenchymal cells in culture. With time, both tritiated and fluorescently tagged labels became localised around the nucleus (Fig. 8a,b) indicative of dynamic uptake and intracellular transport. This uptake was reduced by addition of excess unlabelled ligand suggesting a competitive mechanism. Similar patterns of distribution were found for both [^{3}H]heparin and fluorescent dextran sulphate suggesting that these molecules may share the same routes of entry into the cell. In addition, when cells were incubated in FITC-dextran

Table 1. The effect of heparin on epithelial and mesenchymal cells

Medium	Epithelial cells ($\times10^3$)			Mesenchymal cells ($\times10^3$)		
	No heparin	50 μg/ml heparin	% Change	No heparin	50 μg/ml heparin	% Change
1% FCS depleted of heparin binding proteins	3.2±0.7	13.8±2.5	331	2.8±0.18	2.5±0.22	–11
1% FCS	2.3±0.58	8.4±2.2	265	5.0±1.1	2.2±0.25	–56
1% FCS plus heparin binding proteins	2.4±0.95	9.5±0.68	296	5.8±0.68	2.1±0.24	–64

Heparin (50 μg/ml) was added to epithelial and mesenchymal cells in primary cultures when added either to FCS depleted of heparin binding proteins, or FCS supplemented with these heparin binding proteins. The data are presented as the mean (±s.e.m) number of epithelial and mesenchymal in the cultures 9 days after plating. The percentage change in cell numbers are also expressed in comparison to the same type of medium with no added heparin.

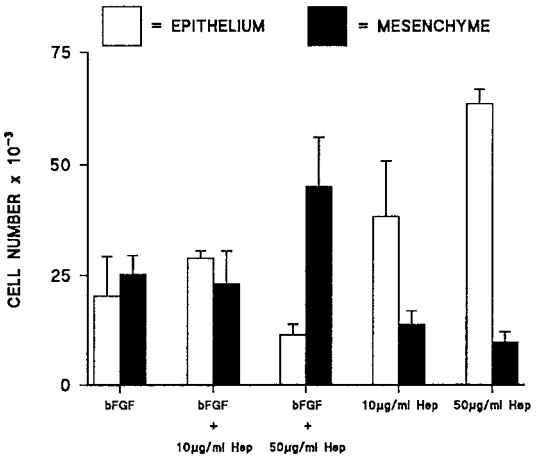

Fig. 6. The effect of heparin (10 and 50 µg/ml) on both epithelial stimulation and mesenchymal inhibition was reversed by 10 ng/ml bFGF. Data represents direct cell counting of epithelium and mesenchyme 9 days after plating.

as an unsulphated macromolecule, a different pattern uptake was observed suggesting that the above distributions were not due to 'cell drinking' phenomena.

What structural characteristics are important to the effects of heparin?

Previous studies (Wright et al., 1989) have shown the inhibitory effect of heparin on smooth muscle cell proliferation to be related to molecular size and charge endowed by the peripheral substitution of anionic molecules (e.g. N- or O-sulphation). Several molecules with differing size and charge were compared to porcine heparin (6-22.5 kDa) in primary culture at equivalent doses (Table 2). Heparins derived from intestinal mucosae of several different species (6-22.5 kDa) all stimulated growth in primary culture (data not shown), whereas a depolymerised derivative of bovine mucosal heparin (3 kDa) had no effect (Table 2). This suggested a size threshold

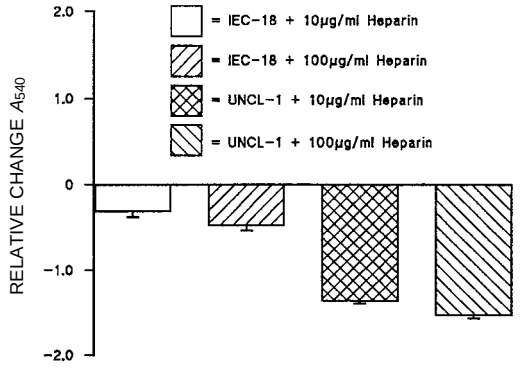

Fig. 7. Effects of porcine heparin (10 and 100 µg/ml) on the proliferation of epithelial (IEC-18) and mesenchymal (UNCL-1) cell lines derived from the suckling rat small intestine grown in 1% FCS. Data represent the change of crystal violet staining intensity in cultures fixed 1 and 4 days after plating and expressed relative to control (untreated) cultures.

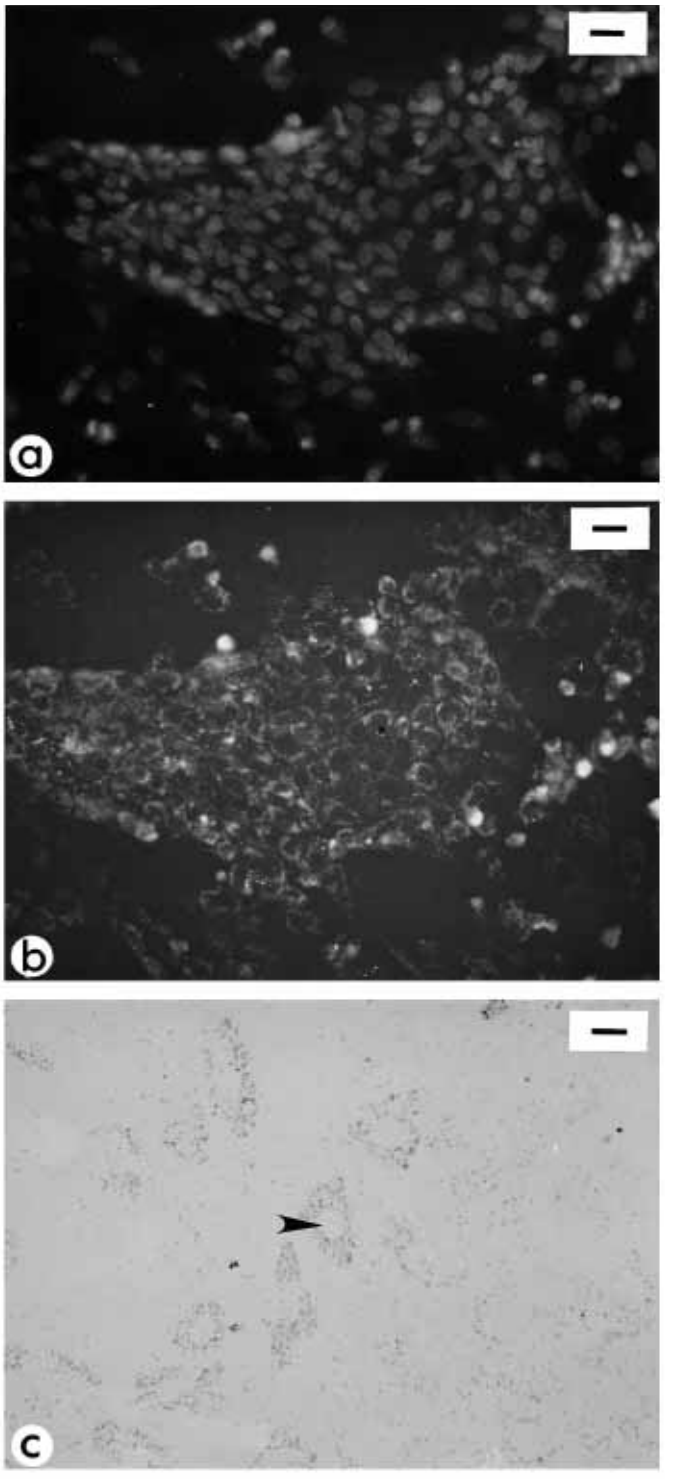

Fig. 8. Intracellular localisation of labelled heparinoids. (a,b) Primary cultures incubated in 10 µg/ml FITC-labelled dextran sulphate (for 48 hours) and counterstained with Hoescht dye 33258. The position of cells was then identified under UV illumination (a) and the perinuclear intracellular vesicles of labelled dextran sulphate under fluorescent microscopy (b). Bars, 30 µm. (c) Primary cultures incubated in 10 µg/ml [^{3}H]heparin (for 3 hours) and radioactive label visualised by autoradiography. The silver grains are shown overlying the cytoplasm, but the nuclei appear label free (arrow marks position of nucleus). Bar, 24 µm.

Table 2. A comparison of the effects of heparinoids (data shown at the 50 μg/ml concentration) with varying charge and sulphation in primary cultures of rat intestinal epithelium

Agent	Mean difference (±s.e.m)
Porcine heparin	0.33±0.04
Low M_r heparin	ND
De-*N*-sulphated heparin	ND
Dextran sulphate	0.39±0.01
Pentosan polysulphate	0.56±0.07
Protamine sulphate	ND

Protamine sulphate (a small basic protein with sulphate substitutions) was also included for comparison with the polyanionic heparinoids. The data represents differences in epithelial cellularity (measured by the binding of crystal violet at low pH) over a six day period. The differences are corrected with respect to control medium and described as ND where the growth was equal to, or less, than control medium.

below which the stimulatory activity is negated. When porcine heparin was modified by de-*N*-sulphation, the stimulatory activity of heparin was also compromised (Table 2). In addition, two structurally dissimilar, sulphated, polyanionic molecules (dextran sulphate and pentosan polysulphate; 8 kDa and 3 kDa, respectively) were shown to mimic the effects of heparin but, protamine sulphate (a small basic protein with sulphate substitutions; size not known) did not (Table 2). These data suggest that polyacidic sulphation of heparin is important for in vitro stimulatory activity.

Does heparin mimic endogenous proteoglycans?

The ability of other GAGS found in the cellular microenvironment to stimulate epithelial proliferation was determined. These molecules included heparan sulphate from porcine (av. 25 kDa) and bovine (size not known) intestinal mucosae, chondroitin-4 and -6 sulphates (av. 30 kDa and 40-80 kDa, respectively), dermatan sulphate (av. 35 kDa) and hyaluronic acid (50-2000 kDa). It should be noted that porcine and bovine heparan sulphates are structurally related to heparin and demonstrate a number of similar in vitro activities (Gallagher et al., 1986; Wright et al., 1988). However, of the glycosaminoglycans tested, only heparin stimulated significant epithelial proliferation (Table 3).

Table 3. A comparison of the effects of different glycosaminoglycan species (data shown at the 50 μg/ml concentration) in primary cultures of rat intestinal epithelium

Agent	Mean difference (±s.e.m)
Porcine heparin	0.33±0.04
Porcine heparan sulphate	0.12±0.02
Bovine heparan sulphate	ND
Chondroitin-4-sulphate	0.06±0.01
Dermatan sulphate	0.04±0.01
Chondroitin-6-sulphate	0.06±0.03
High M_r hyaluronic acid	ND
Low M_r hyaluronic acid	0.11±0.04

The data represents differences in epithelial cellularity (measured by the binding of crystal violet at low pH) over a six day period. The differences are corrected with respect to control medium and described as ND where the growth was equal to, or less, than control medium. The porcine heparin data is shown again for comparison.

DISCUSSION

Heparin has historically been used as an anti-coagulant compound but more recent in vitro work has identified other novel functions for this molecule. These include inhibition of cell proliferation of some epithelial (e.g. rat cervical epithelial cells; Wright et al., 1985) and mesenchymal (intestinal smooth muscle cells; Cochran et al., 1987) cell lines; activities that bear no relation to the anti-coagulant activity of heparin. It has been suggested that this inhibition is due to direct cellular mechanisms involving protein kinase C pathways (Castellot et al., 1989). Other actions of heparin include the potentiation of heparin-binding growth factors (Castellot et al., 1981; Schreiber et al., 1985), interactions with extracellular matrix molecules (Ingham et al., 1990), activation of L-type calcium channels (Knaus et al., 1990), uncoupling of G-proteins (Dasso and Taylor, 1991) and regulation of specific gene expression (Pukac et al., 1992).

In this report, heparin has been identified as a trophic agent for intestinal epithelium in primary culture. Heparin derived from intestinal mucosa of several species elicited a dose-dependent increase of epithelial proliferation and cell number. In addition it inhibited mesenchymal cells that were present in these cultures. Since, the inhibitory activities of heparin on other cell types have been widely reported, this trophic response of intestinal epithelia was an unusual phenomena and consequently, was studied further. In the presence of heparin, the epithelial cells also maintained the expression of alkaline phosphatase and fluid dome formation, suggesting that this agent does not inhibit differentiation. Indeed, when glucocorticoids have been added to the medium with heparin, a time-dependent increase in the expression of alkaline phosphatase (reaching a peak at 24 hours) has been observed (Flint and Evans, 1994).

Heparin consists of repeating disaccharide subunits composed of alternating hexosamine (GlcN) and hexuronic acid (GlcA) residues. The structure is further modified by *O*- and *N*-sulphation and by *N*-acetylation, thus heparin is a polyanionic charged molecule. Removing peripheral *N*-sulphate groups resulted in loss of the activity of heparin in these primary cultures. The importance of a polyacidic charge was also confirmed by the proliferative response of primary intestinal epithelia to dextran sulphate and pentosan polysulphate, two structurally unrelated polysulphated hexose polymers. For heparin, molecular size was also a determinant of activity as depolymerisation resulted in loss of activity. Similar structural motifs are required for heparin-mediated inhibition of proliferation in smooth muscle and cervical epithelial cells i.e. the activity is reduced by de-sulphation and de-polymerisation (Wright et al., 1989).

To investigate the mechanism(s) by which heparin stimulated epithelial proliferation, several direct and indirect modes of activity were examined. Previous work has shown that heparin-like molecules are taken up directly by some types of cells, and carried to a peri-nuclear (e.g. smooth muscle cells; Castellot et al., 1985a) or intra-nuclear location (e.g. hepatocytes; Ishihara et al., 1986). More recently this has been supported by evidence that heparin regulates several early response genes such as *c-fos* and *c-myc* (Pukac et al., 1992) and may act as a nuclear trans-repressor (Busch et al., 1992).

Uptake of labelled [^{3}H]heparin in primary intestinal cultures

resulted in observable label within both epithelial and mesenchymal cells, and was taken up into the cytoplasm and localised around the nucleus after a 24-hour period. This pattern was more obvious using FITC-labelled dextran sulphate, since in autoradiographs silver grains are scattered around the source of β emissions. Incubation with FITC-labelled dextran failed to produce similar distributions and competition with unlabelled heparin or dextran sulphate reduced labelling intensity. Therefore, the uptake of heparinoids into the cytoplasm appears to occur by specific competitive mechanisms, suggesting an intracellular site of action. However, at no time was labelled ligand observed in nuclei. Ion exchange of purified nuclear contents was used previously to identify nuclear heparan [S^{35}]sulphate (Ishihara et al., 1986). Therefore, the methods used to detect labelled heparinoids in this study may be too insensitive to detect low concentrations in nuclei and a nuclear site of action cannot be completely discounted. The use of specific second messenger pathway inhibitors may help to resolve the intracellular activities of heparin upon intestinal epithelial cells.

It is now recognised that indirect actions of heparin may be equally important in its effect on other cellular systems (Fager et al., 1992). Heparin-binding growth factors that enhance proliferation may require heparin-like molecules for receptor binding and their action can be potentiated by heparin (Ornitz et al., 1992). Therefore, heparin may interact with growth factors that act on epithelial cells. Although foetal calf serum is a potential source of heparin-binding molecules, the action of heparin on epithelium was found not to require serum heparin-binding components. However, mesenchymal cells present in primary culture may produce growth factors that interact with heparin (Rosen et al., 1989). We have previously suggested that mesenchymal factors are required for epithelial proliferation in primary cultures (Evans et al., 1992). In contrast, intestinal epithelial lines (IEC-6, 17 and 18) that grow independently of mesenchyme, were not stimulated but partly growth inhibited by heparin (Fig. 7). Inhibitory effects of heparin have also been observed with *homogenous* cultures of cervical keratinocytes (Wright et al., 1985). The requirement for mesenchymal support in these primary cultures, may be the critical factor in stimulation of epithelial proliferation by heparin.

Mesenchymal cells have been shown to produce several HBGFs such as FGFs (Klagsbrun, 1989), keratinocyte growth factor (Rubin et al., 1989) and hepatocyte growth factor (Montesano et al., 1991). In the case of FGFs, heparin potentiates their activity by increasing stability and enhancing ligand-receptor interactions (Damon et al., 1989; Yayon et al., 1991; Ornitz et al., 1992). In addition, intestinal epithelial lines have been shown to proliferate in response to FGFs (Cove and Evans, 1992). However, acidic and basic FGFs did not stimulate primary cultured epithelium when added either alone or in the presence of heparin. Therefore, interactions between exogenous heparin and mesenchyme-produced FGFs could not explain selective stimulation of epithelial proliferation in these cultures. Other types of mesenchyme-derived HBGFs have been shown to elicit epithelium-specific stimulation of proliferation and migration (Rubin et al., 1989; Nakamura, 1991). We have developed mesenchymal cell lines from suckling rat small intestine that express soluble heparin-binding factors and these selectively stimulate proliferation of intestinal epithelium in primary culture (Evans et al., 1993). The identification of these molecules and their possible interactions with heparin are currently under investigation.

Heparan sulphate (HS), a heparin-like molecule, has been located in the basement membrane along the crypt-villus axis (Laurie et al., 1983) and in underlying stromal matrix (Simon-Assmann et al., 1989). However, neither HS, nor any other ECM glycosaminoglycan, were clearly mitogenic in these primary cultures when tested at equivalent doses (0-100 μg/ml) to heparin. Although HS has a similar disaccharide sequence to that of heparin, the charge distribution is very different. In HS, sulphation occurs in clustered regions along the molecule (Gallagher et al., 1985). However in heparin, greater than 80% of the molecule is sulphated resulting in a far higher charge density. As sulphation was identified as a key structural motif for heparin activity, the heterogeneous sulphate distribution in HS may explain the low activity of this molecule in these primary cultures (Table 3).

However, a role for HSPGs in this context cannot be completely discounted. For example, intestinal HS is chemically modified during development (Bouziges et al., 1991), progression of colon carcinomas (Bouziges et al., 1990) and in vitro differentiation of colon carcinoma cell lines (Simon-Assmann et al., 1987; Levy et al., 1988). In addition, 'oversulphated' HS was isolated from pure populations of small intestinal epithelium (Levy et al., 1981). However, comparisons of HSPGs expressed around crypt and villus epithelium have not been made, although there are methods to purify these cell populations (Flint et al., 1991). It would be interesting if highly sulphated HSPGs were expressed within the crypt region of proliferating epithelial cells.

Alternatively, the observations made in this study may reflect a function of mucosal heparin in supporting reconstitution of this epithelium after damage. Tissue regeneration is an organised process involving both epithelium and mesenchyme (Mackenzie and Fusenig, 1983). This requires co-ordination of proliferation, migration and matrix deposition. In vitro studies have shown that heparin (and functionally related heparinoids) can regulate proliferation of epithelial and mesenchymal cells consistent with requirements for wound repair. This needs rapid restoration of the epithelial layer, accompanied by suppression of mesenchymal cell growth.

This idea is supported by other evidence that heparin (or heparin-like molecules) can limit tissue damage and aid repair, preventing vascular atherosclerosis (Karnovsky et al., 1989) and glomerulonephritis (Wright et al., 1988). Vascular smooth muscle or glomerular mesangial cells produce heparin-binding growth factors (Weich et al., 1990; Ishibashi et al., 1992) constitutively. These growth factors stimulate migration and proliferation of associated endothelial or epithelial cells (Castellot et al., 1981, 1985b). In addition, the action of these mesenchyme-derived growth factors is facilitated by heparin-like molecules produced by the overlying endothelial (or epithelial) cells. Furthermore, these heparin-like molecules can inhibit proliferation of the mesenchymal cells. Failure to regulate these processes can contribute to overgrowth of mesenchymal cells and the development of abnormal tissue structures (e.g. formation of atherosclerotic plaques).

The intestinal mucosa is constantly subject to physical and chemical damage, requiring an efficient and rapid repair process. According to the above model, damage to intestinal

mucosa resulting in the release of heparin (e.g. by mast cells), could lead to the inhibition of mesenchymal overgrowth and stimulation of epithelial regeneration. The activity of heparin in these primary cultures may therefore represent an in vitro model of intestinal wound repair requiring further investigation.

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