Characterisation of a laminarin sulphate which inhibits basic fibroblast growth factor binding and endothelial cell proliferation

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SUMMARY

We have evaluated a series of laminarin sulphates with different degrees of sulphation (0.3-2.3) as antagonists of basic fibroblast growth factor (bFGF) and as inhibitors of the bFGF-dependent endothelial cell line FBHE. Inhibition of binding of bFGF by the laminarin sulphates increased with increasing degree of sulphation. Binding of bFGF to low affinity sites on BHK cells was inhibited more strongly than binding to high affinity sites. IC50 values for inhibition of binding to low and high affinity sites by the most highly sulphated laminarin sulphate (LAM S5; degree of sulphation 2.31) were 12±8 μg/ml and 69±66 μg/ml, respectively. LAM S5 dissociated bFGF from low affinity sites on BHK cells but not from high affinity sites. LAM S5 increased the electrophoretic mobility of bFGF indicating that LAM S5 binds directly to bFGF. LAM S5 reduced uptake of bFGF by FBHE cells by 67%. Increasing the degree of sulphation of laminarin sulphates increased the inhibition of bFGF-stimulated DNA synthesis of the endothelial cell line FBHE (IC50 for LAM S5 approx. 1 μg/ml). There was no inhibition of DNA synthesis of FBHE cells by LAM S5 in the presence of 1 μg/ml bFGF indicating that bFGF antagonism is involved in the anti-proliferative activity of this compound. LAM S5 may be of value against diseases associated with bFGF-dependent cell proliferation.

Key words: laminarin sulphate, basic fibroblast growth factor, endothelial

INTRODUCTION

Basic fibroblast growth factor (bFGF) belongs to a family of heparin-binding growth factors, presently comprising nine members (Burgess and Maciag, 1989; Miyamoto et al., 1993). FGFs are mitogenic for a wide range of cell types and also modulate a number of non-mitogenic cell functions such as angiogenesis and wound repair (Baird and Bohlen, 1991). Abnormal production of bFGF is implicated in the pathology of some cancers either by acting as a mitogen for tumour cells or by enhancing tumour neo-vascularisation. Evidence for this involvement includes: (1) human glioblastoma cells grown as xenografts in nude mice are inhibited by an antibody against bFGF (Takahashi et al., 1991); (2) the mouse tumour M5076 is inhibited by an anti-bFGF antibody (Hori et al., 1991); (3) expression of bFGF correlates with tumour stage in human pancreatic cancer (Yamanaka et al., 1993); (4) bFGF synergises with HIV-1 Tat in the induction of Kaposi’s sarcoma (Ensoli et al., 1994). Antagonists of bFGF should therefore be useful in the therapy of cancer and other pathological conditions in which this growth factor is implicated.

Basic FGF binds to both high affinity receptors with tyrosine kinase activity, and to cell surface heparan sulphates which represent low affinity binding sites (see Givol and Yayon, 1992, for recent review). Binding of bFGF to heparan sulphate or heparin was reported to be necessary for subsequent binding to high affinity receptors (Rapraeger et al., 1991; Yayon et al., 1991), although one report demonstrates that high affinity binding can occur in the absence of heparin (Roghani et al., 1994). Heparin-like molecules are, however, necessary for the induction of a biological response by high affinity receptors (Rapraeger et al., 1991; Yayon et al., 1991). Heparin-like molecules may contribute to activation of receptors by bFGF in a number of ways. Heparan sulphates may deliver bFGF in an active conformation to high affinity receptors (Rapraeger et al., 1991; Yayon et al., 1991). It has also been reported that heparin binds directly to FGF receptors indicating that a trimeric complex of heparan sulphate, tyrosine kinase receptor and bFGF may be formed (Kan et al., 1993). Heparin binds many molecules of bFGF and it has been suggested that this is necessary for receptor dimerization and activation as was shown for acidic FGF (Spivak-Kroizman et al., 1994).

One heparan sulphate which potentiates binding of bFGF to high affinity receptors and subsequent biological events is the proteoglycan perlecan (Aviezer et al., 1994a). Several other heparan sulphate proteoglycans, such as syndecan and fibrocollagen, inhibit the restoration of bFGF receptor binding induced by heparin (Aviezer et al., 1994b). Thus, blocking the normal interaction between bFGF and heparin or heparan sulphate is one way to prevent growth stimulation by bFGF. Several small fragments (6-12mer) of heparin have been identified which inhibit both bFGF binding and bFGF-stimulated...
proliferation of adrenocortical endothelial cells (Ishihara et al., 1993; Tyrrell et al., 1993). By contrast, some oligosaccharides >10mer enhance bFGF binding to its high affinity receptor (Ishihara et al., 1993). Both 2-O-sulphate and the negative charge of the carboxy group on L-iduronic acid residues were required for modulation of bFGF activity. Other groups have also shown that fragments with the greatest affinity for bFGF consist of N-sulphated glucosamine units enriched with idurionate-2-sulphate groups (Habuchi et al., 1992; Turnbull et al., 1992). Overall, these results indicate that both charge and size are important determinants of the potency of heparin fragments as bFGF antagonists (Ishihara et al., 1994).

Alternative sources of heparinoids are naturally occurring polysulphated carbohydrates, such as the bacteria-derived sulphated polysaccharide DS-4152 (Nakayama et al., 1993) and carrageenans (Hoffman, 1993), or chemically sulphated glycans, such as pentosan polysulphate. These compounds all selectively antagonise bFGF binding and FGF-dependent proliferation of endothelial cells (Nakayama et al., 1993; Hoffman et al., 1995; Zugmaier et al., 1992). A series of laminarin sulphates with different degrees of sulphation (DS) can be produced by the controlled sulphation of laminarin, a β(1,3)glucan of about 6 kDa (Alban et al., 1992). In this report we have evaluated laminarin sulphates as bFGF antagonists and as anti-proliferative agents. We have also examined the relationship between DS and anti-coagulant activity since anti-coagulant activity is a problem associated with the clinical use of some polysulphated carbohydrates (Pluda et al., 1993).

**MATERIALS AND METHODS**

**Materials**
Laminarin was from Senn (Dielsdorf, Switzerland). Pullulan standards were from Magery-Nagel (Düren, Germany). High molecular weight heparin (H8514; porcine, sodium salt) and pentosan polysulphate (PPS) were from Sigma (Munich, Germany or Poole, UK). [3H]Thymidine and [125I]bFGF were from Amersham International plc (Aylesbury, UK). Unlabelled bFGF was from Bachem (Saffron Walden, UK). Tissue culture materials were from Gibco UK.

**Laminarin sulphates**
Laminarin sulphates were prepared according to Alban et al. (1992) from laminarin. The laminarin used in this study had approximately 10% branching at C-6 and an average degree of polymerization of 35. Briefly, 500 mg laminarin was suspended in 2.5 ml freshly distilled and dried N,N-dimethylformide (DMF) and stirred for 40 minutes at room temperature, then 2 mol pyridine per mol sulphation reagent and dried N,N-dimethylformide (DMF) and stirred for 40 minutes at 80°C. The room temperature, then 2 mol pyridine per mol sulphation reagent and dried N,N-dimethylformide (DMF) and stirred for 40 minutes at 80°C.

**bFGF dissociation**
Cells were grown to confluence in gelatinised 24-well plates and incubated in DMEM containing 0.15% gelatin and laminarin sulphate. The laminarin sulphate was added to the culture plates to a final concentration of 100 μg/ml. After 24 hours, the cells were washed twice with ice-cold PBS and then incubated in DMEM containing 0.15% gelatin, 5 ng/ml [125I]bFGF and laminarin sulphate for 2 hours at 4°C with gentle rocking. The cells were then washed 3 times with PBS at room temperature (1 ml/wash), bFGF bound to low affinity receptors was eluted by 2 washes with 2 M NaCl in 20 mM Hepes, pH 7.5 (1 ml/wash), and bFGF bound to high affinity receptors was eluted by 2 washes with 2 M NaCl in 20 mM sodium acetate, pH 4 (1 ml/wash). During our evaluation of this assay, we found that bFGF binds to gelatinised wells, and some bFGF, resistant to washing with PBS, is eluted by the washes with 2 M NaCl in 20 mM Hepes, pH 7.5. Consequently, cells were only used when confluent for the binding assays in order to avoid binding of bFGF to the culture plates.

**Agarose gel electrophoresis**
The effect of LAM S5 on the electrophoretic mobility of [125I]bFGF was examined by electrophoresis using 1% agarose gels (Isogel; FMC.
Bioproducts Corp., US) in 50 mM Tris-acetate buffer, pH 7.4 (McCaffrey et al., 1989). LAM S5 was incubated with 5 ng/ml $^{125}$I-bFGF in DMEM/0.15% gelatin, pH 7.4, at 4°C for 1 hour. Samples containing loading buffer (6.7% sucrose, 0.04% bromophenol blue) were electrophoresed (150 V/30 mA) on minigels (Mini-Protean II, Bio-Rad, UK) for 2 hours at 4°C. Gels were fixed in 10% trichloroacetic acid for 1 hour, washed free of acid in water and exposed overnight to Fuji RX X-ray film with intensifying screens.

bFGF uptake

bFGF internalisation was determined as described (Roghani and Moscatelli, 1992). Cells were grown to confluence in gelatinised 6-well plates, washed twice with PBS and incubated in DMEM containing 0.15% gelatin and 5 ng/ml $^{125}$I-bFGF and additions as required for 1 hour at 37°C with gentle rocking. After this time, the cells were washed 3 times with PBS, twice with 2 M NaCl in 20 mM Hepes, pH 7.5, and twice with 2 M NaCl in 20 mM sodium acetate, pH 4. Internalised bFGF was released by solubilising the cells in 1 ml 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1. Parallel experiments were carried out at 4°C and specific uptake of bFGF was calculated as uptake at 37°C minus uptake at 4°C.

DNA synthesis

FBHE cells were seeded in 96-well plates at $2\times10^3$ cells/well in DMEM/10% FCS/10 ng/ml bFGF. Following an overnight incubation, the cells were washed twice with DMEM and incubated in bFGF-free medium for 24 hours. The cells were then incubated in fresh medium containing laminarin sulphate in the presence or absence of 10 ng/ml bFGF. DNA synthesis was determined 24 hours later by measuring incorporation of a 6 hour pulse of $^3$H-thymidine ($0.5 \mu$Ci/well) into TCA insoluble material (Dealtry and Balkwill, 1987).

Proliferation of FBHE cells

FBHE cells were plated out at $10^3$ cells/well in DMEM/10% FCS/10 ng/ml bFGF in 96-well plates. Following an overnight incubation, the cells were washed with bFGF-free medium and incubated with laminarin sulphate in medium in the presence or absence of bFGF. Medium was replaced daily and the number of viable adherent cells was determined by trypan blue exclusion using a haemocytometer.

RESULTS

Laminarin sulphates

The characteristics of the laminarin sulphates produced in our syntheses are shown in Table 1. Anti-coagulant activity, as determined by an APTT test or a TT test, increased with increasing degree of sulphation (DS) up to a DS of 1.98 (LAM S4). The most sulphated laminarin derivative, LAM S5, with a DS of 2.31 was less anti-coagulant than LAM S4. Corresponding values in the APTT test for heparin and PPS were 147 and 32.7 USP-U/mg.

bFGF binding

The effect of the laminarin sulphates on bFGF binding was initially examined using BHK cells. These cells express large numbers of high affinity bFGF receptors relative to other normal diploid cells (Neufeld and Gospodarowicz, 1985). It was demonstrated (Moscatelli, 1988) that high and low affinity bFGF binding sites on BHK cells ($K_d$ values 18.4 pM and 2,000 pM, respectively) can be distinguished by eluting bFGF from low affinity, heparin-like, sites with 2 M NaCl, pH 7.5, and from high affinity, bFGF saturable, sites with 2 M NaCl, pH 4. These observations were confirmed in the present study. Thus, binding of $^{125}$I-bFGF to sites elutable with 2 M NaCl, pH 7.5, was reduced by >90% with 100 µg/ml heparin whereas binding was reduced by <5% by 1 µg/ml (55 nM) unlabelled bFGF (27-fold the $K_d$ for low affinity sites). By contrast, binding of $^{125}$I-bFGF to sites elutable with 2 M NaCl, pH 4, was reduced by >85% by 1 µg/ml bFGF (3,000-fold the $K_d$ for the high affinity sites) (data not shown).

Concentrations of laminarin sulphates required to cause 50% inhibition of binding (IC50 values) to low and high affinity sites on BHK cells are summarised in Table 2. Inhibition of bFGF binding to low and high affinity sites by the laminarin sulphates increased with increasing DS, although differences between the three most highly sulphated laminarin sulphates for inhibition of binding to low affinity sites were not statistically significant. IC50 values for inhibition of binding to high affinity sites were 5-20-fold lower than for low affinity sites. Laminarin, the non-sulphated starting material, was the exception to the general relationship between DS and inhibition of bFGF binding. This compound showed some inhibitory

Table 1. Characterisation of laminarin sulphates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Degree of sulphation</th>
<th>$M_r$</th>
<th>$M_r$†</th>
<th>APTT (USP-U/mg)</th>
<th>TT (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminarin</td>
<td>0</td>
<td>-</td>
<td>5,750</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LAM S1</td>
<td>0.3</td>
<td>6,595</td>
<td>10,500</td>
<td>0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>LAM S2</td>
<td>0.64</td>
<td>7,565</td>
<td>15,850</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>LAM S3</td>
<td>1.3</td>
<td>9,485</td>
<td>17,400</td>
<td>19.2</td>
<td>3.1</td>
</tr>
<tr>
<td>LAM S4</td>
<td>1.98</td>
<td>11,400</td>
<td>20,000</td>
<td>36.2</td>
<td>14</td>
</tr>
<tr>
<td>LAM S5</td>
<td>2.31</td>
<td>12,310</td>
<td>20,000</td>
<td>26.2</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* Determined conductimetrically.
† Determined by gel permeation chromatography.

Table 2. Inhibition of bFGF binding to BHK cells by laminarin sulphates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Low affinity sites</th>
<th>High affinity sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminarin</td>
<td>90±88 (n=3)</td>
<td>225±106</td>
</tr>
<tr>
<td>LAM S1</td>
<td>&gt;1,000 (n=2)</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>LAM S2</td>
<td>&gt;1,000 (n=2)</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>LAM S3</td>
<td>24±12 (n=3)</td>
<td>522±109</td>
</tr>
<tr>
<td>LAM S4</td>
<td>16±4 (n=4)</td>
<td>175±35</td>
</tr>
<tr>
<td>LAM S5</td>
<td>12±8 (n=3)</td>
<td>69±66</td>
</tr>
</tbody>
</table>

* Mean ± s.d. from n determinations.
activity against bFGF binding to both low and high affinity receptors (Table 2).

Inhibition of bFGF binding by the most promising bFGF antagonist, LAM S5, was also evaluated using the endothelial cell line FBHE. In preliminary experiments, binding of bFGF to these cells was characterised by sequentially washing off pre-bound bFGF (3 hours at 4°C) with 1-4 M NaCl, pH 7.5, followed by 1-4 M NaCl, pH 4. >98% of bFGF bound to these cells was eluted by 2 M NaCl, pH 7.5. 1 μg/ml bFGF did not reduce the bound bFGF which could be eluted at pH 7.5, whereas heparin was a potent inhibitor of this binding (data not shown). This indicates that, as for BHK cells, the bFGF eluted by 2 M NaCl, pH 7.5, represents binding to low affinity sites. Due to the low level of bFGF which remained bound to FBHE cells after washing with 2 M NaCl, pH 7.5, it was only possible to examine binding to low affinity sites on these cells. Inhibition of this binding by LAM S5 was comparable to inhibition of bFGF binding to low affinity sites on BHK cells (Fig. 1).

**bFGF dissociation**

Dissociation of bFGF was examined after pre-binding [125I]bFGF to cells. A 10 minute incubation with LAM S5 resulted in maximum bFGF dissociation from BHK cells (data not shown). LAM S5 dissociated bFGF in a dose-dependent manner from low affinity sites on BHK cells but not from high affinity sites (Fig. 2a). No bFGF was released from high affinity sites following a 1 hour incubation (data not shown). Heparin also dissociated bFGF from low affinity sites on BHK cells but not from high affinity sites and was slightly more potent than LAM S5 (Fig. 2b). LAM S5 also dissociated bFGF from low affinity sites on FBHE cells at concentrations similar to those which caused dissociation from low affinity sites on BHK cells (compare Fig. 2c with a). As with BHK cells, heparin was more effective than LAM S5 at dissociating bFGF from FBHE cells (Fig. 2c). 100 μg/ml LAM S5 dissociated 76% of total bFGF bound to FBHE cells and 34% of total bFGF bound to BHK cells.

**bFGF/LAM S5 complex**

Changes in the electrophoretic mobility of [125I]bFGF under non-denaturing conditions were used to examine the ability of LAM S5 to directly interact with bFGF. Results are shown in Fig. 3. [125I]bFGF remained at the origin after electrophoresis for 2 hours but migration of [125I]bFGF towards the anode increased when [125I]bFGF was pre-incubated with 100 μg/ml LAM S5. Heparin pre-incubation dramatically increased the migration of [125I]bFGF. In the presence of heparin one band of [125I]bFGF ran with an electrophoretic mobility which was similar to that of bromophenol blue (off the bottom of the gel.

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Fig. 1. Inhibition of bFGF binding by LAM S5. Cells were incubated with LAM S5 and 5 ng/ml [125I] bFGF for 2 hours and the amount of ligand bound to low affinity sites on BHK cells (○), high affinity sites on BHK cells (■) and low affinity sites on FBHE cells (□) was determined.

Fig. 2. Dissociation of pre-bound bFGF by LAM S5. (a,b) BHK cells pre-incubated with bFGF were incubated with LAM S5 (a) or heparin (b) for 10 minutes and the amount of bFGF dissociated (□) and the amounts remaining bound to low (○) and high (△) affinity sites were determined. (c) FBHE cells were pre-incubated with bFGF and bFGF dissociated by LAM S5 (□) or heparin (■) after 10 minutes was determined. Values are from duplicate determinations.
shown in Fig. 3). Laminarin did not significantly increase the electrophoretic mobility of [125I]bFGF.

bFGF internalisation
The effects of LAM S5 on bFGF internalisation are shown in Fig. 4. Specific internalisation of 5 ng/ml [125I]bFGF by BHK cells during a 1 hour incubation at 37°C was reduced by 34% by 100 μg/ml LAM S5 and by 53% by 100 μg/ml heparin (Fig. 4a). The presence of 1 μg/ml unlabelled bFGF reduced internalisation by 72% (Fig. 4a). A combination of 1 μg/ml bFGF and LAM S5 caused 87% inhibition of internalisation (not shown). In contrast to the results with BHK cells, internalisation of bFGF by FBHE cells was inhibited to a greater extent by LAM S5 and heparin than by 1 μg/ml bFGF (Fig. 4b).

FBHE cell proliferation
FBHE cells were used to evaluate the anti-proliferative activities of the laminarin sulphates since it has been reported that low densities of these cells require an exogenous source of bFGF in order to proliferate (Gospodarowicz et al., 1976). Incubating FBHE cells in medium containing bFGF following a 24 hour period in bFGF-free medium resulted in a 7-fold stimulation of DNA synthesis (Fig. 5). Inhibition of the bFGF-stimulated DNA synthesis by the laminarin sulphates increased with increasing DS (Fig. 5). Heparin did not inhibit bFGF-stimulated DNA synthesis (data not shown).

The inhibitory activity of LAM S5 against FBHE DNA synthesis was confirmed in cell proliferation assays. Daily treatment with 10 μg/ml LAM S5 significantly reduced the proliferation of cells grown in the presence of bFGF and also inhibited the low level of proliferation of cells grown in medium not supplemented with bFGF (Fig. 6a). Under these conditions LAM S5 reduced cell proliferation with an IC50 similar to that for inhibition of DNA synthesis (approx. 1 μg/ml) (Fig. 6b).

DISCUSSION

In this study we have identified a highly sulphated laminarin sulphate, LAM S5, which inhibits bFGF binding, dissociates pre-bound bFGF from low affinity sites, inhibits internalisation of bFGF by cells and inhibits the proliferation of the bFGF-dependent endothelial line FBHE.

bFGF antagonism is probably involved in the anti-proliferative action of LAM S5 since a high concentration of bFGF (1 μg/ml) almost completely prevented inhibition of DNA synthesis by LAM S5 (Fig. 7). This suggests that bFGF antagonism is necessary for the inhibition of DNA synthesis by LAM S5.
Binding of LAM S5 to the heparin-binding site on bFGF could account for the ability of LAM S5 to reduce the binding of bFGF to low affinity heparan sulphate sites. In order to inhibit cell proliferation, LAM S5 must prevent activation of high affinity receptors by bFGF, since it is these receptors which are primarily responsible for the induction of a mitogenic response (Bernard et al., 1991). However, LAM S5 was much weaker as an inhibitor of bFGF binding to high affinity sites than as an inhibitor of FBHE proliferation. Several possible mechanisms could allow binding of bFGF to high affinity receptors without subsequent activation. Firstly, it is possible that when LAM S5 binds bFGF it induces a conformational change in bFGF which only weakly reduces its ability to bind to high affinity receptors but more effectively prevents bFGF from initiating a growth response by these receptors. bFGF undergoes a conformational change when bound to heparin or heparan sulphate, and this change is probably necessary for bFGF to activate high affinity receptors (Prestrelski et al., 1992; Yayon et al., 1991). Alternatively, LAM S5 may bind bFGF and present bFGF to high affinity receptors but prevent dimerization and activation of the receptors in a similar manner to the heparin analogue sucrose octasulphate which prevents receptor activation by binding aFGF monomerically and preventing the dimerization necessary for receptor activation (Spivak-Kroizman et al., 1994).

It has been suggested recently that uptake of bFGF via low affinity sites may contribute to the mitogenic activity of bFGF (Rusnati et al., 1993). bFGF can be internalised via both low and high affinity sites on endothelial cells and other cell types (Roghani and Moscatelli, 1992; Rusnati et al., 1993). Some intracellular bFGF in bovine aortic endothelial cells is translocated to the nucleus in a cell cycle-dependent manner where it stimulates the transcription of ribosomal genes (Bouche et al., 1987; Baldin et al., 1990). Intracellular bFGF may be stabilised by complexing with heparan sulphate and so these complexes could be involved in the mitogenic activity of bFGF (Rusnati et al., 1993). In view of these observations, we investigated whether inhibition of bFGF internalisation may be involved in the anti-proliferative activity of LAM S5. Since heparin was a more potent inhibitor of internalisation than LAM S5 but did not inhibit bFGF dependent DNA synthesis, it would appear that modulation of internalisation per se cannot explain the relative anti-proliferative activities of these two agents. However, further work is required to evaluate whether heparin and LAM S5 have different effects on the fate of intracellular bFGF. A substantial amount of bFGF internalisation by FBHE cells during a 1 hour incubation probably occurs via low affinity receptors since the presence of a high concentration of unlabelled bFGF (1 μg/ml) had less of an effect on reducing internalisation of [125I]bFGF than heparin. The reduction by 56% of bFGF internalisation by 100 μg/ml heparin in our experiments was similar to the reduction in internalisation by 10 μg/ml heparin in bovine aortic endothelial cells after a 1 hour incubation reported by Roghani and Moscatelli (1992).
using similar experimental conditions. Internalisation of bFGF by BHK cells was inhibited to a greater extent by 1 μg/ml bFGF than by either LAM S5 or heparin. This suggests that there is a higher proportion of uptake via high affinity sites by BHK cells than by FBHE cells during the first hour of incubation. This may reflect a far higher ratio of high affinity receptors to low affinity receptors on BHK cells than on the endothelial cells (Moscatelli, 1987).

Although low densities of FBHE cells have been reported to require an exogenous source of bFGF in order to proliferate (Gospodarowicz et al., 1976), we noted a low level of proliferation, under our experimental conditions, in the absence of exogenously added bFGF. The independence of these cells from exogenous bFGF increases with increasing cell density (unpublished observations). These observations suggest that FBHE cells may be able to produce bFGF in an autocrine manner as has been shown for other endothelial cells (Schweigerer et al., 1987). bFGF produced by endothelial cells remains cell-associated (Vlodavsky et al., 1987) indicating that dissociation of bFGF may be necessary in order to inhibit autocrine stimulation by this growth factor. LAM S5 rapidly dissociated bFGF from low affinity sites but not from high affinity sites (on BHK cells). However, the proliferation of FBHE cells in the absence of exogenous bFGF was inhibited by LAM S5 suggesting that dissociation of bFGF from low affinity sites may be sufficient to cause some inhibition of autocrine growth.

Although the low sulphated laminarin sulphates LAM S1 and LAM S2 were less active than LAM S5 as inhibitors of bFGF binding, laminarin, the non-sulphated starting material, did cause some inhibition of bFGF binding. The CH₂OH group at C-6 on laminarin is the major site sulphated on LAM S1 and LAM S2 (Alban et al., 1992). Thus a single CH₂OH group appears to contribute more to inhibition of bFGF binding than a single sulphate group. Following sulphation on C-6, the hydroxyl groups at C-2 and C-4 become sulphated and LAM S3, S4 and S5 are sulphated at positions C-2 and C-4 as well as at C-6 (Alban et al., 1992). This sulphation increases the potency of laminarin sulphates as bFGF antagonists.

We determined the anti-coagulant activity of the laminarin sulphates in view of their structural similarities with heparin. Heparin exerts its anti-coagulant activity by interacting with anti-thrombin III. This interaction occurs via highly specific complementary sequences on the two molecules (Lindahl et al., 1984). In the case of aFGF, it has been shown that the ability of heparin fractions to potentiate the activity of this growth factor is independent of binding to antithrombin III (Foschini et al., 1989). bFGF antagonism and anti-coagulant activity can also be dissociated in laminarin sulphates since LAM S5 was as potent an antagonist of bFGF as LAM S4 but had less anti-coagulant activity. Based on the APTT and TT values (Forth and Rummel, 1992), the maximum plasma level of LAM S5 which can be achieved without significantly affecting blood coagulation is 9.5 μg/ml. Corresponding values for heparin and pentosan polysulphate are 1.5 μg/ml and 7.5 μg/ml, respectively.

In conclusion, we have identified LAM S5 as an inhibitor of bFGF-dependent DNA synthesis of FBHE cells. Over-production of bFGF is implicated in neo-vascularisation which occurs in a number of pathological disorders including cancer (Folkman, 1992). LAM S5 has potential in vivo use as an inhibitor of bFGF-dependent angiogenesis since the concentration required to inhibit FBHE cell proliferation is lower than the concentration which is likely to affect blood coagulation.

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