Inhibition of angiogenesis and murine tumour growth by laminarin sulphate

R Hoffman[1,*], DH Paper[2], J Donaldson[1] and H Vogl[2]

[1]Clinical Oncology and Radiotherapeutics Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, UK; [2]Department of Pharmacy, University of Regensburg, 93040 Regensburg, Germany.

Summary LAM S5 is a polysulphated derivative of the glucan laminarin that inhibits basic fibroblast growth factor (bFGF) binding and the bFGF-stimulated proliferation of fetal bovine heart endothelial (FBHE) cells. This report demonstrates that LAM S5 has anti-angiogenic activity, as shown by inhibition of tubule formation by endothelial cells cultured on Matrigel and inhibition of vascularisation of the chick chorioallantoic membrane. In addition, LAM S5 caused a tumour growth delay of the murine RIF-1 tumour of 2.6 days ($P = 0.01$).

Keywords: angiogenesis; laminarin sulphate; anti-tumour

Inhibition of tumour neovascularisation, or angiogenesis, is a promising new strategy for the treatment of cancer (see Scott and Harris, 1994 for recent review). Several polysulphated carbohydrates, such as pentosan polysulphate (PPS), chitin derivatives and the bacteria-derived DS-4152, are anti-angiogenic and inhibit tumour growth in animal models (Zugmaier et al., 1992; Murata et al., 1991; Tanaka et al., 1989). At least some of the anti-tumour activities of these compounds can be attributed to inhibition of heparin-binding angiogenic growth factors produced by tumour cells (Zugmaier et al., 1992; Nakayama et al., 1993). We have recently identified a highly sulphated derivative of the β-1,3-glucan laminarin, designated LAM S5 (molecular weight 12 kDa), which inhibits basic fibroblast growth factor (bFGF) binding and the bFGF-stimulated proliferation of fetal bovine heart endothelial (FBHE) cells (Hoffman et al., 1995a). bFGF is a potent angiogenic factor and is present at elevated levels in the urine of some cancer patients (Nguyen et al., 1994). Elevated expression of bFGF is associated with a poor prognosis for patients with pancreatic cancer (Yamanaka et al., 1993). In this study we have evaluated the anti-angiogenic and anti-tumour activities of LAM S5.

Materials and methods

Reagents

LAM S5 was prepared according to Alban et al. (1992) from laminarin (Senn, Dielsdorf, Switzerland). The laminarin had approximately 10% branching at C-6 and an average degree of polymerisation of 35. Sulphation was carried out by continuous addition of SO_3/pyridine complex dissolved in N,N-dimethylformamide for 4 h at 80°C. Sulphate content of LAM S5 was determined by conductivity (Casu and Gennaro, 1975). The molecular weight of LAM S5 was determined by conductivity (Casu and Gennaro, 1975) and by gel permeation chromatography on a fast protein liquid chromatography (FPLC) system (Pharmacia) with a Superose 12 column.

Matrigel was from Stratech Scientific Ltd (Bedfordshire, UK). Tissue culture materials, excluding fetal calf serum (FCS), were from Gibco BRL (Paisley, UK). Serum, growth factors and other reagents were from Sigma (Poole, UK).

Tissue culture

Fetal bovine heart endothelial (FBHE) cells were grown in gelatinised flasks (0.1% gelatin for 2 h) in Dulbecco's Modified Eagle medium (DMEM) 10% FCS, and were supplied with bFGF (10 ng ml^{-1} every other day). The human microvascular endothelial cell line CDC/EU.HMEC-1 (HMEC-1) (Ades et al., 1992) was kindly supplied by Dr E Ades (Atlanta, GA, USA). HMEC-1 cells were cultured in MCDB 131 medium supplemented with 10% FCS, 10 ng ml^{-1} epidermal growth factor (EGF) and 1 µg ml^{-1} hydrocortisone. RIF-1 (radiation-induced fibrosarcoma) cells were maintained according to the protocol of Twentyman et al. (1980). All media contained antibiotics (100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin).

Endothelial cell tubule formation

Endothelial cells (10^4 cells/well) were suspended in medium and added to Matrigel (37 µl/well) which had been allowed to gel in 96-well plates. Test agent was added and the cells were incubated at 37°C. Cells were examined 12 h later. Experiments were performed in triplicate.

Chorioallantoic membrane (CAM) assay

A modification of a method for the shell-less cultivation of chick embryo was used (Jakobson et al., 1989). Fertilised hen eggs were incubated for 3 days (37°C, 80% humidity). The eggs were then cracked into plastic cradles and 3 days later test compound (in an agarose pellet) was added. The CAMs were examined after a further 1 day. Pellets surrounded by zones of inhibition of vascularisation were scored as positive for inhibition of angiogenesis.

In vitro inhibition of RIF-1 cells

RIF-1 cells were plated down overnight in 96-well plates (10^3 per well) and treated with LAM S5. Cell numbers were determined by an MTT assay 4 days later (Hoffman et al., 1995b).

In vivo inhibition of RIF-1 cells

RIF-1 cells (4×10^5) were injected subcutaneously on the right flank of male C3H/Km mice (day 0). LAM S5 in 0.1 ml of saline was injected intravenously commencing on day 1 (13 mg kg^{-1}; daily 5 days week^{-1}). Tetrahydrocortisol (THC) was administered intraperitoneally commencing on day 1 using a tapering dose of 250 mg kg^{-1} for 4 days, 100 mg kg^{-1} for 3 days, 50 mg kg^{-1} for 4 days and

1 mg kg^{-1} thereafter. Melphalan (formulated according to Honess and Bleehen, 1985) was injected as a single dose (5 mg kg^{-1}) intraperitoneally on day 3. Tumours were measured in three dimensions (a, b, c) and tumour volumes were calculated according to the formula

$$\frac{abc \times \pi}{6}$$

Tumour growth delays, defined as the time in days for treated tumours to reach 300 mm^3 compared with saline controls, were determined from the geometric means of individual tumour growth times and P-values were estimated for a two-tailed t-test. All experiments were carried out in compliance with the UKCCCR guidelines on welfare of animals in research (1988).

Results and discussion

The structure of LAM S5 used in this study is shown in Figure 1. The LAM S5 had a degree of sulphation (average number of sulphates per sugar residue) of 2.31 and a molecular weight determined by conductivity (Casu and Gennaro, 1975) of approximately 12 500 da. The molecular weight determined by gel permeation chromatography was approximately 20 000 da, but this is probably an over-estimate owing to effects of the hydrodynamic volume of LAM S5.

Anti-angiogenic activity of LAM S5 was first evaluated by examining the effect on tubule formation by endothelial cells cultured on the basement membrane material Matrigel. This procedure is an *in vitro* model, which reproduces some of the features of angiogenesis. However, the early steps of angiogenesis–degradation of existing basement membrane, migration through the stromal space and proliferation–are not represented by Matrigel tubule model. Rather, this model represents the later stages of angiogenesis including cell migration and tubule formation. Inhibition of tubule formation by endothelial cells on Matrigel is frequently used to identify agents which may have anti-angiogenic activity (Scott and Harris, 1994; Candal *et al.*, 1994). Migration of the human microvascular endothelial line HMEC-1 was evident 1–2 h after plating on Matrigel, and networks of tubules formed by 8–12 h (Figure 2a). LAM S5 inhibited tubule formation in a dose-dependent manner: 3 μg ml^{-1} LAM S5 prevented the formation of complete networks of tubules and 30 μg ml^{-1} LAM S5 prevented most of the tubule formation, although some cells still migrated and formed clumps (Figure 2b and c). Similar results were obtained with the bovine macrovascular endothelial line FBHE (data not shown).

The anti-angiogenic activity of LAM S5 was further evaluated in the chick chorioallantoic membrane (CAM) assay. This assay, initially developed by Folkman (1975) to study tumour-induced angiogenesis, is now widely used as an

assay for inhibitors of angiogenesis (Folkman and Klagsbrun, 1987). Pellets (10 μg) of LAM S5 inhibited CAM formation on 80% of the eggs (Table I). Pellets (50 μg) of LAM S5
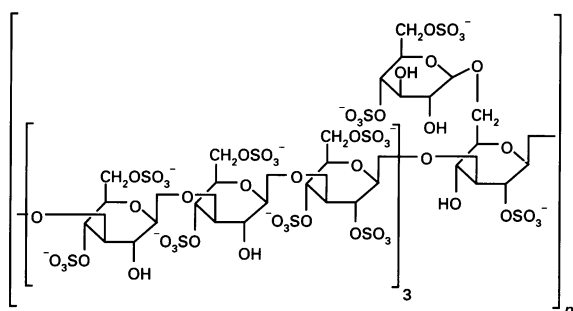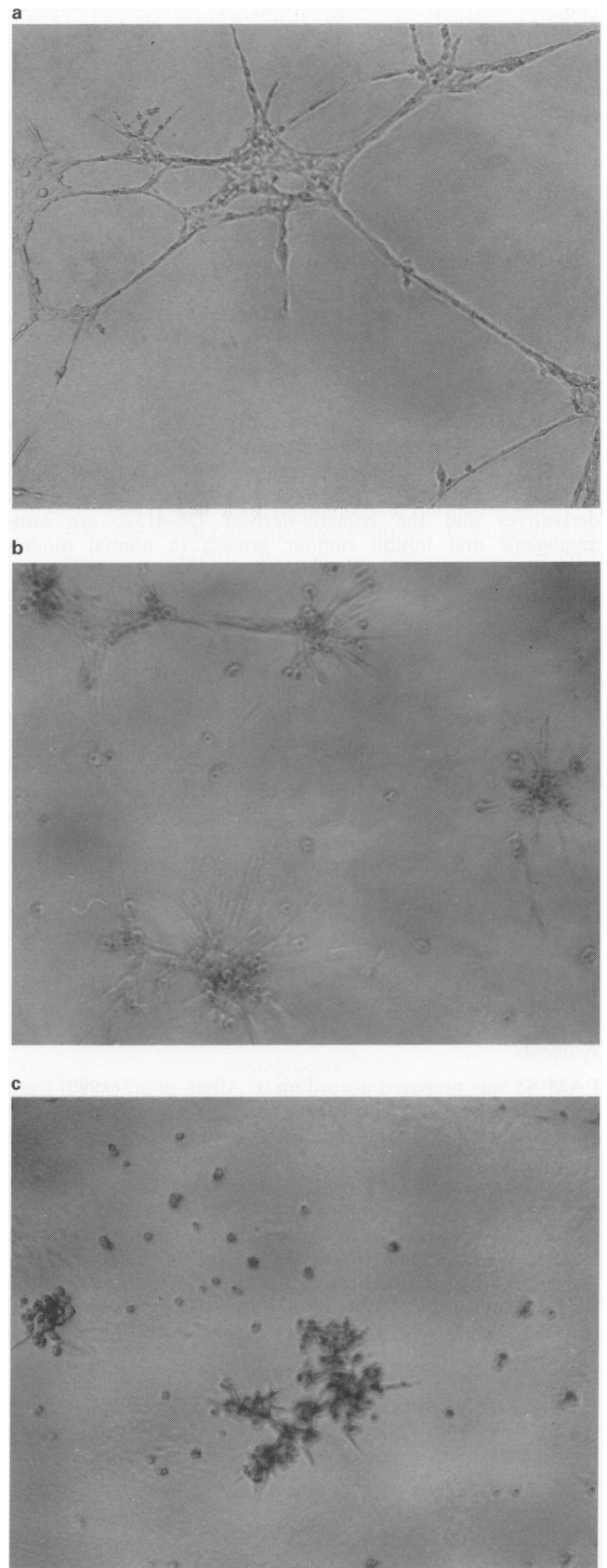

Figure 2 Inhibition of tubule formation by HMEC-1 cells by LAM S5. (a) Control; (b) 3 μg ml^{-1} LAM S5; (c) 30 μg ml^{-1} LAM S5. Cells were plated on Matrigel and observed 12 h later.

Figure 1 Structure of LAM S5.

resulted in inhibition of angiogenesis on all the eggs tested, but this concentration of LAM S5 was associated with increased toxicity to the chick embryos (Table I). Suramin, which has anti-angiogenic activity (Pesenti et al., 1992) and was used as a positive control in our studies, had less anti-angiogenic activity than LAM S5 (Table I).

Several polysulphated carbohydrates with anti-angiogenic activity have also been shown to inhibit tumour growth in animal models (see introduction). LAM S5 was not well tolerated in mice when administered intraperitoneally and caused haemorrhagic deaths. The anticoagulant activity of LAM S5 is probably due to the structural similarity of this compound with heparin. Anticoagulant activity was the dose-limiting toxicity in a recently reported phase I trial with the polysulphated carbohydrate PPS (Pluda et al., 1993). We have estimated, based on the activated partial thromboplastin time (APTT) test, that a maximum plasma concentration of 9.5 μg ml^{-1} LAM S5 is achievable before there is a significant effect on the coagulation system (Hoffman et al., 1995a). LAM S5 was better tolerated when given intravenously. The maximum tolerated dose of LAM S5 given by this route was about 13 mg kg^{-1} on a schedule of 5 × daily week^{-1}. We used this regimen to evaluate the anti-tumour activity of LAM S5 against the murine tumour RIF-1. In the first experiment, LAM S5 was administered alone and in combination with the corticosteroid tetrahydrocortisol (THC). Corticosteroids combined with heparin were shown by Folkman to have anti-angiogenic and anti-tumour activity (Folkman et al., 1983; Crum et al., 1985), and subsequent studies have shown that the anti-tumour activity of some anti-angiogenic heparin-like molecules is enhanced by corticosteroids (Tanaka et al., 1989; Thorpe et al., 1993). LAM S5 alone produced a statistically significant growth delay of RIF-1 tumours relative to control values of about 3 days (Figure 3a and Table II). A combination of THC with LAM S5 produced a growth delay of about 5 days, although this increase in growth delay was not statistically significant relative to LAM S5 alone (Figure 3a and Table II). THC alone had no effect on RIF-1 tumour growth. None of the mice receiving LAM S5, THC or the combination experienced any toxicities or had any significant weight loss (data not shown). THC has no glucocorticoid or mineralocorticoid

activities and thus avoids the toxicity problems of some corticosteroids (unpublished observations; Penhaligon and Camplejohn, 1985).

Anti-angiogenic agents have been reported to potentiate the anti-tumour activities of some cytotoxic drugs (Teicher et al., 1992). Therefore, we studied the effect of LAM S5 in combination with the alkylating agent melphalan. Melphalan has previously been shown to inhibit RIF-1 tumours (Honess and Bleehen, 1985). LAM S5 administered intravenously
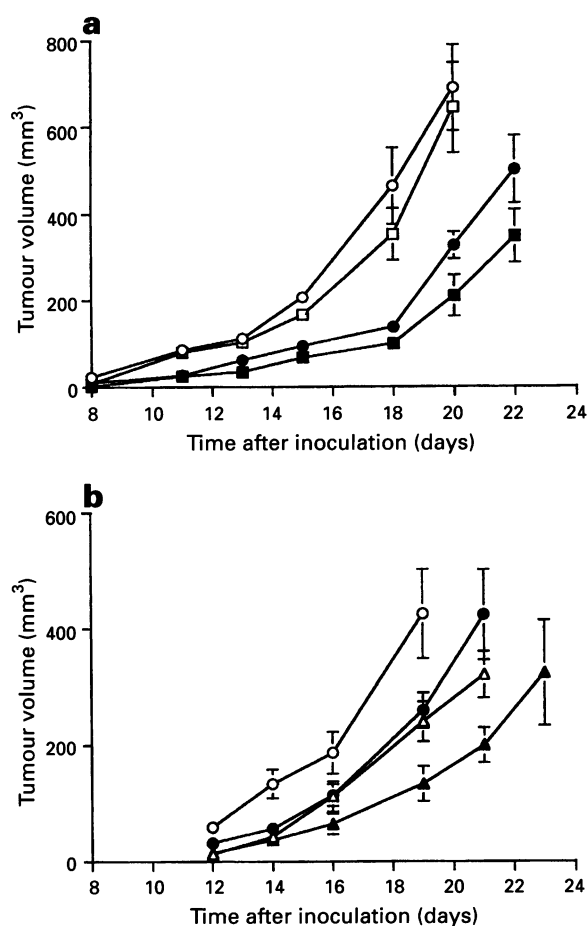
Figure 3 Anti-tumour activity of LAM S5 against the RIF-1 murine tumour. (a) LAM S5 (13 mg kg^{-1}; daily 5 days week^{-1}) (●) or tetrahydrocortisol (tapering dose of 250 mg kg^{-1} to 1 mg kg^{-1}) (□) were administered alone or in combination (■). (b) LAM S5 (13 mg kg^{-1}; daily 5 days week^{-1}) (●) or melphalan (5 mg kg^{-1}; single dose) (△) were administered alone or in combination (▲). Further details in Materials and methods. (○), saline control. Bars represent s.e.

Table I Anti-angiogenic activity of LAM S5 in the chorioallantoic membrane assay

Treatment	Anti-angiogenic effect		Toxicity[1]	
LAM S5 10 μg/pellet	8/10	(80%)	1/11	(9%)
LAM S5 50 μg/pellet	6/6	(100%)	4/10	(40%)
Suramin 50 μg/pellet	6/17	(35%)	3/25	(12%)
Agarose control	1/23	(4%)	2/25	(8%)

[1] Death of the chick embryo

Table II Growth delay of the RIF-1 tumour by LAM S5 alone and combined with tetrahydrocortisol or melphalan

Treatment	Growth delay[a] (geometric mean and ranges on mean)		P value (test vs control)
Experiment 1			
LAM S5 (n = 5)	3.3	(2.2–3.5)	0.002
Tetrahydrocortisol (n = 5)	0.7	(–0.5–2.6)	0.41
LAM S5/tetrahydrocortisol (n = 5)	4.8	(2.3–7.7)	0.005
Experiment 2			
LAM S5 (n = 5)	2.0	(0–4.3)	0.20
Melphalan (n = 7)	2.2	(0.5–4.1)	0.13
LAM S5/melphalan (n = 7)	6.1	(2.5–10.5)	0.01
Experiments 1 and 2			
LAM S5 (n = 10)	2.6	(1.5–3.9)	0.01

[a] Time in days for treated tumours to reach 300 mm^3 compared with saline controls.

(13 mg kg^{-1}; daily 5 days week^{-1}) and a single dose of melphalan administered intraperitoneally (5 mg kg^{-1}) both produced tumour growth delays of about 2 days, but these values are not statistically significant relative to controls (Figure 3b and Table II). A combination of melphalan and LAM S5 resulted in a growth delay of 6 days and this was statistically significant relative to control values (Figure 3b and Table II). No toxicity or weight loss was observed in mice treated with the combination of melphalan and LAM S5. However, two of seven mice died early on (days 7 and 14) in the LAM S5 group. Post-mortems revealed haemorrhaging in the peritoneal cavities.

In summary, these results demonstrate that the poly-sulphated glucan LAM S5 inhibits angiogenesis and has anti-tumour activity against the RIF-1 tumour. The overall tumour growth delay by LAM S5 is 2.6 days ($P = 0.01$) when data from the two in vivo experiments are pooled (Table II). Combining LAM S5 with a corticosteroid or a cytotoxic agent resulted in a slight increase in anti-tumour activity. At present we have not established if the anti-tumour activity of LAM S5 is due to inhibition of tumour

neovascularisation or to a more direct effect on RIF-1 cells. However, endothelial cells are inhibited at a lower concentration of LAM S5 than RIF-1 cells since the IC$_{50}$ value for inhibition of the proliferation of the endothelial line FBHE is 1 μg ml^{-1} LAM S5 (Hoffman et al., 1995a), and 3 μg ml^{-1} LAM S5 starts to inhibit tubule formation of endothelial cells (present results), whereas the proliferation of RIF-1 cells in vitro is inhibited with an IC$_{50}$ of 30 μg ml^{-1} (data not shown). The activity of LAM S5 against other tumours remains to be evaluated. LAM S5 represents a useful lead molecule for developing lower molecular weight derivatives as it is structually a simple polysaccharide composed only of glucose with mainly β-1,3 linkages between the sugars.

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