RESEARCH COMMUNICATION

Carrageenans inhibit growth-factor binding

Richard HOFFMAN

Clinical Oncology and Radiotherapeutics Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

Carrageenans, a family of polysulphated carbohydrates, inhibited binding of basic fibroblast growth factor (bFGF), transforming growth factor β 1 (TGF β 1) and platelet-derived growth factor (PDGF). ι -Carrageenan was the most potent bFGF antagonist ($IC_{50} = 0.4 \pm 0.1 \mu\text{g/ml}$), κ -carrageenan was the most potent PDGF antagonist ($IC_{50} = 1.7 \pm 1.3 \mu\text{g/ml}$) and λ -carrageenan

was the most potent TGF β 1 antagonist ($IC_{50} = 19 \pm 2 \mu\text{g/ml}$). None of the carrageenans, at concentrations up to $200 \mu\text{g/ml}$, inhibited binding of insulin-like growth factor 1 or transforming growth factor α . Carrageenans are selective growth-factor antagonists and have potential for the treatment of disorders associated with the over-production of certain growth factors.

INTRODUCTION

Polypeptide growth factors are multifunctional agents affecting a wide variety of cellular functions. Although growth factors represent attractive targets for drug development, only a few antagonists have so far been identified. One promising group of growth-factor antagonists are polysulphates. Suramin is the best characterized of these compounds and has been shown to inhibit the binding of platelet-derived growth factor (PDGF) (Williams et al., 1984), basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF β) (Coffey et al., 1987), and, to a lesser extent, of insulin-like growth factor 1 (IGF1) (Pollak and Richard, 1990), transforming growth factor α (TGF α) (Hoffman and Cameron, 1992) and epidermal growth factor (EGF) (Coffey et al., 1987). Suramin has shown some clinical activity, but this is frequently associated with significant toxicity (Myers et al., 1992). The polysulphated polysaccharide heparin and related heparin-like molecules modulate the activities of acidic fibroblast growth factor (aFGF) and bFGF (reviewed by Klagsburn, 1990). The activities of FGFs are also modulated by some synthetic polysulphated polysaccharides including dextran sulphate (Tardieu et al., 1992) and pentosan polysulphate, which inhibited Kaposi's-sarcoma-derived growth factor and was active in animal models (Wellstein et al., 1991).

The carrageenans are a family of sulphated polysaccharides isolated from red algae. The backbone structure of carrageenans consists of the repeating disaccharide unit (1 $\rightarrow$ 4)- β -D-galactopyranosyl-(1 $\rightarrow$ 3)- α -D-galactopyranosyl, with 3,6-anhydrogalactose residues commonly present. Carrageenans differ in their patterns and degree of sulphation, and this has marked effects on their physico-chemical properties (Rees et al., 1982). Carrageenans are extensively used in the food industry, and while this is not associated with any acute toxicity, carrageenans can exert a number of physiological effects including modulation of immune function (Vijayakumar and Muthukkaruppan, 1990), anti-viral activity (Girond et al., 1991) and anti-metastatic activity (Coombe et al., 1987). In this report, the effects of carrageenans on the binding of a range of growth factors has been evaluated.

MATERIALS AND METHODS

Carrageenans

All carrageenans were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Carrageenans were made up fresh before each experiment at a concentration of 2 mg/ml in 25 mM Hepes, pH 7.4, heated to 60 °C. The essential features of the carrageenans used in this study are shown in Table 1.

Chemicals

Suramin was a kind gift from Bayer (Newbury, Berks., U.K.). ^{125}I -labelled bFGF, ^{125}I -TGF α and ^{125}I -IGF1 were purchased from Amersham International (Aylesbury, Bucks., U.K.). ^{125}I -PDGF (β -chain homodimer) and ^{125}I -TGF β 1 were purchased from NEN [DuPont (U.K.) Ltd., Stevenage, Herts., U.K.]. Unlabelled growth factors were purchased from Bachem UK (Saffron Walden, Essex, U.K.). All other chemicals were from Sigma.

Cell lines

A431, baby hamster kidney (BHK) clone 13 and Swiss 3T3 cells were purchased from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, Hants., U.K. A431 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/10% (v/v) foetal calf serum (FCS), BHK cells were maintained in MEM/10% (v/v) FCS/5% (w/v) tryptose phosphate broth, and Swiss 3T3 cells were maintained in DMEM/10% (v/v) newborn calf serum (NBCS). L23/P cells (a large-cell adenocarcinoma) were maintained in RPMI/10% (v/v) FCS. All cell lines received antibiotics (100 units/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin).

Growth-factor-binding assays

bFGF

Binding of bFGF to BHK cells was determined by a slight

Table 1 Properties of carrageenans

Properties	Carrageenans		
	κ	ι	λ
Source	<i>Eucheuma cottonii</i>	<i>Eucheuma spinosa</i>	<i>Gigartina aciculata</i> and <i>Gigartina pistillata</i>
Sigma catalogue no.	C 1263	C 4014	C 3889
Ester sulphate (%)	25–30	28–35	32–39
3,6-Anhydro-D-galactose (%)	28–35	0	30
Solubility in cold water	Na ⁺ salt soluble	Na ⁺ salt soluble	All salts soluble
Gelation	Gels most strongly with K ⁺	Gels most strongly with Ca ²⁺	Non-gelling

modification of the method of Moscatelli (1987). BHK cells were plated out in DMEM/10% (v/v) FCS at 1×10^5 cells/well (1 ml/well) in 24-well plates and left for 2 days until they were > 90% confluent. Longer periods of growth resulted in significant loss of cells during subsequent washing steps. The cells were incubated in DMEM containing 0.15% gelatin at 37 °C for 2 h and then washed twice with ice-cold PBS. Binding experiments were carried out in binding buffer (bicarbonate-free DMEM/0.15% gelatin/25 mM Hepes, pH 7.4) containing 0.1 nM 125 I-bFGF at 4 °C for 3 h on a reciprocal shaker. Cells were then washed three times with ice-cold PBS (1 ml per wash). Total binding was determined by measuring ligand released after a 10 min extraction with 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1 (0.2 ml).

PDGF

Binding of PDGF to Swiss 3T3 cells was essentially as described by Bowen-Pope and Ross (1985). Briefly, cells were plated out at 1×10^5 cells/well in 24-well plates, grown to confluence (4 to 5 days) and washed once in rinse buffer (PBS, pH 7.4, containing 1 mM CaCl₂ and 0.1% BSA). The cells were incubated with 40 pM 125 I-PDGF and test substance, or an excess of unlabelled PDGF in binding buffer (bicarbonate-free DMEM/25 mM Hepes, pH 7.4/0.25% BSA) for 3 h at 4 °C on a reciprocal shaker. Cells were washed three times with rinse buffer, solubilized with 1% (v/v) Triton X-100/0.1% BSA in distilled water (0.3 ml/well) and the radioactivity of the solubilisate was counted with a gamma counter.

TGF β 1

Binding of TGF β 1 to Swiss 3T3 cells was determined by the method of Wakefield (1987). Briefly, cells were plated out at 5×10^4 cells/well in 24-well plates and grown for 2 days until approx. 70% confluent. Longer growth periods were avoided, since it has been reported (Wakefield, 1987) that the affinity of the TGF β receptor decreases in quiescent cells. Cells were washed with binding buffer (bicarbonate-free DMEM/0.1% BSA/25 mM Hepes, pH 7.4) and incubated with 20 pM 125 I-TGF β 1 and test compound, or an excess of unlabelled ligand for 2 h at room temperature. Cells were then washed with ice-cold binding buffer and solubilized with 1% (v/v) Triton X-100/10% (v/v) glycerol/25 mM Hepes, pH 7.4 (400 μ l/well). The radioactivity of the solubilisate was counted with a gamma counter.

IGF1

L23/P cells were plated out at 1×10^5 cells/well in 24-well plates and grown to 90% confluency (24 h). Cells were washed once with binding buffer (bicarbonate-free DMEM/0.1% BSA/

25 mM Hepes, pH 7.4) and incubated with binding buffer containing 0.1 nM 125 I-IGF1 and test substance, or excess unlabelled ligand for 90 min at 4 °C. Cells were washed four times with ice-cold binding buffer, solubilized with 1 M NaOH and the radioactivity of the solubilisate was counted with a gamma counter.

TGF α

A431 cells were plated out at 4×10^4 cells/well in 96-well microtitre plates and allowed to grow to 90% confluency (24 h). Medium was flicked off and the cells were washed twice (150 μ l/wash) with ice-cold binding buffer (bicarbonate-free DMEM containing 0.1% BSA and 25 mM Hepes, pH 7.4). Test compound was incubated with 1 nM 125 I-TGF α in binding buffer (100 μ l/well) for 1 h at 4 °C. Non-specific binding was determined in the presence of a 500-fold excess of unlabelled ligand. Cells were washed rapidly three times with ice-cold binding buffer and solubilized with 1 M NaOH. Radioactivity of the solubilisate was counted with a gamma counter.

Growth-factor-binding experiments were performed three times with duplicate determinations in each experiment.

RESULTS

The activities of the carrageenans as growth-factor antagonists were investigated by examining their abilities to modulate binding of radiolabelled growth factors to cells. Carrageenans were mixed with ligand just before addition to the cells. Pre-incubation of carrageenans with ligand for periods of up to 2 h did not affect subsequent inhibition of growth-factor binding (results not shown). All of the carrageenans tested inhibited binding of bFGF to BHK cells, with ι -carrageenan being the most potent inhibitor. The results of a typical experiment are shown in Figure 1. The results are expressed in terms of inhibition of total binding rather than specific binding (total minus non-specific), since the carrageenans were able to reduce binding to below non-specific binding values. (Non-specific binding represented approx. 45% of total binding as determined using 50 nM bFGF, a 500-fold excess over labelled bFGF.) BHK cells express both low- and high-affinity receptors (Moscatelli, 1987) and we have found that the low-affinity sites are not saturated even in the presence of 1 μ M unlabelled bFGF (R. Hoffman, unpublished work). Thus the apparent high level of non-specific binding probably represents binding of bFGF to the low-affinity binding sites, which are not all blocked by 50 nM unlabelled bFGF.

The carrageenans inhibited the binding of several other growth factors, but binding was never reduced to values below those determined in the presence of a 500-fold excess of unlabelled ligand. Inhibition of specific binding of PDGF to Swiss 3T3 cells

Figure 1 Inhibition of bFGF binding to BHK cells

Binding of 0.1 nM 125 I-bFGF to BHK cells in the presence of ι -carrageenan (●), κ -carrageenan (■) or λ -carrageenan (▲) was determined after a 3 h incubation at 4 °C.

Figure 2 Inhibition of PDGF binding to Swiss 3T3 cells

Binding of 40 pM 125 I-PDGF to Swiss 3T3 cells in the presence of carrageenans was determined after a 3 h incubation at 4 °C. Symbols are as described in the legend of Figure 1.

is shown in Figure 2. Interestingly, the order of potency was different from that for inhibition of bFGF binding. For inhibition of PDGF binding, κ -carrageenan and λ -carrageenan were more potent antagonists than ι -carrageenan.

The effects of carrageenans on specific binding of TGF β 1 were examined using Swiss 3T3 cells. The activity of the carrageenans as TGF β 1 antagonists was significantly weaker than the activity of these compounds as inhibitors of bFGF or PDGF binding (Figure 3).

The effects of carrageenans on specific binding of IGF1 were examined using L23/P cells. Non-specific binding was 10% of total binding. λ -Carrageenan, at concentrations up to 250 μ g/ml, did not significantly inhibit specific binding of IGF1 to L23/P cells (Figure 4). ι -Carrageenan and κ -carrageenan at 200 μ g/ml did not inhibit binding of IGF1 (results not shown). Suramin, a known antagonist of IGF1 binding (Pollack and Richard, 1990), was used as a positive control in this experiment and caused 100% inhibition of IGF1 binding at a final concentration of 200 μ g/ml (results not shown). ι -Carrageenan at concentrations up to 200 μ g/ml had no effect on TGF α binding to A431 cells (Figure 4). (Non-specific binding was 12%.) Neither λ -carrageenan nor κ -carrageenan at 200 μ g/ml inhibited TGF α binding (results not shown). Suramin was used as a positive control in this experiment (Hoffman and Cameron, 1992) and inhibited binding of TGF α with an IC_{50} value of 200 μ g/ml.

A summary of IC_{50} -value determinations is shown in Table 2.

Figure 3 Effects of carrageenans on TGF β 1 binding to Swiss 3T3 cells

Binding of 20 pM 125 I-TGF β 1 to Swiss 3T3 cells in the presence of carrageenans was determined after a 2 h incubation at room temperature. Symbols are as described in the legend of Figure 1.

Figure 4 Effects of carrageenans on IGF1 and TGF α binding

Binding of 0.1 nM 125 I-IGF1 to L23/P cells in the presence of λ -carrageenan was determined after 90 min at 4 °C. Binding of 1 nM 125 I-TGF α to A431 cells in the presence of ι -carrageenan was determined after 1 h at 4 °C. Symbols are as described in the legend of Figure 1.

Table 2 Inhibition of growth-factor binding by carrageenans

Values represent mean concentrations $\pm$ S.D. ($n = 3$) required to reduce specific binding (for PDGF, TGF β 1, IGF1, TGF α) or total binding (for bFGF) to 50% of control values. See the Materials and methods section for full details.

Growth factor	IC_{50} value (μ g/ml)		
	ι -Carrageenan	κ -Carrageenan	λ -Carrageenan
bFGF	0.4 ± 0.1	7.7 ± 2.5	2.4 ± 1.0
PDGF	11.7 ± 5.5	1.7 ± 1.3	2.3 ± 1.4
TGF β 1	36 ± 18	102 ± 42	19 ± 2
IGF1	> 200	> 200	> 200
TGF α	> 200	> 200	> 200

DISCUSSION

The carrageenans represent a new group of polysulphated carbohydrates which antagonize the binding of some growth factors. All three types of carrageenans examined in this study were similar in as much as they all showed some activity as

inhibitors of the binding of bFGF, PDGF and TGF β 1 and did not show activity as inhibitors of the binding of IGF1 or TGF α . For the most potent carrageenan, there is a difference in IC₅₀ values of at least 600-fold between values for the most strongly and most weakly inhibited growth factor. This difference in IC₅₀ values is significantly greater than for suramin, which inhibits all of these growth factors with an approx. 20-fold difference between IC₅₀ values for the most potently inhibited growth factors, such as bFGF, and the most weakly inhibited, such as EGF (Coffey et al., 1987). Thus the carrageenans show more discrimination as inhibitors of growth-factor binding than does suramin.

The precise mechanism by which polysulphates inhibit the interaction between growth factors and their receptors has not been determined. The ability of suramin to inhibit the interaction between PDGF and its receptor appears to be due to binding of suramin to PDGF rather than the PDGF receptor (Hosang, 1985). FGFs characteristically bind to heparin, and the structural similarity of polysulphated carbohydrates with heparin suggests that polysulphated carbohydrates may interact with the heparin-binding domain on FGFs. BHK cells express both heparin-like low-affinity receptors and high-affinity receptors (Moscatelli, 1987). Both PDGF and TGF β 1 bind to heparin (Vlodavsky et al., 1987; McCaffrey et al., 1992), and this may explain the abilities of the carrageenans to inhibit binding of these growth factors. To the best of the author's knowledge, there are no reports of either TGF α or IGF1 binding to heparin, and neither of these growth factors were inhibited by the carrageenans.

Of particular interest in the present study is the observation that the carrageenans showed some selectivity in their inhibition of growth-factor binding and that no one carrageenan was, overall, the most potent growth-factor antagonist. The interaction between polysulphated carbohydrates and proteins has been shown, in some instances, to display a remarkable degree of selectivity, the best-defined example being the interaction between heparin and anti-thrombin III (Van Boeckel et al., 1991). The structural differences between carrageenans in terms of their degrees of sulphation and carbohydrate backbone indicates that this may confer on them an ability to interact selectively with domains on growth factors.

The aberrant production of growth factors is associated with a large number of pathological conditions. For example, bFGF production is implicated in the proliferation and neo-vascularization of tumours (Baird and Bohlen, 1991). The potent activity of *l*-carrageenan as an antagonist of bFGF indicates that this carrageenan is a potentially useful anti-cancer agent. This is currently under investigation.

I thank Jane Donaldson and David Sykes for some technical assistance, and Dr. Peter Twentyman for reading the manuscript.

REFERENCES

- Baird, A. and Bohlen, P. (1991) in *Peptide Growth Factors and Their Receptors* I (Sporn, M. B. and Roberts, A. B., eds.), pp. 419–472, Springer Verlag, New York
- Bowen-Pope, D. F. and Ross, R. (1985) *Methods Enzymol.* **109**, 69–100
- Coffey, R. J., Jr., Leof, E. B., Shipley, G. D. and Moses, H. L. (1987) *J. Cell. Physiol.* **132**, 143–148
- Coombe, D. R., Parish, C. R., Ramshaw, I. A. and Snowden, J. M. (1987) *Int. J. Cancer* **39**, 82–88
- Girond, S., Crance, J. M., Van-Cuyck-Gandre, H., Renaudet, J. and Delionce, R. (1991) *Res. Virol.* **142**, 261–270
- Hoffman, R. and Cameron, L. (1992) *Anal. Biochem.* **203**, 70–75
- Hosang, M. (1985) *J. Cell. Biochem.* **29**, 265–273
- Klagsburn, M. (1990) *Curr. Opin. Cell Biol.* **2**, 857–863
- McCaffrey, T. A., Falcone, D. J. and Du, B. (1992) *J. Cell. Physiol.* **152**, 430–440
- Moscatelli, D. (1987) *J. Cell. Physiol.* **131**, 123–130
- Myers, C., Cooper, M., Stein, C., LaRocca, R., Walther, M. M., Weiss, G., Choyke, P., Dawson, N., Steinberg, S., Uhrich, M. M. et al. (1992) *J. Clin. Oncol.* **10**, 881–889
- Pollak, M. and Richard, M. (1990) *J. Natl. Cancer Inst.* **82**, 1349–1352
- Rees, D. A., Morris, E. R., Thom, D. and Madden, J. K. (1982) in *The Polysaccharides* (Aspinall, G. O., ed.), vol. 1, Academic Press, New York and London
- Tardieu, M., Gamby, C., Avramoglou, T., Jozefonvicz, J. and Barritault, D. (1992) *J. Cell. Physiol.* **150**, 194–203
- Van Boeckel, C. A. A., Grootenhuis, P. D. J. and Haasnoot, C. A. G. (1991) *Trends Pharmacol. Sci.* **12**, 241–243
- Vijayakumar, R. K. and Muthukkaruppan, V. R. (1990) *Immunol. Invest.* **19**, 163–183
- Vlodavsky, I., Fridman, R., Sullivan, R., Sasse, J. and Klagsburn, M. (1987) *J. Cell. Physiol.* **131**, 402–408
- Wakefield, L. M. (1987) *Methods Enzymol.* **146**, 167–173
- Wellstein, A., Zugmaier, J. A., Califano, J. A., III, Kern, F., Paik, S. and Lippman, M. E. (1991) *J. Natl. Cancer Inst.* **83**, 716–720
- Williams, L. T., Tremble, P. M., Lavin, M. F. and Sunday, M. E. (1984) *J. Biol. Chem.* **259**, 5287–5294