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Antiangiogenic effect of sulphated and nonsulphated glycosaminoglycans and polysaccharides in the chick embryo chorioallantoic membrane

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The inhibiting effect of sulphated and nonsulphated glycosaminoglycans and polysaccharides on the normal outgrowth of capillaries was tested in the chick embryo chorioallantoic membrane (CAM) with and without the presence of hydrocortisone. An antiangiogenic response to 50 μg of heparin and heparan sulphate (without hydrocortisone present) was observed in 38.8% and 23.1% of the CAMs, respectively, while the antiangiogenic response rate for dermatan sulphate, chondroitin sulphate A or C, hyaluronic acid and keratan sulphate was 15.9–0%. All sulphated homopolysaccharides tested were more effective than the naturally occurring glycosaminoglycans. Nonsulphated dextran and (methyl) cellulose had no antiangiogenic effect, while largely desulphated heparin retained such an effect. Hydrocortisone generally improved the antiangiogenic effect, a 100% response was obtained when it was combined with cellulose sulphate or fucoidan (polyfucose sulphate derived from marine algae), but the antiangiogenic effect of the largely desulphated heparin was unaffected by the presence of hydrocortisone. The results show that different polysulphated polysaccharides also have an antiangiogenic effect, without the addition of corticosteroids. The effect was apparently independent of their degree of sulphation, but the glycosidic structure may be of critical importance.

Keywords: angiogenesis, glycosaminoglycans, polysaccharides, hydrocortisone, capillaries, chorioallantoic membrane

Angiogenesis, the growth of new blood capillaries, is an important process in the development of tissues and organs. The angiogenic process is complex, and several factors, including the presence of heparin, have been shown to affect the formation of new capillaries [1]. Heparin promotes the proliferation [2] and locomotion of capillary endothelial cells *in vitro* [3], and enhances angiogenesis induced by tumour extract in the chorioallantoic membrane (CAM) of chick embryos [4]. Heparin plus cortisone decreases thymidine uptake by endothelial cells *in vitro* (but not by tumour cells) [5], and Folkman *et al.* [6] have reported that heparin plus cortisone inhibits normal angiogenesis in the CAM. Folkman *et al.* have also observed that treatment with this drug combination can inhibit the growth of, and even result in disappearance of, malignant tumours in mice [6].

We have recently shown that heparin and heparan sulphate can inhibit normal angiogenesis in the chick embryo CAM in the absence of hydrocortisone, contrary to several previous reports [7]. We have therefore expanded our studies to investigate the antiangiogenic effects of some modified glycosaminoglycans and artifically sulphated polysaccharides. The results show that sulphation of glycosaminoglycans and polysaccharides increases or induces an antiangiogenic effect, in contrast to the effects seen with unmodified analogues. However, compared with unmodified heparin, desulphated heparin largely retains its inhibiting effect on normal angiogenesis, while nonsulphated hyaluronic acid, dextran and (methylated) cellulose are lacking such an effect.

Materials and methods

Bioassay

The CAM of chick embryos was used to bioassay the antiangiogenic effect as described by Folkman *et al.* [6]. In brief, fertilized eggs (Shaver Starcross 288, Linköpings Kontrollhönseri, Linköping, Sweden), incubated for three days at 37°C, were explanted into plastic cups [8] and then incubated for three more days at 37°C and 3% CO_2 at 100% humidity before being used for the experiments.

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Methyl cellulose disks

The drugs to be tested were incorporated in small methyl cellulose disks as described by Crum *et al.* [9]. 10 µl of a 0.45% solution of methyl cellulose (Sigma) dissolved in redistilled water was dispensed onto the tips of teflon rods (diameter 3 mm) and dried to form disks which were then stripped off and placed on the CAM.

Drugs

The following test substances were included in various amounts in the methyl cellulose disks: heparin, *N*-acetylheparin, heparin fragment (Fragmin^R), and *N*-acetylheparin fragment (derived from Fragmin^R) were gifts from Kabi-Vitrum AB, Sweden; largely desulphated heparin, heparan sulphate from human aorta, purified chondroitin sulphate A (CSA) and dermatan sulphate were gifts from Professor Ulf Lindahl, Uppsala (biochemical data on these substances are given in [10]); keratan sulphate, chondroitin sulphate C (CSC) (Sigma Chemical Co., St. Louis, MO, USA); hypersulphated CSA (mean mol. wt 9000) (Luitpold-Werk, Finland); hyaluronic acid sulphate (mean mol. wt 16 600), xylan sulphate (mean mol. wt 5600) and dextran sulphate (mean mol. wt. 65 600) were gifts from Pharmacia AB, Sweden; dextran sulphate (mean mol. wt 500 000), fucoidan (mean mol. wt 100 000) (Sigma); cellulose sulphate (Serva, Germany); Dextran T-70^R, Dextran 500^R, hyaluronic acid (Healon^R) (Pharmacia AB, Sweden). When hydrocortisone was used, 60 µg of hydrocortisone-21-phosphate (Sigma) was included in the disks. Plain methyl cellulose disks, without addition of drugs, were used as controls.

Evaluation of the antiangiogenic response

The antiangiogenic response was checked under a dissection microscope 48 h after the disks has been placed on the CAM. The response was graded as positive if no capillaries were visible in a zone that extended beyond the margin of the methyl cellulose disk (> 3 mm). No avascular zone, or an avascular zone equal in size to or smaller than the overlying disk, was graded as a negative response.

At least ten eggs (n = 10–28) were evaluated for each individual set of drug-containing disks, and several sets of disks (with different drugs) were usually tested simultaneously. In order to avoid biased decisions the evaluation was performed in a masked fashion: the chick embryos were coded and examined randomly, and the investigator was thus not aware of which drug the individual disk contained until the examination of all CAMs had been completed.

Results and discussion

The antiangiogenic response rates of the different sulphated glycosaminoglycans and polysaccharides with or without hydrocortisone are shown in Tables 1 and 2. In general, the antiangiogenic response rates increased with higher amounts of test substance in the disks. The response rates were higher when hydrocortisone was included in the disks. It is obvious that the artificially sulphated glycosaminoglycans and polysaccharides were more effective as inhibitors of angiogenesis than the unmodified analogues (Tables 1 and 2). Dextran sulphate, fucoidan and cellulose sulphate are particularly effective as inhibitors of normal angiogenesis in the presence

Table 1. Antiangiogenic response to heparin and other glycosaminoglycans in the chick embryo chorioallantoic membrane with or without hydrocortisone (as per cent of tested embryos).

	Amount of applied drug (µg)					
	Without hydrocortisone			*With hydrocortisone*[b]		
Drug[a]	0.5	5	50	0.5	5	50
Heparin	25.5 (2)[c]	26.3 (2)	38.8 (21)	31.1 (2)	40.0 (2)	45.7 (22)
N-Ac-heparin	NT[d]	NT	27.3 (1)	NT	NT	67.4 (1)
Desulphated heparin	NT	3.9 (2)	23.4 (5)	NT	NT	21.4 (5)
Heparin fragment	NT	NT	41.6 (2)	NT	NT	56.0 (2)
N-Ac-heparin fragment	NT	17.8 (1)	57.1 (2)	NT	NT	82.4 (1)
Heparan sulph.	NT	0.0 (1)	23.1 (2)	NT	8.7 (1)	33.3 (2)
Hyaluronic acid	NT	NT	12.2 (2)	NT	NT	0 (1)
HA sulph.	NT	12.0 (1)	45.7 (2)	NT	NT	64.3 (1)
Keratan sulph.	NT	9.5 (1)	0.0 (1)	NT	21.0 (1)	14.3 (1)
CSA	NT	5.3 (1)	6.5 (2)	NT	15.4 (1)	12.2 (2)
Hypersulph. CSA	NT	25.0 (1)	31.3 (1)	NT	43.5 (1)	50.0 (1)
Dermatan sulph.	NT	0.0 (1)	15.9 (2)	NT	NT	4.5 (1)
CSC	NT	17.4 (1)	13.6 (2)	NT	5.6 (1)	29.6 (2)

[a] Heparin fragment = Fragmin^R. HA sulph. = hyaluronic acid sulphate. CSA = chondroitin sulphate type A (purified 4S units). CSC = chondroitin sulphate type C (mainly 6S units).
[b] 60 µg of hydrocortisone-21-phosphate.
[c] Figure within brackets = number of experiments, 10–28 embryos per experiment.
[d] NT = not tested.

Table 2. Antiangiogenic response to sulphated polysaccharides in the chick embryo chorioallantoic membrane with or without hydrocortisone (as per cent of tested embryos).

		Amount of applied drug (μg)					
		Without hydrocortisone			*With hydrocortisone*[c]		
Drug	*MW*[a] *DS*[b]	0.5	5	50	0.5	5	50
Dextran sulph.	65.6 1.6	10.0 (1)[f]	40.0 (1)	62.1 (1)	23.8 (1)	75.0 (3)	83.3 (2)
Dextran T-70		NT[e]	NT	0.0 (1)	NT	NT	5.9 (1)
Xylan sulph.	5.6 2.1	NT	21.4 (2)	37.5 (1)	NT	42.1 (1)	69.6 (2)
Fucoidan	100 NK[d]	NT	30.8 (1)	44.0 (1)	NT	NT	100 (1)
Cellulose sulph.	NK NK	4.4 (1)	50.0 (1)	77.8 (1)	19.2 (1)	76.2 (1)	100 (1)

[a] MW = mean molecular weight × 10^3.
[b] DS = degree of sulphation (average number of sulphate groups per monosaccharide subunit).
[c] 60 μg of hydrocortisone-21-phosphate.
[d] NK = not known.
[e] NT = not tested.
[f] Figures within brackets = number of experiments, 10–28 embryos per experiment.

of hydrocortisone. The mean (± SD) antiangiogenic response rate for plain methyl cellulose disks was 2.5 ± 2.9 (n = 19).

The mechanism behind the inhibiting effect of sulphated polysaccharides on the normal angiogenesis in the CAM is unknown. Heparin combined with cortisone may inhibit collagenous protein synthesis in the CAM [11] and cause dissolution of the basement membrane of growing capillaries, leaving basement membranes of large vessels intact [12]. We have found that heparin alone has a significant antiangiogenic effect, which is enhanced in an additive manner by hydrocortisone [7]. Hydrocortisone itself has a weak antiangiogenic effect in the CAM, the response rate being about 10% [7]. This was also reported by Sakamoto *et al.* [13], who found that hydrocortisone prevented DNA synthesis in endothelial cells and that heparin promoted nonspecific binding of hydrocortisone to growing endothelial cells [14]. Heparin thus enhances the suppressive effect of hydrocortisone on the endothelial cell growth.

An antiangiogenic effect of heparin fragments or other sulphated polysaccharides in the presence of corticosteroids has been reported previously. Folkman *et al.* [6] found that heparin fragments as short as hexasaccharides inhibit angiogenesis in the presence of cortisone but had no such effect when tested alone. Inoue *et al.* [15] reported that some sulphated polysaccharide–peptidoglycan complexes inhibited embryonic angiogenesis with or without cortisone. Folkman *et al.* [16] have also found that a sulphated cyclodextrin had an antiangiogenic effect when combined with cortisone, being more than 100 times more potent than heparin. This synthetic compound, however, was inactive when tested alone. Herbert *et al.* [17] have reported that xylan sulphate and heparin have a strong antiproliferative effect on cultured bovine aortic endothelial cells in the presence of fibroblast growth factors, while the opposite effect was observed with cultured human umbilical vein endothelial cells.

The present results show that not only heparin [7] but also various modified heparins, heparan sulphate and sulphated polysaccharides have an inhibiting effect on the normal growth of capillaries. It may be noted that heparan sulphate appears to be present on all endothelial cells in all tissues [18] and in vascular basement membranes [19]. The presence of heparan sulphate in the capillary walls may thus have a dual significance, being important both for the local control of angiogenesis and for protecting the endothelial cell surface against thrombus formation [18]. The effect of heparin on angiogenesis is independent of the presence of antithrombin binding sequences in the molecules since the modified (i.e., *N*-acetylated) heparins lacking affinity for antithrombin also show a substantial antiangiogenic activity (Table 1).

Sulphated homopolysaccharides appear to have several interesting effects in experimental situations, for example anti-viral effects [20, 21], inhibition of autoimmune disease [22], sperm–egg fusion [23, 24] or tumour metastasis [25], and immunomodulating effects [26, 27]. The present results show that they may also inhibit normal angiogenesis. In the case of fucoidan, this effect may be exercised by disrupting the cell-to-cell contacts between endothelial cells [28].

Generally, the addition of sulphate groups to the polysaccharide seems to increase its antiangiogenic effect. Within certain limits the number of sulphate groups (per monosaccharide unit) does not seem to be critical since xylan sulphate, with a degree of sulphation (DS) of 2.1, appears

to be less potent than dextran sulphate with a DS of 1.6 (Table 2). Moreover, there was no obvious correlation between the antiangiogenic effect and the DS (ranging from 0.7 to 1.75) of dextran sulphate (data not shown).

It may also be noted that the sulphated homopolysaccharides in Table 2 (except for xylan sulphate) have more pronounced antiangiogenic effects than the sulphated heterosaccharides (Table 1). This indicates that the molecular structure of the polysaccharide carrying the sulphate groups may be important for the antiangiogenic efficiency. The present data do not permit detailed conclusions as to the significance of the differences between the saccharide structures. However, of those nonsulphated polysaccharides tested, the largely desulphated heparin was the only one that showed a significant antiangiogenic effect and, somewhat surprisingly, this effect was not affected by the presence of hydrocortisone (Table 1). The significance of this finding is now under further investigation in our laboratory.

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