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HEPARAN SULPHATE IS A POTENT INHIBITOR OF DNA SYNTHESIS IN VITRO

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SUMMARY: The inhibition by glycosaminoglycans of DNA synthesis in vitro has been studied. The marked inhibition caused by heparin was reduced by chemical modification: analogues containing either N-sulphate or O-sulphate groups alone were still inhibitory, those containing either no sulphate groups or free amino groups were not. Of the widely distributed glycosaminoglycans only heparan sulphate was a strong inhibitor of DNA synthesis. The results indicate that high inhibitory activity to DNA polymerase, depends on specific structural features of the heparin family of glycosaminoglycans and is not simply related to gross charge density, although this plays a role.

Glycosaminoglycans, found predominantly at the cell surface and in the extracellular matrix, have been implicated in tissue organisation, cell-cell adhesion and cell locomotion (1). However they have also been reported to occur in the nucleus (2-6) and one glycosaminoglycan (heparin) has been found to have two contrasting effects on nucleic acid metabolism which has been widely used in studies of transcription and replication: in assays utilizing native DNA templates, heparin inhibits the activity of DNA polymerases (7-10) and blocks the initiation of RNA polymerase (11-14), presumably by binding to the enzymes (15+16). In assays with isolated chromatin or whole nuclei however, DNA (17-20) and RNA (21,22) synthesis are increased in the presence of heparin with simultaneous destabilisation of chromatin (23,24) and release of histone and free DNA, (17-19, 21,23) however large scale destabilisation is not required for stimulation of template activity (20).

ABBREVIATIONS: deSH - Completely desulphated heparin; deNSH - de-N-sulphated heparin; NAcdeSH and NAcdeNSH - N-acetylated deSH and deNSH respectively; ReNSdeSH - re-N-sulphated deSH.

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Heparin is thought to be a differentiation product only of mast cells and is not found in other cell types. In contrast, heparan sulphate (a closely related glycosaminoglycan) is widely distributed (1) and its metabolism has been found to be altered after transformation (25-30), when the normal controls governing DNA synthesis are lost. We have examined the structural requirements for inhibition of DNA polymerase by heparin and the possibility that heparan sulphate also is an inhibitor of DNA polymerase in assays with activated DNA.

MATERIALS AND METHODS

Preparation and Assay of Glycosaminoglycans - The pyridine salt of heparin was desulphated by solvolysis in dimethyl sulphoxide containing either 5% water for 1½h at 50°C to remove N-sulphate groups only (31) - deNSH, or with 10% water for 7h at 100°C to remove the majority of both N- and O-sulphate groups (32) - deSH. Free amino groups in samples of each product were acetylated (31) - NAcdeNSH and NAcdeSH - and in a sample of deSH were re-N-sulphated (33) - ReNSdeSH. Heparan sulphate from cultures of control cells, 21OC and 213CSC (27), and from both a simian virus 40 transformed clone, 215CSC (27), and a highly tumourigenic variant cell line, 219CT (30), both derived from 21OC, was isolated by the procedure previously reported (27). The procedure included ion exchange chromatography and chondroitinase ABC digestion to remove residual amounts of contaminating glycosaminoglycans, however for these experiments the whole cell sheet, without trypsin treatment was used as source of the glycosaminoglycans. Hyaluronic acid chondroitin sulphates A and C and Dermatan sulphate were obtained from Miles Laboratories. Aliquots of each glycosaminoglycans were assayed for hexosamine (34), uronic acid (35) and sulphate (36). The percentage of amino groups in the heparin analogues which were either unsubstituted or N-sulphated was determined by the yield of anhydromannose residues as described (40), but treating directly with nitrous acid without prior hydrolysis to remove acetyl groups. Under these conditions only N-sulphated or unsubstituted amino groups of hexosamines react (unpublished observations), in contrast to all hexosamines including those with N-acetylated amino groups under the published conditions (40). These results, together with the sulphate to hexosamine ratios, substantiate the designations of the heparin derivatives, and their degree of sulphation was supported by electrophoresis in pH 1 (results not shown).

Preparation and Assay of DNA polymerase - DNA polymerase was prepared from thymus glands of 3 week old Wistar Furth rats. The tissue was washed in cold 50mM Tris-Cl (pH 7.6), 25mM KCl, 5mM $MgCl_2$ containing 0.25M sucrose and then homogenised in 4 volumes of the same solution. The homogenate was used to prepare a $105,000g_{av}$ supernatant which was then dialysed against 50mM KPi buffer (pH 7.2); 1mM 2-mercaptoethanol; 40% v/v glycerol, and stored at -20°C. Assays for DNA polymerase-α were carried out essentially as described previously (37) but using 0.1mM [^{3}H]dTTP at 0.1 Ci/nmole (Radiochemical Centre, Amersham) and 20µg activated calf thymus DNA per assay. The control value for 1 hour incubations (100% activity) was equivalent to 550 units of enzyme activity (where 1 unit = 1 picomole dTTP incorporated per hour).

RESULTS AND DISCUSSION

Addition of a very small amount of heparin to a cytoplasmic preparation of DNA polymerase caused marked inhibition of the enzymatic activity (Fig. 1). The enzyme was almost completely inactive when the ratio of heparin to DNA was as low as 1:20 (w/w). Sulphate groups in heparin were essential for this inhibition as NAcdeSH (which has carboxyl groups but very few sulphate groups) caused no inhibition at all at the highest level tested (7 µg hexosamine per assay). Two other analogues were also not inhibitory (summarized in Table 1) - deSH (in which the carboxyl group is balanced by the free amino group) and deNSH (which has O-sulphate groups in addition to the carboxyl and free amino groups). Both the O-sulphated (NAcdeNSH) and N-sulphated (ReNSdeSH) analogues however were strongly inhibitory to DNA polymerase (FIg. 1). As these analogues have similar degrees of sulphation (Table 1), the different degrees of inhibition suggest that the N-sulphate group was slightly more important that the O-sulphate groups in the inhibitory activity of heparin.

Heparan sulphate from all four cell lines tested, was strongly inhibitory to the polymerase, with fifty per cent inhibition occurring at about 6-8 µg of glucosamine equivalents in the assay (Fig. 1). Only small differences were seen between the different heparan sulphate samples with heparan sulphate from the tumour and SV40 transformed cell lines (219CT and 215CSC) being slightly more inhibitory than that from the control clones (210C and 213CSC). Factors other than gross charge are important for the inihibtory activity, as 1) the heparin analogues ReNSdeSH and NAcdeNSH, which had only slightly higher sulphate to glucosamine ratios than the heparan sulphate samples (Table 1) caused fifty per cent inhibition at about 0.5 and 1 µg of glucosamine equivalents respectively, and 2) only low inhibition was observed with chondroitin sulphates A and C (results for chondroitin sulphate C only shown in Fig. 1) and dermatan sulphate (Fig. 1). The three latter

Table 1. Chemical composition of Glycosaminoglycans Investigated for Ability to Inhibit DNA Polymerase.

Glycosaminoglycan	Uronic Acid	Sulphate	Unsubstituted or N sulphated amino groups	Inhibitory Activity
	mole ratios to hexosamine		%	
Heparin	1.4	2.4	86	+++
deNSH	1.3	1.1	97	-
NAcdeNSH	1.4	1.3	2	++
deSH	1.3	0.2	89	-
NAcdeSH	1.4	0.3	2	-
ReNSdeSH	1.2	1.0	91	++
Heparan sulphate from				
210C	1.0	0.9[+]	47[+]	+
213CSC	0.9	ND[*]	ND	+
215CSC	1.1	0.8[+]	47[+]	+
219CT	1.0	0.8[+]	50[+]	+
Hyaluronic acid	1.2	0.1	ND	-
Chondroitin sulphate A	1.2	1.0	ND	-
Chondroitin sulphate C	1.2	1.0	ND	-
Dermatan sulphate	1.1	1.0	ND	-

[+]These results were obtained by analysis of metabolically labelled heparan sulphates (30)

[*]ND - not determined

glycosaminoglycans all have slightly higher sulphate to hexosamine ratios than the heparan sulphate samples (Table 1). The unsulphated glycosaminoglycan, hyaluronic acid, caused no inhibition of DNA polymerase and even caused a slight stimulation (Fig. 1). These results could not be attributed to contaminating enzyme activities, as some of the assays were repeated with a partially purified preparation of DNA polymerase-α from rat spleen (38) and identical results were obtained.

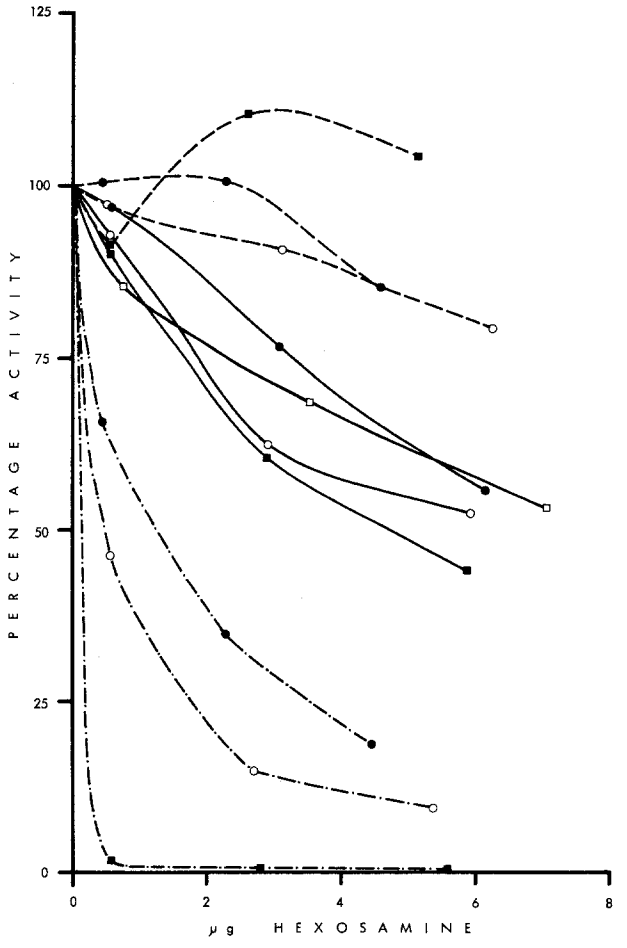

Fig. 1. Activity of rat thymus cytoplasmic DNA polymerase in the presence of various glycosaminoglycans and analogues.

Enzyme activity is plotted as a percentage of the control value, against the amount of each glycosaminoglycan added (expressed as μg hexosamine/assay) Heparin, ■—·— ; NAcdeNSH, ●—·— ; ReNSdeSH, O—·— ; heparan sulphate from control clones 210C ●—— ; and 213CSC □——; and from the transformed clone 215CSC O—— ; and tumour line 219CT ■—— ; chondroitin sulphate C ● ---; dermatan sulphate O ---; and hyaluronic acid ■--- .

From this evidence it is clear that sulphate groups are required, but that other structural features are important in the inhibition of DNA polymerase by glycosaminoglycans. One difference between the heparin analogues and heparan sulphate is that the former have a higher percentage of iduronic acid residues. However the presence of a high ratio of iduronic to glucuronic acid in a sulphated glycosaminoglycan is not sufficient to cause

inhibition of DNA polymerase as dermatan sulphate was only a weak inhibitor (Fig. 1). In summary, these results indicate that high inhibitory activity to DNA polymerase depends on structural features of the heparin family of glycosaminoglycans.

The significance of these observations depends on whether or not interactions between heparan sulphate and DNA polymerase or other nucleic acid metabolising enzymes occur within an intact cell under normal physiological conditions. Of critical importance to this question is the subcellular localisation of the two components. Heparan sulphate is conventionally thought of as a component of the cell surface. However it has been found to be present also in the nucleus (2-6) as well as in other subcellular organelles. DNA polymerase-α until recently has been found predominantly in the cytoplasmic fraction. It is only with the application of unconventional fractionation procedures that the majority of this enzyme has been detected in the nucleus (39,40) - the localisation of heparan sulphate is such procedures has not been reported. Our results raise the possibility that heparan sulphate (which is found in most cell types) may play a role in the control of DNA synthesis or in the activation or repression of genes during development. In fact it has been suggested that a heparin-like proteoglycan present in sea urchin embryos may play such a role during the early stages of development (22,24). In this respect, it is relevant that similar changes in heparan sulphate metabolism to the changes reported for tumour (28,30) and virally transformed cells (25-27,29,30) have been reported to occur during the development of mouse embryos (41). If heparan sulphate were to play such a role, this would provide a means for linking events at the cell surface, where heparan sulphate is thought to have an important function, with events in the nucleus.

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REFERENCES

1. Kraemer, P.M. (1979) in "Surface of Normal and Malignant Cells", Hynes, R.O. (ed.), Wiley, New York, pp 149-198.
2. Bhavanandan, V.P. and Davidson, E.A. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2032-2036.
3. Stein, G.S., Roberts, R.M., Davis, J.L., Head, W.J., Stein, J.L., Thrall, C.L., Van Veen, J. and Welch, D.W. (1975) Nature 258, 639-641.
4. Margolis, R.K., Crockett, C.P., Kiang, W.L. and Margolis, R.U. (1976) Biochim. Biophys. Acta 451, 465-469.
5. Fromme, H.G., Buddecke, E., v. Figura, K. and Kresse, H. (1976) Exp. Cell Res. 102, 445-449.
6. Saiga, H. (1977) J. Fac. Sci. (Univ. Tokyo Sect. 4) 14, 1-10.
7. Lazurus, L.H. and Kitron, N. (1973) J. Mol. Biol. 81, 529-534.
8. Lazurus, L.H. and Kitron, N. (1974) Arch. Biochem. Biophys. 164, 414-419.
9. DiCioccio, R.A. and Sahai Srivastava, B.I. (1978) Cancer Res. 38, 2401-2407.
10. Sato, N.L. (1979) Acta Medica et Biologica 26, 155-162.
11. Cox, R.F. (1973) Eur. J. Biochem. 39, 49-61.
12. Monroy, G., Jacquet, M., Groner, Y. and Hurwitz, J. (1975) Cold Spring Harb. Symp. Quant. Biol. 39, 1033-1041.
13. Pfeffer, S.R., Stahl, S.J. and Chamberlin, M.J. (1977) J. Biol. Chem. 252, 5403-5407.
14. Coupar, B.E.H. and Chesterton, C.J. (1977) Eur. J. Biochem. 79, 525-533.
15. Brennessel, B.A., Buhrer, D.P., Gottlieb, A.A. (1978) Anal. Biochem. 87, 411-417.
16. Sternbach, H., Engelhardt, R. and Lezius, A.G. (1975) Eur. J. Biochem. 60, 51-55.
17. Cook, R.T. and Aikawa, M. (1973) Exp. Cell Res. 78, 257-270.
18. Arnold, E.A., Yawn, D.H., Brown, D.G., Wyllie, R.C., Coffey, D.S. (1972) J. Cell Biol. 53, 737-757.
19. Seki, S. and Oda, T. (1977) Biochim. Biophys. Acta 479, 391-399.
20. Smith, M.R. and Cook, R.T. (1977) Exp. Cell Res. 110, 15-23.
21. Chambon, P., Ramuz, M., Mandel, P. and Doly, J. (1968) Biochim. Biophys. Acta 157, 504-519.
22. Kinoshita, S. (1971) Exp. Cell Res. 64, 403-411.
23. Ansevin, A.T., Macdonald, K.K., Smith, C.E. and Hnilica, L.S. (1975) J. Biol. Chem. 250, 281-289.
24. Kinoshita, S. (1976) Exp. Cell Res. 102, 153-161.
25. Underhill, C.B. and Keller, J.M. (1975) Biochem. Biophys. Res. Commun. 63, 448-454.
26. Underhill, C.B. and Keller, J.M. (1977) J. Cell Physiol. 90, 53-60.
27. Winterbourne, D.J. and Mora, P.T. (1978) J. Biol. Chem. 253, 5109-5120.
28. Nakamura, N., Hurst, R.E. and West, S.S. (1978) Biochim. Biophys. Acta 538, 445-457.
29. Keller, K.L., Keller, J.M. and Moy, J.N. (1980) Biochemistry 19, 2529-2536.

30. Winterbourne, D.J. and Mora, P.T. (1981) J. Biol. Chem. (in the press).

31. Inoue, Y. and Nagasawa, K. (1976) Carbohydr. Res. 46, 87-95.

32. Nagasawa, K., Inoue, Y. and Kamata, T. (1977) Carbohydr. Res. 58, 47-55.

33. Shively, J.E. and Conrad, H.E. (1976) Biochemistry 15, 3932-3942.

34. Smith, R.L. and Gilkerson, E. (1979) Anal. Biochem. 98, 478-480.

35. Bitter, T. and Muir, H.M. (1962) Anal. Biochem. 4, 330-334.

36. Terho, T.T. and Hartiala, K. (1971) Anal. Biochem. 41, 471-476.

37. Salisbury, J.G., O'Connor, P.J. and Saffhill, R. (1978) Biochim. Biophys. Acta 517, 181-185.

38. Salisbury, J.G. (1976) Ph.D. Thesis, Univ. Manchester.

39. Lynch, W.E., Surrey, S. and Lieberman, I. (1975) J. Biol. Chem. 250, 8179-8183.

40. Herrick, G., Spear, B.B., Veomett, G. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1136-1139.

41. Keller, K.L., Underhill, C.B. and Keller, J.M. (1978) Biochim. Biophys. Acta 540, 431-442.