Effect of heparin and liver heparan sulphate on interaction of HepG2-derived transcription factors and their *cis*-acting elements: altered potential of hepatocellular carcinoma heparan sulphate

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Proteoglycan assembly in malignant tumours is subject to profound changes. The significance of these alterations is not well understood; especially, their role in nuclear regulation is a topic for debate. The capacity of heparin and liver carcinoma heparan sulphate (HS) to alter DNA–transcription factor interactions has been studied to provide further evidence concerning the regulatory potential of glycosaminoglycan (GAG) in the nucleus. Experiments both *in vitro* and *in vivo* indicated that heparin and HS are capable of inhibiting the interaction of transcription factors with their consensus oligonucleotide elements. Among five transcription factors studied, AP-1, SP-1, ETS-1 and nuclear factor κ B proved to be sensitive to heparin

and heparan sulphate, whereas TFIID was hardly inhibited in either *in vitro* or *in vivo* systems. Interestingly, HS from peritumoral liver was five times more effective than heparin. Liver carcinoma HS was less effective than liver HS, but its activity was comparable with that of heparin. These results indicate that the structural differences of GAG chains strongly influence their biological behaviour. The loss of their recognized functional activity in malignant tumours might promote the development of uncontrolled growth and gene expression favouring the neoplastic process.

Key words: DNA–protein interaction, proteoglycans, regulation.

INTRODUCTION

In recent years, several data have indicated that proteoglycans (PGs) on the cell surface and in the extracellular matrix (ECM) serve not only for structural support. The functional activity of PGs are partly attributed to their glycosaminoglycan (GAG) components. The composition and structure of these molecules are quite stable under normal conditions [1,2]. However, these features are changed permanently during the course of embryogenesis, and fast remodelling occurs during pathological events. Alteration of PG assembly in malignant tumours was observed as much as 20 years ago [3–5]. It was demonstrated that both the size and the charge of tumourous heparan sulphate (HS) differ from that of normal tissue [3], and this also holds true for dermatan sulphate and chondroitin sulphate (CS) molecules [4,5]. Besides altered assembly in the Golgi apparatus, heparanase activity can also modify the HS chains, as described previously in Caco-2 colon cells [6]. The biological consequences of these alterations have hardly been assessed, but an increasing number of functional studies are attempting to understand their impact on physiological and pathological events. Structural analysis of GAG chains revealed that iduronate-rich, highly sulphated hexasaccharide units of heparin and HS are responsible for their protein-binding properties. These structures are required for growth factor binding, as described for basic fibroblast growth factor (bFGF) and hepatocyte growth factor [7–9]. Interestingly, the great majority of heparin-binding molecules are active in signalling and in cellular regulation. These proteins have a

positive charge to which their interaction with heparin has mainly been attributed. However, evidence suggesting the necessity of a well-determined sugar moiety for this protein-binding function is also accumulating [10].

Intracellular action of heparin or HS predicts their presence inside the cells. Internalization of heparin into the cell and nucleus has been demonstrated on several previous occasions. We also found in a recent study that heparin is transported to the nuclei of U937 leukaemia cells, where it co-localizes with bFGF [11]. The presence of HS in the nuclei of hepatocytes was described in 1987 [12]. It has been proposed that cell-surface HS PGs are subject to insulin-mediated processing in the ECM, followed by their internalization into the cells. The majority of these HS PGs are catabolized in lysosomes, but a proportion of them escapes this pathway, and the free HS chains enter the nucleus [12,13]. In hepatoma cell culture the nuclear HS level increases 3-fold at the time of contact inhibition [14]. GAG internalization, together with a particular protein, supposedly protects the protein against degradative enzymes [15]. These observations also suggest that GAG chains of PGs may control cell nuclear events.

However, the functional role of GAGs inside the nucleus still remains to be elucidated. Several candidate proteins regulated by heparin or HS have also been described. Transcription factors, such as casein kinase II, midkines and histones, were reported as target molecules [10,16,17]. Inhibition of kinase activity might lead to decreased phosphorylation of nuclear proteins, such as topoisomerase II or p53. Topoisomerase I is directly inhibited by

Abbreviations used: bFGF, basic fibroblast growth factor; CS, chondroitin sulphate; ECM, extracellular matrix; GAG, glycosaminoglycan; HCC, hepatocellular carcinoma; HS, heparan sulphate; NF κ B, nuclear factor κ B; SEAP, serum alkaline phosphatase; PG, proteoglycan.

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heparin and HS from peritumoral liver, but its activity is not effected by HS either from hepatocellular carcinoma (HCC) or of commercial origin [11,18].

c-Jun/c-Fos-mediated transcription was repressed by heparin in a reporter gene assay [19]. This was the first demonstration of the direct effect of heparin on the protein kinase C/mitogen-activated protein kinase/Jun/Fos/AP-1 signal transduction pathway. Au et al. [20] found that heparin induces post-translational modification of JunB.

In the present study, we attempted to find further support for the regulatory role of heparin and HS, focusing especially on the differences observed between HS isolated from either normal or neoplastic liver. The effects of these GAGs have been compared in terms of AP-1, SP-1, TFIID, ETS-1 and nuclear factor κ B (NF κ B) transcription factor–DNA interactions in HepG2 cells. An attempt has been made to prove that HS structures in the liver have considerable impact on regulatory events taking place in the nucleus.

MATERIALS AND METHODS

Chemicals

Tissue-culture media and sera were obtained from Gibco BRL (Eggenstein, Germany) and Serotec (Kidlington, Oxford, U.K.). Sodium–heparin from pig intestinal mucosa with an average M_r of 15000 was the product of Boehringer Ingelheim Serva (Gagny, France). Chemicals for PAGE were purchased from Sigma (Deisenhofen, Germany).

Consensus oligonucleotides (AP-1, TFIID) were bought from Promega (Mannheim, Germany); SP-1 and NF κ B were purchased from Stratagene (Heidelberg, Germany). The ETS-1 binding sequence was designed according to previously published data [21], and MWG Biotech Göttingen (Germany) synthesized the oligonucleotide sequence.

Cell culture and treatments

HepG2 HCC was a standard American Tissue Type Culture Collection cell line. Passages between 11 and 14 were used for the experiments. Cells were plated at a density of 250000 cells/ml, and were cultured in Dulbecco's modified Eagle's medium in 5% (v/v) fetal bovine serum. Nuclear proteins were extracted from the culture on days 5–7.

For GAG treatments, cells were seeded in 24-well plates at a density of 250000 cells/ml, and grown for 24 h. Subsequently, the serum was replaced with 5% (w/v) albumin. On the third day, the cells were exposed to 20 or 100 μ g/ml heparin, or 20 μ g/ml isolated liver HS for 24 h.

Isolation and purification of glycosaminoglycans

Isolation and purification of GAGs has been described previously [22]. Briefly, liver tissues were pulverized in liquid nitrogen. This step was followed by lipid extraction in chloroform/methanol. The samples were then delipidated and dehydrated by serial washing in chloroform/methanol, and acetone-dried under nitrogen. The dry powder was dissolved in Tris/HCl buffer, pH 7.9, and digested with 1 mg/ml Pronase B (Serva) at 50 °C for 2–3 days. β -Elimination of GAG chains was accomplished by 0.5 M NaOH treatment for 4 h at room temperature. The remnants of macromolecules were removed by trichloroacetic acid precipitation. The supernatant was dialysed against water, then precipitated with cetylpyridinium chloride. These purification steps (trichloroacetic acid and cetylpyridinium chloride precipitation) were repeated until the samples became protein-

free. The yields of GAGs were determined by dimethyl-Methylene Blue colorimetry at 525 nm, using mixed GAGs or HS as standards [23]. HSs were purified by treatment with chondroitinase ABC (Seikagaku, Ijamsville, MD, U.S.A.) [24]. The composition of the isolated GAG, and the purity of HS, was determined by cellulose acetate zone electrophoresis and PAGE, where the samples were stained with Alcian-Blue 8GX. The absence of any protein was confirmed with Coomassie Brilliant Blue R250 (Sigma) staining of the polyacrylamide gels.

Proliferation assay

HepG2 cells (1×10^5 or 2.5×10^5 /ml) were plated and treated with heparin or GAG for 24 or 48 h respectively, as described above. Cellular proliferation was followed by determination of the increase of DNA content with the Hoechst fluorochrome in an Ascent Fluoroscan at a range of 355–460 nm.

Preparation of nuclear extracts

Nuclear protein extract was prepared as follows: 2×10^6 cells were washed in 10 ml of Tris-buffered saline (20 mM Tris/HCl and 150 mM NaCl, pH 7.5), and collected in Eppendorf tubes. After washing the cells, they were incubated in 500 μ l of ice-cold extraction buffer (20 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM PMSF, pH 7.8) for 15 min. Subsequently, 30 μ l of 10% (v/v) Nonidet P40 was added to each tube and mixed vigorously for 10 s. Nuclei were pelleted by centrifugation (12000 rev./min; 13700 g) for 30 s, and extracted in 50 μ l of ice-cold extraction buffer containing 400 mM NaCl for 15 min at 4 °C. The nuclear debris was removed by centrifugation, and the supernatant was used as a nuclear extract. Protein concentration was determined with Coomassie Assay reagent (Pierce, Rockford, IL, U.S.A.).

Gel-shift reactions

Double-stranded consensus sequences for transcription factor DNA-binding sites (3.5 pmol) were end-labelled with [γ - 32 P]-dATP using T4 polynucleotide kinase (Amersham, Braunschweig, Germany). The labelling reaction was performed for 10 min at 37 °C, and then stopped by the addition of 0.2 M EDTA. Unincorporated nucleotides were removed by using Qiagen tip 20, according to the manufacturer's instructions.

Gel-shift reactions were performed in a volume of 30 μ l, comprising 15 μ l of $2 \times$ binding buffer (40 mM Hepes, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 0.5 mM PMSF, pH 7.5) containing 2 μ g of nuclear protein, 8000 c.p.m. of radiolabelled oligonucleotide, and non-labelled competitor oligonucleotide or GAG adjusted to the final volume with extraction buffer containing 400 mM NaCl. Labelled oligonucleotides were added after 10 min of preincubation. The reaction was performed at room temperature for 30 min, and the samples were run on 4% non-denaturing (acrylamide/bisacrylamide, 30:1, w/v) gels in $0.5 \times$ TBE buffer (where $1 \times$ TBE = 45 mM Tris/borate/1 mM EDTA) at 170 V (in a Bio-Rad Protean II chamber) for 110 min at 4 °C. Gels were exposed to Kodak X-Omat film for autoradiography.

Heparin labelling with biotin

Heparin was dissolved in 100 mM sodium acetate, pH 5.5, and oxidized with 10 mM sodium periodate for 10 min. The oxidized heparin was separated from sodium periodate on a Sephadex G25 column (Pharmacia, Orsay, France). Oxidized heparin was incubated overnight with 2 mg/ml long-arm biotinylated

hydrazine (Vector Laboratories, Peterborough, U.K.). The biotinylated heparin was purified on a Sephadex G25 column.

AP-1 reporter gene assay

HepG2 cells (3×10^5 /ml) were plated on to 24-well plates. After attachment overnight, the cells were adapted to serum-free medium as described above, and were transfected with pAP-1-serum alkaline phosphatase (SEAP) construct (Clontech, Heidelberg, Germany) using the calcium-phosphate precipitation method [25]. Transfection was performed over 4 h before shock with glycerol. After transfection, the cells were treated with 100 or 200 μ g/ml heparin, or 50 μ g/ml GAG samples, for 36 h. Secreted placental alkaline phosphatase activity was measured by using the SEAP detection kit (Roche Biochemicals, Zurich, Switzerland).

Evaluation of results and sequence analysis

Oligonucleotide and competitor sequence similarity was analysed by using the Bestfit program of the GCG package. The sequence analysis was performed on ICGEB bioinformatic servers in Trieste, Italy.

Densitometric analysis of gel-shift assays was performed by using an Eagle EYE II still-video system (Stratagene) one-dimensional scan software. The densitometric data were analysed with statistical and regression methods (EXCEL).

RESULTS

Transcription factor activity in HepG2 hepatocellular carcinoma cells

To establish the basis for further experimentation, the amount of the five transcription factors present in 2- μ g nuclear extracts was determined on the basis of their DNA binding after 6 h, and after 1, 2, 3, 4 and 5 days, by gel-shift assay. End-labelled consensus sequences for AP-1-, c-ETS-1-, SP-1-, NF κ B- and TFIID-responsive elements were used.

We identified DNA-binding protein activity for TFIID from the first day in culture, but the other factors were only represented in minor amounts until the fifth day. Therefore our experiments were performed with HepG2 cells 5–7 days after setting up the cultures. At that time, nuclear extracts contained sufficient amounts of AP-1, SP-1 and TFIID to saturate 10 fmol of labelled consensus oligonucleotides, which was not the case for ETS-1 and NF κ B (Table 1).

Table 1 Saturation of 10 fmol of labelled consensus oligonucleotides by transcription factors present in 2 μ g nuclear extract of HepG2 cells

Cell nuclear protein extract was isolated from HepG2 cells 5 days after plating. Using labelled oligonucleotides, the DNA-binding activities were determined for the transcription factors AP-1, ETS-1, SP-1, TFIID and NF κ B. The Table shows the mean results $\pm$ S.D. of three independent experiments.

Transcription factor	Protein-bound labelled oligonucleotide (%)
AP-1	95.2 $\pm$ 4.53
ETS-1	57.5 $\pm$ 4.51
SP-1	98.2 $\pm$ 2.22
TFIID	98.5 $\pm$ 1.89
NF κ B	31.9 $\pm$ 17.82

Figure 1 Determination of the specificity of electrophoretic mobility shifts in HepG2 cell nuclear extracts

The quantity of non-labelled consensus oligonucleotides that completely inhibit the mobility shift resulting from the interaction of 10 fmol of end-labelled AP-1, ETS-1, SP-1, TFIID, NF κ B sequences and 2 μ g of cell nuclear extracts was determined. (A) A representative experiment is shown from three independent ones. Lane 1, negative control (no nuclear extract); lane 2, positive control (mobility shift of 2 μ g of nuclear extract); lanes 3–5, 50, 100 or 500 fmol, respectively, of unlabelled added oligonucleotide. (B) Determination of the molar excess of unlabelled oligonucleotides that is sufficient for the complete inhibition of the mobility shift reaction for the subsequent transcription factors. The bars represent the means $\pm$ S.D. for three independent experiments.

Specificity of the DNA–transcription factor interactions

To determine the specificity of DNA–protein interaction, labelled oligonucleotides were competed with a 20–100-fold excess of unlabelled ones. Figure 1 shows the amount of unlabelled oligonucleotides (200–1000 fmol) that completely abrogated the binding of particular labelled consensus oligonucleotides. The highest amount was needed for AP-1; the lowest amount was for TFIID.

The inhibitory effect of unspecific oligonucleotides on DNA–protein interactions was also assessed. Here, depending on the sequence similarities of unspecific oligonucleotides, at least 20 pmol of double-stranded, 70% similar oligonucleotide was required to completely abolish AP-1, SP-1, TFIID and NF κ B DNA binding (2000 $\times$ excess). In general, non-specific oligonucleotides had to be added in 1000–5000-fold excess.

Effect of heparin on DNA–transcription factor interaction

Inhibitory action of commercial heparin depended on the type of consensus oligonucleotides actually used, but the effect was dose-dependent. Under our reaction conditions, heparin caused complete inhibition of DNA–protein interaction in amounts as follows: AP-1, 167.75 pmol; ETS-1, 187.93 pmol; SP-1, 217.76 pmol; and TFIID, 365.29 pmol (Figure 2). The considerable difference between sensitivity of AP-1 and TFIID for heparin indicates an interaction that is not only charge-related. Similar inhibitory action was detected when the HepG2 cells were treated with heparin in culture. HepG2 cells were exposed to 20 or 100 μ g/ml heparin for 24 h. The results in Figure 3 show the concentration-dependent decrease of DNA-bound AP-1, SP-1, NF κ B and ETS-1 proteins after heparin treatment of the

Figure 2 Effect of heparin on mobility-shift reactions

The quantity of heparin that completely inhibited the mobility shift of AP-1, ETS-1, SP-1 and TFIID was determined using 10 fmol of labelled consensus oligonucleotides and 2 µg of cell nuclear extracts from HepG2 cells cultured for 5 days. **(A)** Representative gels are shown from three independent experiments: lane 1, negative control (no nuclear extract); lane 2, positive control (2 µg of nuclear extract); lanes 3–5, 20, 40 or 60 pmol of heparin added, respectively, to the reaction mix. **(B)** Determination of the amount of heparin that completely abrogated the DNA binding of the transcription factors. The bars are calculated means $\pm$ S.D. from five independent experiments.

Figure 3 DNA–transcription factor interactions in HepG2 cells after treatment with heparin for 24 h

HepG2 cells (5×10^5) were plated in six-well plates at 2.5×10^5 cells/ml density. After 1 day for attachment, the cells were cultivated further in serum-free medium. At 48 h, the cells were treated with 20 or 100 µg/ml heparin for 24 h. Neither the serum-free condition nor the heparin treatment influenced cell growth significantly (proven with proliferation methods). At the end of the treatment, nuclei were isolated, and the 0.4 M NaCl-soluble fractions were extracted as described in the Materials and methods section. The band-shift reactions were performed with 2 µg of nuclear protein, and 10 fmol of labelled oligonucleotide. **(A)** Representative gels for AP-1, ETS-1, NFkB, SP-1 and TFIID after heparin treatment of HepG2 cells. Lane 1, negative control for mobility shift without nuclear extract; lane 2, positive control with nuclear extract from non-treated cells; lanes 3 and 4, positive reactions after treatment with 20 and 100 µg of nuclear protein respectively. **(B)** The decrease in the amount of transcription factors available to bind to the consensus oligonucleotides, after treatment with 100 µg/ml heparin. The DNA binding is expressed as a percentage of the control values. All columns represent the means $\pm$ S.D. for three independent experiments.

Figure 4 Uptake of liver HS into HepG2 cells

HepG2 cells (2.5×10^5 /ml) were plated on cover slips in six-well plates. On day 3, cells were treated with biotinylated heparin or liver HS (the same samples as used in the mobility-shift assays) at 10 µg/ml for 4 h. The cover slips were fixed with methanol/acetone, and the biotin signal was detected with streptavidin–fluorescein. The cell nuclei were stained with propidium iodide. The majority of cells contained labelled material, mainly with cytoplasmic localization, but a modest amount of nuclear representation was also observed. For a clearer view, the picture is shown as a negative. Black dots represent biotin-labelled liver HS.

Figure 5 Analysis of isolated GAG specimens

(A) Representative electrophoretic pictures on cellulose acetate of GAG samples. The bands, as shown from the bottom, are HS, dermatan sulphate and CS respectively. Lane 1, GAG from normal liver; lane 2, GAG from peritumoral liver; lane 3, GAG from an HCC sample; lane 4, peritumoral liver HS after chondroitinase ABC digestion; lane 5, HCC HS after chondroitinase ABC digestion. **(B)** Electrophoresis of GAG samples on an SDS/10% polyacrylamide gel. The gel was fixed, stained with Alcian Blue, and then stained with Coomassie Brilliant Blue R250. The latter dye did not detect any protein contamination in the GAG samples. The molecular-mass markers stained with Coomassie Blue: the molecular mass of the marker proteins are shown (in kDa) on the right-hand side of the gel. Lane 1, HP (Serva); lane 2, HP (Sigma); lane 3, HS (Seikagaku); lane 4, peritumoral liver HS; lane 5, HCC HS (the same patient as in lane 4); lane 6, peritumoral liver HS; lane 7, HCC HS (same patient as in lane 6); lane 8, normal liver HS. Note the larger size of the liver HS specimens.

HepG2 cells. TFIID oligonucleotide–protein interactions were not affected significantly by the treatments.

Since cellular uptake of GAGs is still a matter of debate, we treated the HepG2 cells with biotin-labelled heparin or liver HS

Figure 6 Effect of liver HS DNA–transcription factor interactions

(A) Representative mobility-shift gels for AP-1, SP-1, TFIID and ETS-1 transcription factors. The reactions were performed in accordance with the standard method. With the exception of ETS-1, lanes 1 show the negative control (no extract added); lanes 2, positive control; lanes 3–5, 3, 6 and 12 pmol respectively of added peritumoral liver HS; lanes 6–8, 3, 6 and 12 pmol respectively of liver tumour HS. In the case of ETS-1, lane 1 shows the negative control (no extract added); lane 2, positive control; lanes 3–5, 16, 32 and 48 pmol, respectively, of added peritumoral liver HS; lanes 6–8: 16, 32 and 48 pmol, respectively, of added liver tumour HS. (B) The histogram shows the quantity of peritumoral liver HS and hepatocellular carcinoma HS that completely abrogated the DNA binding of the transcription factors. The bars are calculated means $\pm$ S.D. from three independent experiments.

to prove their presence in the cells. The detection was carried out by streptavidin fluorescein. Using a confocal laser-scanning microscope, the majority of cells was found to contain labelled material, mainly within their cytoplasm, but modest amounts were also observed in the nuclei, especially in the case of liver HS (Figure 4).

Characterization of GAGs from liver and HCC

The GAG composition of individual tumorous and non-tumorous samples differed, but had several common features [4]. In general, the GAG content increased in both HCCs and peritumoral tissues, and thus more GAG could be isolated from them than from the normal liver. HS and, to a lesser extent, dermatan sulphate were the GAGs in normal and peritumoral liver. In contrast, liver cancers were characterized by the increase in CS and HS content (Figure 5). In a series of samples, the cellulose acetate electrophoresis did not show any change in the mobility of GAGs. On the other hand, upon PAGE the average mass of tumorous HS was generally larger than that derived from peritumoral tissue, and both surpassed the size of commercial heparin and HS.

Effect of peritumoral liver and HCC-derived heparan sulphate on transcription factor–DNA interaction

HS from peritumoral liver and cancer was used to inhibit gel shifts with AP-1, SP-1 and TFIID. HS from peritumoral liver decreased the binding of AP-1, SP-1 and TFIID in a dose-dependent manner. All of the three transcription factors showed a similar sensitivity for peritumoral HS, which was much more effective than heparin (Figure 6). HSs from tumour tissues had less, or no effect on the interaction. Surprisingly, the opposite tendency was identified with the ETS-1–DNA interaction, where

Table 2 Effect of heparin and liver HS on the AP-1-regulated transcriptional activity of HepG2 cells

HepG2 cells (3×10^5 /ml) were plated on 24-well plates. After 1 day of serum-free adaptation, the cells were transfected for 4 h with pAP-1-SEAP by using the calcium-phosphate precipitation method. After transfection, cells were treated with 100 or 200 μ g/ml heparin or 50 μ g/ml GAG from tumorous, peritumoral or normal liver for 36 h. Reporter gene activity was measured with the alkaline phosphatase chemiluminescence method. Heparin and liver HS specimens decreased significantly ($P < 0.05$ by Student's *t* test analysis) the AP-1-dependent transcriptional activity, whereas tumorous HS did not show a significant effect ($P = 0.3032$). The results are means $\pm$ S.D. for four independent experiments.

Treatment	Relative luminescence	<i>P</i>	<i>n</i>
Control	$(1.78 \times 10^6) \pm (1.33 \times 10^6)$	< 12	
Heparin (100 μ g/ml)	$(7.26 \times 10^5) \pm (1.67 \times 10^5)$	< 0.04	6
Heparin (200 μ g/ml)	$(6.62 \times 10^5) \pm (1.77 \times 10^5)$	< 0.03	6
HCC GAG (50 μ g/ml)	$(1.48 \times 10^6) \pm (5.55 \times 10^5)$	< 0.30	6
Normal liver GAG (50 μ g/ml)	$(3.70 \times 10^5) \pm (2.09 \times 10^5)$	< 0.01	6
Peritumoral liver GAG (50 μ g/ml)	$(1.37 \times 10^5) \pm (0.56 \times 10^5)$	< 0.001	6

the tumorous HS was more effective. These findings provided further support for the notion that transcription factors are sensitive not only to charge, but also to structural motifs present in HS.

AP-1 reporter gene assay

To confirm our finding, AP-1 reporter gene assay was performed. HepG2 cells were transfected for 4 h with pAP-1-SEAP construct by the calcium-phosphate method. Subsequently, the cells were treated with 100 or 200 μ g/ml heparin, and 50 μ g/ml liver and liver carcinoma GAGs for 36 h. The effect of GAGs on the AP-1-mediated pathway was assessed by the changes in alkaline phosphatase activity of the reporter gene. As Table 2 demon-

strates, both heparin and liver HS decreased the alkaline phosphatase production, whereas no change was observed after treatment with HS of carcinoma origin. It is noteworthy that the effect of liver-derived HS was higher again than that of heparin.

DISCUSSION

Heparin-like molecules are able to regulate cellular functions on several levels [10]. The objective of the present study was to find data that confirmed further the biological significance of GAGs in the regulation of nuclear functions. Furthermore, we wanted to demonstrate the decreased, or lost, regulatory capacities of HS produced by malignant tumours.

The possible role of heparin-like molecules in the regulation of nuclear functions has been debated for a long time. The experimental results of Ishihara et al. [12–14] suggested such an effect of HS in liver and hepatoma cell nuclei. Several other authors have provided data which showed nuclear localization of GAGs and the high affinity of nuclear proteins for binding heparin [26,27]. The regulatory role of heparin in the proliferation of smooth-muscle cells is also well known. Furthermore, Busch et al. [19] demonstrated that heparin and other sulphated polysaccharides travel to cell nuclei and regulate c-Jun/c-Fos/AP-1 transactivation; they proposed that this regulation could be a general one, and is particularly pronounced in smooth-muscle cells of blood vessels. Results have also been obtained concerning the role of sulphated polysaccharides in transcriptional regulation, either alone or together with other factors [28–31]; their regulatory potential on hepatoma cells has also been described previously [32,33]. Recently, Margolis and colleagues demonstrated the presence of biglycan and glypican in the nuclei of neurons and glioma cells [34]. We observed the inhibitory effect of liver HS on topoisomerase I, which was decreased or lost in hepatoma HS. Topoisomerase-I inhibitory action of HS and heparin was abrogated by bFGF [11].

Previously, we demonstrated that the expression of PGs changes in liver diseases [35–37]. Liver cancer was characterized by a decrease in syndecan and an increase in perlecan. Consequently, a considerable proportion of isolated HCC HS was derived from perlecan. The structural differences between the sugar chains of the two PGs might explain the functional disturbances we observed. The fact that interaction of bFGF with perlecan HS was pro-angiogenic, whereas that with syndecan HS was anti-angiogenic, supports this possibility [38–41]. Moreover, during the course of tumour progression, further alterations can be expected. Generally, undersulphated HS was characteristic for malignant tumours [42–44].

At this time, neither the structural requirements for a particular action nor the mechanism of nuclear uptake of heparin can be specified. However, we were able to demonstrate heparin and liver HS in the nuclei of several cell types [11], which argues for a direct transcriptional regulatory role of GAGs. In our experiments, paired-liver HCC HS specimens were compared. The HS of carcinoma samples was always less effective, but the extent of the decrease varied. This could be explained by the PG pattern of particular samples, because the ratio of perlecan and syndecan PGs proved to be different by immunohistochemistry.

Conflicting data have been published on the role of heparin in tumour progression and therapy [45–50]. This contradiction could be explained by the need for a well-defined HS structure, which is required to induce certain biological effects. Therefore we surmise that a better understanding of the relationship between structure and function of HS molecules will provide a new framework in which to explain the role of GAGs in malignant transformation and progression. Comparison of active and

inactive HS moieties from liver specimens would be a reasonable approach to study this problem. Therapeutically, heparin was considered to be the most effective GAG. It is known that rat liver produces HS with heparin-like structural features [51]. In our previous studies on inhibition by topoisomerases-I and -II, we also found heparin to be the most effective [11]. However, in transcription factor–DNA interaction studies, the inhibitory potential of liver HS was about five times higher than that of heparin. This, together with the reporter gene assay on HepG2 cells, supports our hypothesis that specific oligosaccharide structures are needed to exert a particular effect, such as inhibition of DNA–transcription factor interactions.

This study provided evidence that peritumoral liver HS can be a potent factor in transcriptional regulation. Consequently, it is feasible that production and transport of altered GAG moieties into the nuclei of liver cancer cells may have a significant impact on nuclear functions.

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