Stimulation of heparan sulphate synthesis in cultured rat hepatocytes by (+)-catechin

Wolfgang SINN,* Perumana R. SUDHAKARAN† and Kurt VON FIGURA*

* *Physiologisch-Chemisches Institut der Universität Münster, and † Institut für Arterioskleroseforschung an der Universität Münster, 44 Münster, Federal Republic of Germany*

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The secretion of heparan sulphate by cultured rat hepatocytes was increased in the presence of (+)-catechin. The increase was due to a new species of heparan sulphate that lacked the carbohydrate–protein linkage between xylose and serine in normal heparan sulphate proteoglycan. The mean molecular weight of this heparan sulphate varied between 6300 and 9500, was not affected by treatment with alkali or Pronase and was 2–3-fold lower than that of chains released from heparan sulphate proteoglycan. After digestion with Pronase, only a minor fraction of chains contained serine, and after treatment with alkali and NaB^3H_4 reduction less than 5% of the chains exposed [^3H]xylitol at the reducing terminals. These results suggested that (+)-catechin or metabolites of it acted as acceptors of heparan sulphate synthesis. In cultures treated with cycloheximide, synthesis of heparan sulphate decreased to less than 5%. (+)-Catechin could restore the heparan sulphate synthesis to almost normal values. The (+)-catechin-induced heparan sulphate was secreted. Only a small fraction was incorporated into the plasma membrane or other cellular compartments. This may indicate that the protein core is essential for association of heparan sulphate with cellular compartments.

Rat liver hepatocytes are distinguished from other cell types in culture by their almost exclusive synthesis of one type of glycosaminoglycan, e.g. heparan sulphate (Oldberg *et al.*, 1977; Prinz *et al.*, 1980; Sudhakaran *et al.*, 1980). Cultured rat hepatocytes maintain their capability to synthesize heparan sulphate and to distribute it into various cellular compartments and into the medium for several days. The fraction secreted into the medium as a proteoglycan comprises less than one-fifth of total heparan sulphate. About twice as much becomes associated with the cell surface (Prinz *et al.*, 1980). The heparan sulphate isolated from plasma membrane consists of three or four heparan sulphate chains attached to the protein core (Oldberg *et al.*, 1979; Mutoh *et al.*, 1980). Heparan sulphate initiated on artificial acceptors such as *p*-nitrophenyl β -D-xyloside does not associate with the cell membrane (Sudhakaran *et al.*, 1981). Part of the intracellular heparan sulphate is associated with fractions enriched in lysosomal marker enzymes. This heparan sulphate is mostly devoid of protein core and in the process of degradation. Another fraction of intracellular heparan sulphate is associated with the microsomal fraction. This heparan

sulphate is attached to protein and behaves differently from extracellular heparan sulphate (W. Sinn, unpublished work).

The biosynthesis of heparan sulphate by cultured rat hepatocytes is regulated by cyclic AMP at the level of transfer of xylose to the protein core (Sudhakaran *et al.*, 1980). In the absence of synthesis of protein core, artificial β -xylosides partially restore the synthesis of heparan sulphate (Sudhakaran *et al.*, 1981).

The flavonoid (+)-catechin [Catergen, (+)-cyanidol-3] has been introduced in the therapy of certain liver diseases, i.e. acute hepatitis, chronic and toxic hepatopathies (Bertelli, 1975). In addition, (+)-catechin has been used as a therapeutic drug in various genetic disorders affecting mesenchymal tissues, such as Marfan syndrome, Ehlers–Danlos syndrome type V and osteogenesis imperfecta (Francis *et al.*, 1976; Stöss *et al.*, 1979). The biochemical mode of action of (+)-catechin is not fully understood. Prevention of lipid peroxidation and radical-scavenging properties, prevention of lipid peroxidation as a consequence of glutathione depletion, inhibition of several enzymes, such as histidine decarboxylase, prostaglandin synthetase

and cyclic AMP phosphodiesterase, and decrease in membrane permeability may contribute to the pharmacological and biochemical activity of the drug (for review see Hennings, 1979).

We now report on the effect of (+)-catechin on heparan sulphate biosynthesis in cultured rat hepatocytes. (+)-Catechin stimulates the synthesis and secretion of heparan sulphate. The stimulation is due to the formation of heparan sulphate chains that are most probably initiated on (+)-catechin itself or metabolites thereof. In the absence of protein core synthesis, (+)-catechin restores heparan sulphate biosynthesis to almost normal values. The (+)-catechin-induced heparan sulphate is diverted from the cellular compartment and is largely secreted.

Materials and methods

Materials

[6-³H]Glucosamine hydrochloride (sp. radioactivity 19 Ci/mmol) was obtained from New England Nuclear Chemicals, Dreieich, Germany, and [³⁵S]sulphate (carrier-free, 5 Ci/mg of S), L-[3-³H]-serine (sp. radioactivity 11 Ci/mmol), [1-¹⁴C]glycine (sp. radioactivity 40 μCi/mmol) and NaB³H₄ (sp. radioactivity 7 Ci/mmol) were from Amersham, Braunschweig, Germany. Sephacryl S-300, Sephadex G-50 (fine grade) and Sephadex G-25 (fine grade) were from Pharmacia, Freiburg, Germany, and DEAE-cellulose (DE-52) was from Whatman, Maidstone, Kent, U.K. AG-1-X2 (Cl⁻ form) and AG-50-W-X8 (H⁺ form) resins were from Bio-Rad, München, Germany. Collagenase (EC 3.4.24.3) and chondroitinase ABC (EC 4.2.2.4) were from Sigma, München, Germany, and Pronase (EC 3.2.24.4) was from Merck, Darmstadt, Germany. Chondroitin sulphates of known mean molecular weight were a gift from Dr. A. Wasteson, Biomedicum, Uppsala, Sweden. (+)-Catechin was obtained from Zyma, München, Germany.

Cell culture

Hepatocytes were isolated from rat liver by the collagenase perfusion method of Seglen (1976) and maintained as monolayers in culture exactly as described previously (Ullrich *et al.*, 1979; Sudhakaran *et al.*, 1980). Experiments were started 20–24 h after seeding in 60 mm plastic dishes (Becton Dickinson, Heidelberg, Germany).

Determination of labelled heparan sulphate

Incorporation of [³⁵S]sulphate or [6-³H]-glucosamine into intracellular cell-surface-associated and secreted heparan sulphate was determined as described previously (Sudhakaran *et al.*, 1980). Unless otherwise stated the concentration of (+)-catechin dissolved directly in the media was 200 μg/ml and the incubation time was 6 h. Viability was not

affected by incubating hepatocytes in the presence of 200 μg of (+)-catechin/ml for up to 24 h, as indicated by exclusion of Trypan Blue.

Double-labelled [³H,³⁵S]heparan sulphate was prepared from the media of 15 dishes incubated in the presence of 50 μCi of [3-³H]serine/ml and of 0.5 μCi of [³⁵S]sulphate/ml. The [³H,³⁵S]heparan sulphate was isolated by Pronase digestion and precipitation with cetylpyridinium chloride (Sudhakaran *et al.*, 1980), and subjected to chromatography on DEAE-cellulose and Sephacryl S-300 (see below).

For analysis of the reducing end groups, heparan sulphate was isolated from the media of 200 dishes incubated in the presence of 0.05 μCi of [³⁵S]-sulphate/ml. The medium was concentrated by ultrafiltration and extensively dialysed against water. Half of the material was subjected to β-elimination (see below). The samples were then separately chromatographed on DEAE-cellulose and Sephacryl S-300 (see below). From controls all [³⁵S]heparan sulphate was pooled, whereas from (+)-catechin-treated cultures only the low-molecular-weight species eluted with *K_{av}* values between 0.33 and 0.68 was pooled and precipitated in 80% (v/v) ethanol containing 1% (w/v) potassium acetate.

Characterization of heparan sulphate

Digestion with chondroitinase ABC and separation of products by paper chromatography were done as described by Saito *et al.* (1968), and nitrous acid degradation at pH 1.5 was performed as described by Shively & Conrad (1976). Heparan sulphate chains were liberated from the proteoglycan by β-elimination at 37°C in 0.15 M-NaOH for 4 h. Chromatography over a 0.5 cm × 3 cm column of AG-1-X2 resin was done with a linear salt gradient made from 60 g of 6 M-urea and 60 g of 2.5 M-NaCl in 6 M-urea. Chromatography on a 0.5 cm × 3 cm column of DEAE-cellulose, equilibrated with 0.1 M-Tris/HCl buffer, pH 7.4, containing 6 M-urea, was done with a linear salt gradient made from 50 g of equilibration buffer and 50 g of 1.0 M-NaCl in the equilibration buffer.

Distribution of the molecular size of heparan sulphate was determined by gel filtration over Sephacryl S-300 columns (1 cm × 98 cm) in 50 mM-Tris/HCl buffer, pH 7.4, containing 4 M-guanidinium chloride.

Analysis of reducing end groups

[³⁵S]Heparan sulphate (for preparation see above) corresponding to 0.05 μmol of uronic acid was treated with 1.7 μmol of NaB³H₄ (sp. radioactivity 1 Ci/μmol) as described by Ögren & Lindahl (1975). The reduced samples were subjected to gel filtration on Sephadex G-25 and precipitation with cetylpyridinium chloride with the aid of 0.5 mg of

carrier chondroitin 4-sulphate. The analysis for [^3H]xylitol, [^3H]hexosaminitol and [^3H]aldonic acids was done as described previously (Ögren & Lindahl, 1975; Klein *et al.*, 1976).

Other methods

Incorporation of [^{14}C]glycine into trichloroacetic acid-precipitable material of the cell layer and the secretions was determined as described previously (von Figura *et al.*, 1979). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Radioactivity was determined in a 2450 B liquid-scintillation counter (Packard, Frankfurt, Germany), with Instagel (Packard) as scintillation medium.

Results

Effect of (+)-catechin on heparan sulphate synthesis

In the presence of 200 μg of (+)-catechin/ml, the incorporation of [^{35}S]sulphate into heparan sulphate was slightly increased. Analysis of the distribution of heparan sulphate into the cellular, the cell-surface-associated and the extracellular pool demonstrated that (+)-catechin stimulated 3.3-fold the incorporation of [^{35}S]sulphate into the heparan sulphate secreted, whereas the incorporation of [^{35}S]sulphate into cellular and cell-surface-associated heparan sulphate was even slightly diminished (Table 1). The stimulation of [^{35}S]heparan sulphate secretion was dose-dependent in the range 20–200 μg of (+)-catechin/ml (Fig. 1), observed as early as 2 h after incubation in the presence of the compound and reversible within 24 h after removal of (+)-catechin (results not shown). The incorporation of [6- ^3H]glucosamine into secreted heparan sulphate was stimulated in the presence of (+)-catechin to a similar extent as that of [^{35}S]sulphate, indicating that the amount and not only sulphation of heparan sulphate was increased. The decreased amounts of intracellular and cell-surface-associated heparan sulphate are not due to increased breakdown. During chase period for 24 h in the absence or in the presence of (+)-catechin 70% and 79% respectively of the cellular heparan sulphate were degraded.

Characterization of heparan sulphate synthesized in the presence of (+)-catechin

The ^{35}S -labelled polysaccharide secreted in the presence of (+)-catechin was characterized as heparan sulphate by its resistance towards chondroitinase ABC and susceptibility to degradation by nitrous acid. The molecular-weight distribution of degradation products after nitrous acid treatment was not affected by (+)-catechin (results not shown). Heparan sulphate chains released by β -elimination from the secreted ^{35}S -labelled polysaccharide of both control and (+)-catechin-treated cultures were eluted from AG-1-X2 resin columns at a salt concentration of 1.25 M-NaCl, as observed previously (Sudhakran *et al.*, 1980).

A major difference was found for the molecular-weight distribution of heparan sulphate se-

Fig. 1. Dose-dependence on (+)-catechin of [^{35}S]sulphate incorporation into extracellular heparan sulphate. Hepatocytes were incubated for 6 h at 37°C in the presence of up to 500 $\mu\text{g/ml}$ of (+)-catechin. Experimental details are indicated in the text. Bars indicate the range of duplicate cultures.

Table 1. Effect of (+)-catechin on [^{35}S]sulphate incorporation into heparan sulphate

Experimental details are indicated in the text. The values represent the means and ranges of duplicates of one experiment. In three different experiments the [^{35}S]sulphate incorporation into secreted heparan sulphate was 3.8-fold (2.5–5.4) and that into total heparan sulphate 1.4-fold (1.2–1.6) that of controls (ranges in parentheses).

Addition	$10^{-3} \times [^{35}\text{S}] \text{Heparan sulphate (c.p.m./mg of cell protein)}$			
	Medium	Cell surface	Cell layer	Total
None (control)	65 ± 8	118 ± 15	130 ± 10	313 ± 6
(+)-Catechin (200 $\mu\text{g/ml}$)	196 ± 1	74 ± 4	109 ± 7	379 ± 3

creted by control and by (+)-catechin-treated cultures. Most of the heparan sulphate secreted by controls was eluted near the void volume of a Sephacryl S-300 column. In secretions of (+)-catechin-treated hepatocytes this molecular-weight species represented only the minor fraction, whereas most of the heparan sulphate was eluted with a K_{av} below 0.5 (Fig. 2).

The high-molecular-weight heparan sulphate of controls and (+)-catechin-treated hepatocytes behaved as a proteoglycan. Treatment with alkali or

Fig. 2. Gel chromatography on Sephacryl S-300 of $[^{35}\text{S}]$ heparan sulphate

$[^{35}\text{S}]$ Heparan sulphate was isolated from the media of controls (a) and of (+)-catechin-treated cultures (b) and chromatographed before (○) and after (●) β -elimination. Experimental details are indicated in the text. The column was calibrated with Dextran Blue (V_{DB}), chondroitin sulphates with mean molecular weights of 19000 (V_{19}) and 5600 ($V_{5.6}$) and $^3\text{H}_2\text{O}$ (V_{T_2O}).

Pronase released heparan sulphate chains with a mean molecular weight of 19000. The mean molecular weight of the major heparan sulphate species secreted by (+)-catechin-treated cultures

Fig. 3. Gel chromatography on Sephacryl S-300 of $[^3\text{H},^{35}\text{S}]$ heparan sulphate

$[^3\text{H},^{35}\text{S}]$ Heparan sulphate was isolated from the media of controls (a) and of (+)-catechin-treated hepatocytes (b) incubated in the presence of $[3\text{-}^3\text{H}]$ serine and $[^{35}\text{S}]$ sulphate. For the isolation procedure, which included extensive proteolysis and ion-exchange chromatography, see the Materials and methods section. After chromatography on a Sephacryl S-300 column (0.45 cm $\times$ 98 cm) the fractions were analysed for ^3H (●) and ^{35}S radioactivity (○). The $^3\text{H}/^{35}\text{S}$ ratios of heparan sulphate from controls (▲) and (+)-catechin-treated cultures (△) are shown in (c). For reasons not understood, the $^3\text{H}/^{35}\text{S}$ ratio in controls did not increase with K_{av} . Contamination with other ^3H -labelled material is unlikely, since ^3H and ^{35}S radioactivity co-eluted at the preceding chromatography on DEAE-cellulose (see the Materials and methods section). The column was calibrated with Dextran Blue (V_{DB}) and $^3\text{H}_2\text{O}$ (V_{T_2O}).

varied between 6300 and 9500 and was not affected by treatment with alkali or Pronase (Fig. 2).

To exclude that heparan sulphate proteoglycan is degraded after secretion by proteinases or endoglycosidases in (+)-catechin-treated cultures, [^{35}S]-heparan sulphate secreted by controls was incubated with conditioned medium of (+)-catechin-treated cultures. The molecular-weight distribution of [^{35}S]-heparan sulphate was not affected by this treatment.

Analysis of the reducing end of heparan sulphate

The analysis of the molecular-weight distribution before and after β -elimination could not exclude that the heparan sulphate secreted by (+)-catechin-treated cultures was attached to small peptides. Heparan sulphate chains are linked via xylose to serine residues to the protein core (Mutoh *et al.*, 1980). The presence of the carbohydrate-protein linkage region was assayed by determination of the $^3\text{H}/^{35}\text{S}$ ratio of heparan sulphate secreted by hepatocytes grown in the presence of [$3\text{-}^3\text{H}$]serine and [^{35}S]sulphate and degraded with Pronase. In (+)-catechin-treated cultures the ratio was 3.3-fold lower than in controls. The difference was more striking for the shorter heparan sulphate chains that are predominantly formed in the presence of (+)-catechin and that had $^3\text{H}/^{35}\text{S}$ ratios up to 40-fold lower than control heparan sulphate chains of the same size (Fig. 3). Further evidence for the lack of *O*-glycosidically attached hydroxy amino acids in (+)-catechin-induced heparan sulphate was provided by the analysis of the reducing terminal of heparan sulphate chains obtained after β -elimination and reduction with NaB^3H_4 . In chains liberated from the heparan sulphate proteoglycan [^3H]xylitol represents the major neutral sugar alcohol (Fig. 4). In the low-molecular-weight heparan sulphate chains secreted by (+)-catechin-treated hepatocytes [^3H]xylitol was barely detectable and the [^3H]xylitol/[^{35}S]sulphate ratio was more than 11-fold lower than in controls. Taking into account the 2–3-fold lower average molecular weight of the (+)-catechin-induced chains, this result indicated that less than 5% of the chains had xylose at the reducing terminal. [^3H]Aldonic acid and [^3H]glucosaminitol were not detectable either in heparan sulphate from controls or in that from (+)-catechin-treated cultures. Thus the low-molecular-weight heparan sulphate secreted in the presence of (+)-catechin appears neither to be the product of endoglycosidases nor to be initiated on protein.

Effect of (+)-catechin on heparan sulphate synthesis in cycloheximide-treated hepatocytes

The analysis of the reducing terminal of heparan sulphate secreted in the presence of (+)-catechin suggested that these chains were initiated on

Fig. 4. Characterization of [^3H]xylitol [^{35}S]heparan sulphate secreted by controls (a) and by (+)-catechin-treated cultures (b) was treated with NaB^3H_4 before (.....) and after (—) β -elimination. The neutral sugar fraction was obtained after hydrolysis in 1M-HCl for 16h at 100°C and chromatography over AG-50-W-X8 and AG-1-X2 resin (Klein *et al.*, 1976). The neutral sugar fraction obtained from equal amounts of heparan sulphate (based on ^{35}S radioactivity) were subjected to chromatography on Whatman 3MM paper in butan-1-ol/acetone/water (2:7:1, by vol.) for 16h. As standards [^3H]glucitol (Glc-ol), [^3H]galactitol (Gal-ol) and [^3H]xylitol (Xyl-ol) were used.

(+)-catechin or metabolites thereof. It was therefore assayed whether in the absence of protein synthesis (+)-catechin would allow the synthesis of heparan sulphate. In the presence of 1mM-cycloheximide, synthesis of heparan sulphate was diminished to less than 5% of that of controls. The remaining heparan sulphate secreted behaves as a proteoglycan (Sudhakaran *et al.*, 1981). In the presence of (+)-catechin, the heparan sulphate synthesis in hepatocytes treated with cycloheximide was restored to almost normal values (Table 2). However, the distribution of the heparan sulphate synthesized under these conditions differs markedly from that of controls. More than 80% of total [^{35}S]heparan sulphate was secreted, whereas in controls less than 20% was recovered in the medium. The molecular-weight distribution of (+)-catechin-induced heparan sulphate in the presence of cycloheximide was the same as that in the absence of cycloheximide.

Table 2. Effects of (+)-catechin on incorporation of [^{35}S]sulphate into heparan sulphate and of [$1\text{-}^{14}\text{C}$]glycine into trichloroacetic acid-precipitable material in cycloheximide-treated hepatocytes

Hepatocytes were incubated for 30 min in the presence of 1 mM-cycloheximide before either [^{35}S]sulphate or [$1\text{-}^{14}\text{C}$]glycine was added, either with or without (+)-catechin. Experimental details are indicated in the text. The values represent the means and ranges of duplicates.

Cycloheximide (1 mM)	(+)-Catechin (200 $\mu\text{g}/\text{ml}$)	$10^{-3} \times [^{35}\text{S}]\text{Heparan sulphate (c.p.m./mg of cell protein)}$				$10^{-3} \times ^{14}\text{C-labelled material}^*$ (c.p.m./mg of cell protein)
		Medium	Cell surface	Cell layer	Total	
—	—	59 $\pm$ 9	105 $\pm$ 10	116 $\pm$ 10	280 $\pm$ 5	1461 $\pm$ 4
—	+	316 $\pm$ 3	71 $\pm$ 4	66 $\pm$ 6	453 $\pm$ 4	999 $\pm$ 1
+	—	6 $\pm$ 30	3 $\pm$ 22	3 $\pm$ 2	12 $\pm$ 20	223 $\pm$ 30
+	+	170 $\pm$ 5	10 $\pm$ 5	22 $\pm$ 9	202 $\pm$ 7	95 $\pm$ 1

* Incorporation of [$1\text{-}^{14}\text{C}$]glycine into cellular and secreted acid-precipitable material was determined and the sum of both is given.

Discussion

The analysis of the (+)-catechin-induced heparan sulphate suggested that in rat hepatocytes (+)-catechin or metabolite(s) of (+)-catechin act as acceptor for heparan sulphate synthesis. This assumption is based on the observation that (+)-catechin-induced heparan sulphate (i) was of low molecular weight and not affected by β -elimination or digestion with Pronase, (ii) was characterized by low xylose and serine contents and (iii) exposed none of the known constituents of the carbohydrate-protein linkage at the reducing terminal. Furthermore (+)-catechin induced the synthesis of heparan sulphate in the absence of protein synthesis. The covalent linkage of (+)-catechin to heparan sulphate could not be demonstrated directly. The amounts of heparan sulphate synthesized were too low, and radioactive (+)-catechin of sufficiently high specific radioactivity to demonstrate the initiation of heparan sulphate on (+)-catechin was not available.

The effect of (+)-catechin on heparan sulphate synthesis was not seen in human skin fibroblasts and chicken chondrocytes (P. R. Sudhakaran, unpublished work). This may indicate that a liver-specific metabolite of (+)-catechin is the acceptor or that the specificity of one of the glycosyltransferases involved in chain initiation of heparan sulphate is less absolute in hepatocytes than in other cell types. The galactosyltransferase that transfers galactose to xylose is known to act on various artificial xylosides and to have less rigid substrate requirements than the other glycosyltransferases involved in the formation of the carbohydrate-protein linkage region (Okayama *et al.*, 1973; Schwartz *et al.*, 1974; Galligani *et al.*, 1975; Robinson *et al.*, 1975). (+)-Catechin, which contains five hydroxyl groups (I), has no obvious structural similarity with serine (threonine) or the carbohydrates in the carbohydrate-protein linkage region GlcUA-Gal-Gal-Xyl,

(I) (+)-Catechin

but some of its metabolites are converted into glucuronides (Das, 1971).

Whereas β -xylosides are used as artificial acceptors of heparan sulphate synthesis by hepatocytes only in the absence of protein synthesis (Sudhakaran *et al.*, 1981), (+)-catechin induced heparan sulphate synthesis in the absence and in the presence of protein synthesis. Unlike (+)-catechin-induced chains, β -xyloside-induced chains were only slightly shorter than chains initiated on core protein but contained twice as much sulphate groups per carbohydrate (Sudhakaran *et al.*, 1981). It seems noteworthy that in the presence of (+)-catechin the secretion of heparan sulphate proteoglycan was not inhibited and the amounts of cell-associated heparan sulphate were only moderately decreased.

At present it is unknown whether (+)-catechin initiates heparan sulphate synthesis *in vivo* also and whether the action in heparan sulphate synthesis in hepatocytes is related to the therapeutic effect of (+)-catechin discussed for various diseases affecting liver (Hennings, 1979).

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