Effects of aging on the synthesis of antithrombin-binding sites on heparin chains and heparan sulphate chains in the rat

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[³⁵S]Heparin proteoglycans were isolated from the skins and peritoneal mast cells of male rats aged 2 to 22 months. Their [³⁵S]heparin chains were separated on antithrombin–agarose into fractions with high and low affinities for antithrombin. In skin, the proportion of ³⁵S-labelled high-affinity heparin chains declined from 23 % at 2 months to 8 % at 12 months and did not change significantly between 12 and 22 months. In peritoneal mast cells, the proportion of ³⁵S-labelled high-affinity heparin chains increased from 14 % at 2 months to 21 % at 4 months and then did not vary significantly until 15 months of age. By 21 months a consistent and significant decline to 8 % occurred. The

structures of high-affinity heparin proteoglycans did not change with age. Their decreased proportions, without change in their structure, may indicate that they are produced by a unique subset of mast cells, the proportion of which declines with age. [³⁵S]heparan sulphate chains were isolated from skins and brains of rats in the same age range and fractionated on antithrombin–agarose. There were no significant variations in the proportions of ³⁵S-labelled high-affinity heparan sulphate chains in skin (10 %) or brain (24 %) between 4 and 22 months of age.

INTRODUCTION

Heparin proteoglycans (PGs) are produced by serosal (connective tissue type) mast cells (MCs). Rat skin heparin proteoglycan preparations are mixtures of PGs with low binding affinity for antithrombin III (LA-PGs) in which all the polysaccharide chains have low binding affinity for antithrombin III (AT) and therefore very low specific anticoagulant activities (LA-chains), and PGs with high binding affinity for AT (HA-PGs) in which some of the polysaccharide chains have high binding affinity for AT (HA-chains) [1,2]. HA-chains have one to five sites with high binding affinity for AT (AT-binding sites, ATBSs) and corresponding degrees of high specific anticoagulant activity [3]. The unique structure of the ATBS has been elucidated by Lindahl and coworkers (see, for example, [4]).

Heparan sulphates (HSs), less highly sulphated glycosaminoglycans with many structural similarities to heparins [5], used to be considered devoid of anticoagulant activity. A report of relatively low anticoagulant activity in some HS preparations [6] stimulated studies that led to the isolation of HS with high affinity for antithrombin (HA-HS), containing specific ‘marker’ components of the ATBS sequence, originally considered unique to HA-heparins [7,8].

HS is synthesized by almost all mammalian cells, but only a few normal cell types produce HA-HS. Rosenberg and coworkers (see, for example, [9]) have shown that between 1 % and 10 % of the HS produced by cultured endothelial cells and fibroblasts is HA-HS. A study of the incorporation of [³⁵S]sulphate into HS in various organs of young adult rats [10] found proportions of ³⁵S-labelled HA-HS (expressed as a percentage of total HS radioactivity) ranging from 4.7 % (kidneys) to 21.5 % (brain). These values are percentages of the total HS produced by all cell types in these organs. The proportions of HA-HS made by the specific HA-HS-synthesizing cell types in these organs must be substantially higher than the above total organ values, which suggests that ATBS synthesis can occur more efficiently *in vivo* than *in vitro*.

The mechanisms controlling ATBS synthesis *in vivo* are not yet known. *In vitro*, ATBS synthesis on heparin chains by mouse mastocytoma cells [11] and on HS chains by endothelial cells [12] is stimulated by sodium butyrate, an agent that can induce or modulate gene transcription in many cell types (see, for example, [13]). Recent work on HSPG synthesis suggests that ‘regulatory components’ in the Golgi apparatus may coordinate the activities of the enzymes involved in synthesizing the specific monosaccharide sequence of an ATBS [14,15].

Past studies of rat skin heparin PGs reported from this laboratory have used male Wistar rats in the weight range 250–330 g, corresponding to an age range of ~ 2–3 months. The proportion of HA-heparin PGs in different preparations has ranged from 60 % [1] to 32 % [16], with most values being in the 40–50 % range. In more recent unpublished work, heparin PGs were prepared from the combined skins of 6 ‘retired breeder’ male Wistar rats in the weight range 500–550 g (estimated age 8 months). The proportion of HA-heparin PGs was only 21 %, which suggested that the efficiency of synthesis of HA-heparin in skin MCs might decline with age. The present study was planned primarily to test this hypothesis by using Fischer 344 rats, which are preferred over the Wistar strain for aging studies [17]. The opportunity was also taken to analyse HA-heparin and HA-HS from other sources in the same animals. Heparin was recovered from skin and from MCs in the peritoneal cavity. HS was recovered from skin and also from brain, because brain had the highest proportion of HA-HS chains in young adult Wistar rats studied earlier [10].

MATERIALS AND METHODS

Animals

Forty-four male Fischer 344 rats of known ages obtained from Harlan Sprague–Dawley (Indianapolis, IN, U.S.A.) were assigned to 11 age groups, ranging from 2 to 22 months. They were supplied with standard chow and tap water *ad libitum*. Twenty-eight rats from which skin, brain and peritoneal mast

cell glycosaminoglycans (GAGs) were to be recovered each received an intraperitoneal injection of ~ 8 mCi of $\text{H}_2^{35}\text{SO}_4$ in 1 ml of 0.15 M NaCl. Sixteen rats in the age range 16–21 months from which only the peritoneal mast cell GAGs were to be recovered each received ~ 2 mCi of $\text{H}_2^{35}\text{SO}_4$. Animals were killed 24 h after injection, by decapitation after sedation with pentobarbital.

Purification of peritoneal mast cells

Peritoneal cavities were rinsed with approximately 3 ml per 100 g body weight of a solution containing 0.15 M NaCl, 2 mM trisodium citrate and bovine serum albumin (2 g/l) adjusted to pH 7.4 with 1 M NaOH and warmed to 37 °C. Mast cells were purified by a modification of the method of Shanahan et al. [18], using a discontinuous gradient of Percoll (Pharmacia). An isotonic stock solution of '100%' Percoll (as defined by the manufacturer) was prepared by mixing 90 ml of Percoll with 10 ml of $10\times$ Hanks solution without CaCl_2 or MgCl_2 (Gibco), adding NaHCO_3 (35 mg) and adjusting the pH from 7.8 to 7.4 with H_3PO_4 (5 μl). Solutions containing 30% and 75% Percoll were prepared by dilution with $1\times$ Hanks solution containing NaHCO_3 (350 mg/l) and BSA (1 g/l), pH 7.4. Gradients were prepared in 17×100 mm polystyrene tubes (Falcon). A 75% Percoll solution (1 ml) was over-layered with 2 ml of 30% Percoll, and peritoneal washings (10 ml) were layered over the gradient. Centrifuging for 15 min at 600 g at ambient temperature pelleted the MC at the bottom of the tube and retained all other cell types at or above the 30%/75% Percoll interface. The mast cells were resuspended in peritoneal wash solution and an aliquot was stained with an equal volume of Toluidine Blue solution (0.5 mg/ml in 0.15 M NaCl). MC purity was consistently > 95%, the main contaminants being erythrocytes.

The following experimental procedures have been described in detail previously [10].

Isolation of [^{35}S]heparin PGs and [^{35}S]heparan sulphate chains from skin

Individual solvent-extracted skins were digested with Pronase and the insoluble material remaining washed with 2 M NaCl containing dextran sulphate (0.2 mg/ml). The total sulphated glycosaminoglycans (GAGs) were precipitated with cetyl pyridinium chloride in 0.4 M NaCl, solubilized in 2.4 M NaCl and precipitated with ethanol. They were then incubated with chondroitin ABC lyase (0.2 units per ml). Undigested GAGs were precipitated with ethanol, dried, incubated with crude pancreatic DNAase (40 Kunitz units per ml) and fractionated by elution from DEAE-Sephacel with a linear NaCl concentration gradient at pH 2.5. This procedure separates heparin completely from less highly sulphated GAGs. The action of chondroitin ABC lyase is partly inhibited by heparin [19]. Therefore a peak containing [^{35}S]HS chains and incompletely degraded galactosaminoglycans eluted immediately before the heparin peak (see Figure 1 in [10]). The HS-containing material was recovered by precipitation with ethanol and incubated with chondroitin ABC lyase again. [^{35}S]HS eluted in the void volume when the incubation mixture was chromatographed on Sephadex G-25. It was recovered by precipitation with ethanol. Heparin PGs, the predominant form of heparin in rat skin, have Pronase-resistant peptide cores [20] and were recovered after gel filtration on Sepharose CL-4B [21] by precipitation with ethanol. Half of each heparin PG preparation was treated with NaOH to yield heparin chains [20].

Isolation of [^{35}S]heparan sulphate chains from brain

The procedure was as described above for skin, with the exception that a single digestion with chondroitin ABC lyase sufficed, as there is no heparin in brain.

The purity of [^{35}S]heparan sulphate products was assessed by agarose gel electrophoresis in 0.03 M barium acetate [10] and by the pH 1.5 nitrous acid degradation reaction [22].

Heparin from skin and HS from skin and brain were determined quantitatively by metachromasia with 1,9-dimethyl Methylene Blue [23], using commercial pig mucosal heparin and chondroitin sulphate from whale cartilage as standards.

Isolation of [^{35}S]heparin proteoglycans from peritoneal mast cells

NaCl was added to each MC suspension to a concentration of 1 M. Ethanol (3 volumes) was then added. After standing overnight at 4 °C, a precipitate was recovered by centrifuging (4800 g at 0 °C for 2 h), washed with ethanol and dried at 4 °C. This product was digested with Pronase and the insoluble material remaining washed with 2 M NaCl containing dextran sulphate (0.2 mg/ml). Commercial pig mucosal heparin (1 mg) was added as a carrier. The wash was filtered through a 0.2 μm cellulose acetate filter and the NaCl concentration of the combined digest and wash was adjusted to 0.2 M before fractionation on DEAE-Sephacel. [^{35}S]Heparin was recovered by precipitation with ethanol and fractionated by gel filtration on Sepharose CL-4B [21]. Part of each proteoglycan preparation was treated with NaOH to yield heparin chains.

Fractionations on antithrombin-agarose

Each heparin chain product was separated into LA- and HA-heparin chain fractions by elution from antithrombin-agarose with a discontinuous NaCl concentration gradient (see, for example, [16]). Radioactivity was determined by β -scintillation counting in Complete Phase Combining System (PCS; Amersham).

Several intact skin heparin PG products were fractionated on AT-agarose. The HA-PGs were recovered and treated with NaOH. The resultant chains were mixed with samples of ^3H -end-labelled HA-heparin chains [21], prepared from the skin of a two-month-old rat, and refractionated on antithrombin-agarose (see Figure 3).

Each [^{35}S]HS chains product was separated into fractions with no affinity, low affinity and high affinity for antithrombin, as defined previously, by elution from a column of antithrombin-agarose with a discontinuous NaCl concentration gradient (see Figure 3 in [10]).

Statistical analyses

Data were analysed with the SigmaStat program (Jandel Scientific, San Raphael, CA, U.S.A.). The distribution of the percentage of HA-heparin chain values was normalized with the arcsin square-root transform. Data distribution was then tested for normality with the Kolmogorov-Smirnov test and for equal variance about age-group means by the Levene median test. Acceptable data were tested by one-way analyses of variance, combined with Student-Newman-Keuls multiple-comparison tests. Student's *t*-test was used for post-hoc comparisons between age-group mean values. $P < 0.05$ was accepted as statistically significant in all tests.

Figure 1 Separations of ^{35}S -labelled LA- and HA-heparin chains, prepared from total rat skin ^{35}S heparin proteoglycans, by gradient elution from a column of antithrombin-agarose

○, Chains from a 2-month-old rat; ●, chains from a 19-month-old rat. The broken line shows the NaCl concentration gradient.

Figure 2 The proportions of HA-heparin chains in the total heparin proteoglycans from skin and peritoneal mast cells of male rats aged 2–22 months

Proportions of HA-heparin chains, determined by fractionations of total ^{35}S heparin chains on antithrombin-agarose, are expressed as percentages of total ^{35}S heparin chain radioactivity. The histograms show age-group mean values $\pm$ S.D. for skin (solid bars) and peritoneal mast cells (hatched bars). $n = 4$ for each age group.

RESULTS

Aging effect on skin heparin PGs

Gel filtration on Sepharose CL-4B showed that PGs constituted $\sim 90\%$ of the total ^{35}S heparin radioactivity in skin at all ages (results not shown; see, for example, Figure 1 in [21]). The mean values for total (i.e. labelled and unlabelled) heparin PG concentrations for rats aged 2, 8, 12 and 22 months (means $\pm$ S.D.) were 146 ± 17 , 140 ± 19 , 142 ± 29 and 130 ± 24 μg heparin per g dry, defatted, shaved skin respectively. There were no statistically significant differences between age groups.

Figure 3 Fractionations of skin ^{35}S heparin chains, prepared from ^{35}S HA-heparin PGs and mixed with ^3H HA-heparin chains, by gradient elution from a column of antithrombin-agarose

○, ^{35}S -labelled chains (LA- and HA-) from rat skin HA-PGs; ●, ^3H HA-chains (contaminated with $\sim 5\%$ LA-chains) from the skin of a 2-month-old rat. The ^{35}S -labelled products are from rats aged 2 months (a), 8 months (b) and 15 months (c). The same NaCl concentration gradient (represented by the dashed line in a) was applied in all three fractionations.

In Figure 1, two examples of separations of mixtures of ^{35}S -labelled LA-chains and HA-chains (prepared from the total skin heparin PGs) on a column of AT-agarose illustrate the marked difference in the proportions of ^{35}S HA-heparin chains synthesized at 2 months of age (22% of total heparin PG radioactivity) and at 19 months (6%). This type of analysis was applied to 28 rats in all, ranging in age from 2 months to 22 months. The data are summarized in Figure 2. All the data for skin heparin showed normal distribution and variance. The age-group mean percentages of HA-heparin chains in skin decreased from 22.8% at 2 months to 15.9% at 4 months, 11.2% at 8 months and 7.9% at 12 months. Each decrease with increasing age was statistically significant. There were no significant differences between the mean values at 12, 15, 19 and 22 months.

Figure 3 shows fractionations of ^{35}S -labelled chains from the HA-PGs of rats aged 2, 8 and 15 months. The proportions of ^{35}S HA-chains, expressed as percentages of total HA-PG radioactivity, were 42.4%, 41.2% and 41.4% respectively. Each mixture of ^{35}S -labelled chains was co-chromatographed with an aliquot of ^3H -end-labelled HA-chains prepared from the skin of a two-month-old rat. The precise co-elution of ^3H - and ^{35}S -labelled HA-chains in a NaCl concentration gradient illustrates their essentially identical average ATBS densities. This approach has been used previously to demonstrate variability of ATBS density on HA-HS chains [10].

Table 1 The concentrations of HS and proportions of [³⁵S]HA-HS in brains and skins of male rats aged 2–22 months

Concentrations of HS, determined with dimethyl Methylene Blue [23], are expressed per g of moist brain weight and per g of dry, defatted, shaved skin. Proportions of [³⁵S]HA-HS, determined by fractionation on antithrombin-agarose, are expressed as percentages of total [³⁵S]HS radioactivity. All values are means $\pm$ S.D. $n = 4$ for each age group. Abbreviation used: ND, not determined.

Age (months)	Brain		Skin	
	[HS] (μ g/g)	HA-HS (%)	[HS] (μ g/g)	HA-HS (%)
2	ND	29.0 $\pm$ 1.4	ND	9.0 $\pm$ 0.5
4	22.8 $\pm$ 2.1	24.7 $\pm$ 1.4	128 $\pm$ 12	9.6 $\pm$ 0.7
8	21.2 $\pm$ 1.7	24.2 $\pm$ 1.7	120 $\pm$ 14	9.6 $\pm$ 1.4
12	ND	ND	ND	9.5 $\pm$ 0.5
15	20.9 $\pm$ 1.7	22.9 $\pm$ 2.1	110 $\pm$ 25	9.7 $\pm$ 0.5
19	ND	ND	ND	9.2 $\pm$ 0.6
22	21.9 $\pm$ 1.6	23.3 $\pm$ 2.0	117 $\pm$ 25	10.4 $\pm$ 0.5

Aging effect on peritoneal mast cell heparin PGs

Gel filtration on Sepharose CL-4B showed that PGs constituted at least 95 % of the total [³⁵S]heparin radioactivity in peritoneal MCs at all ages (results not shown). The use of carrier heparin precluded the determination of peritoneal MC heparin mass. The data for peritoneal MC [³⁵S]HA-heparin chain proportions, expressed as percentages of the total [³⁵S]heparin proteoglycan radioactivity, are summarized in Figure 2 to facilitate comparison with the skin heparin data.

The data for peritoneal heparins at 2, 4, 8, 12, 15, 21 and 22 months were distributed normally. Statistical analyses of these data showed a significant increase in the mean percentage of HA-heparin chains from 14.1 % at 2 months to 21.1 % at 4 months, with no significant differences between group mean values at 4, 8, 12 and 15 months. The decrease from 21.7 % at 15 months to 8.1 % at 21 months and the subsequent decrease to 5.4 % at 22 months were both statistically significant.

The total data for peritoneal heparins failed the test for normal distribution. This was due to the marked variability between individual values at 16, 17, 19 and 20 months, as indicated by the large standard deviations for these age groups in Figure 2. For example, values for HA-heparin chain proportions at 20 months ranged from 5.1 % to 21.0 %. This suggests that there are large individual variations in the age at which the efficiency of ATBS synthesis declines in peritoneal MC.

There were no significant differences between the mean percentages of HA-heparin chains in skin at 2 months and in peritoneal MCs at 4, 8, 12 and 15 months.

Student's *t*-tests of differences in the mean percentages of HA-heparin chains between skin and peritoneal heparins from the same age group showed significant differences at 2, 4, 8, 12 and 15 months, but not at 22 months.

Lack of an aging effect on heparan sulphate chains in skin and brain

Agarose gel electrophoresis and degradation with nitrous acid confirmed that the purification of HS chains from skin was impaired by the presence of heparin. A second digestion with chondroitin ABC lyase in the absence of heparin (i.e. after separation on DEAE-Sephacel) gave pure skin HS. Brain HS was free from contamination by other GAGs after one incubation with chondroitin ABC lyase.

The data for concentrations of HS and proportions of [³⁵S]HA-HS in brains and skins are presented in Table 1. HS concentrations were determined from 4 to 22 months of age. There were no statistically significant differences between mean age-group values for brains or skins.

The proportions of [³⁵S]HA-HS in the purified HS products, which are expressed as percentages of total [³⁵S]HS radioactivity, were determined from 2 to 22 months of age. For brain HA-HS, the group mean value at 2 months (29.0 %) was significantly higher than in all other age groups tested. There were no significant differences between any of the other age groups (overall mean value 23.8 %). For skin HA-HS, there were no statistically significant differences between any of the age groups (overall mean value 9.6 %).

DISCUSSION

This study's main objective was to determine the effects of aging on the proportions of newly synthesized heparin chains and HS chains containing ATBSs. As heparin PGs are Pronase-resistant [20,24], it was also possible to determine the proportions of HA-heparin chains in newly synthesized HA-heparin PGs.

The concentrations of heparin PGs in the skins of male Fischer 344 rats did not decline with age. This finding is consistent with an earlier report that skin heparin concentrations did not decline with age in male Wistar rats [25]. However, the present study showed that one specific aspect of rat skin heparin PG biosynthesis, ATBS synthesis, was markedly age-dependent. After the onset of puberty at 2 months, HA-heparin chain synthesis in rat skin declined by 65 % by 12 months and was then maintained at a constant lower level until 22 months, the oldest age studied. (The median life span of the male Fischer 344 rat is approximately 24 months [17].)

Although the proportion of HA-heparin PGs synthesized in skin declined with increasing age, HA-heparin PG structure did not change significantly. Figure 3 shows that the proportions and average ATBS densities of HA-chains from the HA-heparin PGs of rats aged 2, 8 and 15 months were essentially identical.

In contrast with skin MC heparin, the efficiency of ATBS synthesis on heparin chains in rat peritoneal MC increased in early adult life, from 2 to 4 months of age, when it attained the level expressed in skin MC at 2 months. This level was maintained from 4 months until 15 months, by which time ATBS synthesis in skin MC was at its lowest level. Between 16 months and 20 months there were marked differences between peritoneal HA-heparin chain proportions in rats in the same age group. A decline in HA-heparin chain levels that was both marked and consistent was not seen until 21 months of age.

The synthesis of the specific monosaccharide sequence of an ATBS may require 'regulatory components' in the Golgi apparatus to coordinate the activities of the enzymes involved [14,15]. The final stage in the synthesis of HA-heparin chains by mouse mastocytoma cells is believed to be the 3-*O*-sulphation of the internal glucosamine-*N*-sulphate residue of the ATBS pentasaccharide sequence [26]. The variations in proportions of HA-heparins from different sources of heparin in rats [27] indicate that the expression and/or regulation of the 3-*O*-sulphotransferase that completes the biosynthesis of HA-heparin chains is tissue-specific. The present study shows that age is another important factor affecting HA-heparin synthesis.

Interpretation of the observed age-related changes is hindered by our incomplete understanding of heparin PG biosynthesis in MCs. The MCs in rat skin make a high proportion of LA-PGs plus a wide range of different HA-PG species [16], but we do not

know whether each individual MC produces a single type of PG or a mixture of PG species. One possible explanation of an age-dependent decrease in the proportion of HA-heparin PGs synthesized, without a change in HA-heparin PG structure, is that there is a unique subset of HA-heparin PG-producing MCs that maintains its HA-heparin-synthesizing phenotype throughout the lifespan. In this model, the proportion of MCs with this phenotype declines to a basal level in skin by 1 year of age, but is maintained at a high constant level in the peritoneal cavity until at least 15 months of age. This site-specific difference presumably correlates with localized differences in the expression of microenvironmental factors required to maintain the phenotype. Stem cell factor and interleukin 3 are cytokines required to maintain the heparin-synthesizing phenotype of the serosal MC *in vitro* [28], but the question of the regulation of ATBS synthesis has not been specifically addressed.

In contrast with the age-dependent changes in HA-heparin synthesis, the efficiency of expression of the ATBS sequence on HA-HS chains remained unchanged in brain from 4 months to 22 months and in skin from 2 months to 22 months of age. The present work confirms that HA-heparin synthesis and HA-HS synthesis are controlled independently. It has been shown earlier, using young adult (2 to 3 month-old) male Wistar rats, that source-dependent variations in HA-heparin proportions [27] do not correlate with source-dependent variations in HA-HS proportions [10].

It was shown recently that the four members of the syndecan family of HS-containing transmembrane proteoglycans, which were probed for the extracellular domains of their core proteins, are expressed in distinct cell-specific and tissue-specific patterns in mice [29]. HA-HS chains are components of ryoducan, the syndecan synthesized by rat microvascular endothelial cells [30]. The current work shows that tissue-specific patterns of HA-HS expression are maintained in the rat throughout adult life.

The presence of endogenous heparin in normal plasma has never been demonstrated convincingly. There is evidence that HA-HS on the luminal surface of endothelial cells can promote thrombin-antithrombin complex formation [31], but recent studies with perfused rat hearts [32] suggest that neutralization of thrombin by thrombomodulin is the primary anticoagulant mechanism on the vascular endothelium. It is also now clear that most of the HA-HS produced by endothelial cells is on their abluminal surfaces or in adjacent subendothelial basement membrane [33]. Antithrombin III is known to permeate to extravascular spaces [34] and its association with subendothelial basement membrane, which can be abrogated by treatment with heparitinase or heparinase, has been demonstrated [33,35].

It has been proposed that HA-HS [33,36] and HA-heparin [37] both act as extravascular anticoagulants. HA-HS produced by endothelial cells and also by fibroblasts [38,39] could provide a basal level of extravascular anticoagulation. HA-heparin is stored intracellularly in MC granules and could be released rapidly and intermittently as required, providing a high localized level of anticoagulation to counteract the procoagulant activity of macrophages at inflammation sites [37].

Currently, one can only speculate on the relative importance of HA-heparin and HA-HS as physiological anticoagulants. The body's total potential anticoagulant activity from HA-heparin and HA-HS has been estimated in the young adult male Wistar rat at 53 units of anti-factor IIa activity and 60 units of anti-factor Xa activity [10]. HA-heparin accounts for 83% of the potential anti-factor IIa activity and 73% of the potential anti-factor Xa activity. Therefore loss of skin HA-heparin with age

lowers the body's content of ATBS-based anticoagulant activity markedly in the rat, because skin is the main source of heparin in this species [27]. The possible loss of endogenous anticoagulant activity due to aging makes this an important area for further studies (a) to characterize the mechanisms controlling ATBS synthesis and (b) to establish unequivocally the roles of HA-HS and HA-heparin as endogenous anticoagulants.

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