



Title	Analysis of the structure and neuritogenic activity of chondroitin sulfate/dermatan sulfate hybrid chains from porcine fetal membranes
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6 **Analysis of the Structure and Neuritogenic Activity of Chondroitin**
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8 **Sulfate/Dermatan Sulfate Hybrid Chains from Porcine Fetal**
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10 **Membranes**

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16 **Taishi Hashiguchi, Shuji Mizumoto, Shuhei Yamada, and Kazuyuki Sugahara**
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22 **Abstract** The amniotic membrane (AM) is the innermost layer of fetal membranes and
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24 possesses various biological activities. Although the mechanism underlying these
25
26 biological activities remains unclear, unique components seem to be involved. AM
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28 contains various extracellular matrix components such as type I collagen, laminin,
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30 fibronectin, hyaluronic acid, and proteoglycans bearing chondroitin sulfate/dermatan
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32 sulfate (CS/DS) glycosaminoglycan side chains. To elucidate the function of CS/DS in
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34 AM, the structure and bioactivity of the CS/DS chains from porcine fetal membranes
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36 (FM-CS/DS) were investigated. A compositional analysis using various chondroitinases
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38 revealed that the characteristic DS domain comprised of iduronic acid-containing
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40 disaccharide units is embedded in FM-CS/DS, along with predominant disaccharide
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42 units, GlcA-GalNAc, GlcA-GalNAc(4-*O*-sulfate), and GlcA-GalNAc (6-*O*-sulfate),
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44 where GlcA and GalNAc represent D-glucuronic acid and *N*-acetyl-D-galactosamine,
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46 respectively. The average molecular size of FM-CS/DS chains was unusually large and
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48 estimated to be 250 - 300 kDa. The FM-CS/DS chains showed neurite
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3 outgrowth-promoting activity with a dendrite-like morphology, which was eliminated
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5 by digestion with chondroitinase ABC of the CS/DS chains. This activity was
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7 suppressed by antibodies against growth factors including pleiotrophin, midkine, and
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9 fibroblast growth factor-2. The binding of these growth factors to FM-CS/DS was also
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11 demonstrated by surface plasmon resonance spectroscopy.
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19 **Keywords** Chondroitin sulfate, Dermatan sulfate, L-Iduronic acid, Amniotic membrane,
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21 Fetal membranes, Neurite outgrowth-promoting activity.
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27 **Abbreviations**

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29 GAG, glycosaminoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan
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31 sulfate; KS, keratan sulfate; HA, hyaluronic acid; AM, amniotic membrane; FM, fetal
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33 membranes; IdoA, L-iduronic acid; GlcA, D-glucuronic acid; GalNAc,
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35 N-acetyl-D-galactosamine; FGF, fibroblast growth factor-2; PTN, pleiotrophin; MK,
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37 midkine; HGF, hepatocyte growth factor; P-ORN, poly-DL-ornithine; HPLC, high
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39 performance liquid chromatography; CSase, chondroitinase; NOP, neurite
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41 outgrowth-promoting; 2AB, 2-aminobenzamide; Δ HexA,
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43 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid; IU, international units;
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3 **Introduction**
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6 Glycosaminoglycans (GAGs) are involved in the regulation of various
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8 biological processes such as cell proliferation, differentiation, and migration, cell-cell
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10 recognition, extracellular matrix deposition, and tissue morphogenesis [1-4]. GAGs
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12 include chondroitin sulfate/dermatan sulfate (CS/DS), heparan sulfate (HS)/heparin,
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14 keratan sulfate (KS), and hyaluronic acid (HA). CS/DS chains are found ubiquitously in
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16 extracellular matrices and at cell surfaces. The sugar backbone of CS/DS consists of
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18 repeating disaccharide units: D-glucuronic acid (GlcA) β /L-iduronic acid
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20 (IdoA) α 1-3*N*-acetyl-D-galactosamine (GalNAc). During chain elongation in the
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22 biosynthesis of CS/DS, the disaccharide units are modified by specific sulfotransferases
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24 at C-2 of GlcA/IdoA, and C-4 and/or C-6 of GalNAc in various combinations, and
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26 display enormous structural diversity producing characteristic sulfation patterns critical
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28 for the binding to a variety of functional proteins [4]. This structural variability of
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30 GAGs is the basis for a wide range of their biological activities.
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41 CS/DS plays crucial roles in various biological events such as the
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43 development of the central nervous system [4], wound healing [5, 6], and growth factor
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45 signaling [3, 7]. Among these events, the involvement of CS/DS in the development of
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47 the central nervous system has attracted attention from a therapeutic point of view for
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49 the potential application of CS/DS to nerve regeneration. Several studies have shown
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51 that the sulfation pattern of brain CS/DS alters during development, characterized by an
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53 increase in 4-*O*-sulfation and a decrease in 6-*O*-sulfation in the whole brain [8], as well
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3 as a dramatic increase in the proportion of IdoA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate)
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5 units in the cerebellum [9]. The proportion of oversulfated disaccharides and the
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7 presence of IdoA are crucial factors for the neurite outgrowth-promoting (NOP) activity
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9 of CS/DS [10-14]. CS/DS chains from embryonic pig brains contain a higher proportion
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11 of IdoA than those prepared from adult pig brains [12]. They promote the outgrowth of
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13 neurites of embryonic mouse hippocampal neurons in culture by capturing at least
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15 endogenous pleiotrophin (PTN) produced by non-neuronal cells (glial cells) and
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17 hepatocyte growth factor (HGF), and then presenting them to neuronal cells to promote
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19 neuritogenesis [4, 15, 16].
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27 The amniotic membrane (AM) is the innermost layer of fetal membranes
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29 (FMs) and possesses various biological activities. Since AM reduces ocular surface
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31 inflammation and scarring, and promotes rapid epithelialization, it has been applied to
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33 the treatment of several ocular disorders [17]. The proliferation of corneal epithelial
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35 cells was promoted when cultivated on human AM [18]. In addition, both mouse and
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37 human embryonic stem cells efficiently differentiate into neural precursors when
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39 cultured on human AM matrix components [19]. Although the mechanism underlying
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41 the biological activities of AM remains unclear, unique components seem to be involved.
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43 AM contains various extracellular matrix components such as type I collagen, laminin,
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45 fibronectin, HA, and proteoglycans including CS, HS, and KS chains [20]. Since GAGs
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47 have been implicated in various biological processes as described above, we
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49 hypothesized that GAGs in AM may play a major role in the biological activities of AM.
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3 Notably, CS/DS in AM may be involved in the induction of the differentiation of
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5 embryonic stem cells into neuronal precursors. Hence, in this study GAGs were isolated
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7 from porcine FMs containing amnions, and their structure and NOP activity were
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9 analyzed.
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16 **Materials and methods**

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19 *Materials* The following sugars and enzymes were purchased from Seikagaku Corp.
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21 (Tokyo, Japan): CS-E from squid cartilage, standard unsaturated disaccharides,
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23 chondroitinase (CSase) ABC (EC 4.2.2.4) from *Proteus vulgaris*, CSase AC-I (EC
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25 4.2.2.5) from *Flavobacterium heparinum*, CSase AC-II (EC 4.2.2.5) from *Arthrobacter*
26
27 *aurescens*, keratanase II (EC 3.2.1.) from *Bacillus sp.*, and hyaluronidase (EC 4.2.2.1)
28
29 from *Streptomyces hyalurolyticus*. CSase B (EC 4.2.2.19), and heparinases (Hepases) I
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31 (EC 4.2.2.7) and III (EC 4.2.2.8) from *Flavobacterium heparinum* were purchased from
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33 IBEX Technologies (Montreal, Canada). Porcine FMs were obtained from Hokudo Corp.
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35 (Hokkaido, Japan). Pregnant ddY mice were purchased from SLC Inc. (Shizuoka,
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37 Japan). Poly-DL-ornithine (P-ORN), anti-neurofilament and anti-HGF-antibodies, and
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39 serum immunoglobulins from mouse and goat, were purchased from Sigma. Actinase E
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41 was obtained from Kaken Pharmaceutical Co. (Tokyo, Japan).
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43 Anti-microtubule-associated protein 2 was purchased from Leico Technologies Inc. (St.
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45 Louis, MO). Recombinant human (rh)-PTN, rh-fibroblast growth factor-2 (FGF-2), and
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47 rh-HGF were purchased from Wako Corp. (Osaka, Japan). Anti-FGF-2 was from
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3 Upstate Biotechnology, Inc. (New York, NY). Anti-PTN and anti-midkine (MK)
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5 antibodies, and rh-MK were purchased from R&D Systems (Minneapolis, MN). Accell
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7 Plus QMA anion-exchange cartridges were obtained from Waters Corp. Dialysis
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9 membranes were purchased from Spectrum laboratories Corp. (Rancho Dominguez,
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19 *Extraction of the GAG-peptide fraction from porcine FMs* Porcine FMs (350 g in wet
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21 weight) were cut into pieces with scissors, boiled in 650 ml of water for 10 min to
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23 inactivate proteases and glycosidases, cooled, and digested with 3 g of actinase E in a
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25 total volume of 900 ml of 0.1 M borate-sodium buffer, pH 8.0, containing 10 mM
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27 calcium chloride as described previously [21]. The digestion was carried out at 60 °C
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29 for 96 h, and an additional 1.5 g each of the enzyme was added after 24, 48, and 72 h.
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31 Following incubation, the sample was precipitated with 5% trichloroacetic acid and
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33 centrifuged. The supernatant was treated with diethylether to remove trichloroacetic
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35 acid. The aqueous phase was neutralized using 1 M sodium carbonate, and treated with
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37 80% ethanol containing 1% sodium acetate at 4 °C overnight. The resultant precipitate
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39 was reconstituted in 160 ml of water, and subjected to anion-exchange chromatography
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41 on an Accell QMA Plus cartridge (Waters, Milford, MA), which was eluted stepwise
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43 with 50 mM phosphate buffers, pH 6.0, containing 0.15, 1.0, and 2.0 M NaCl [21]. Each
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45 eluted fraction was desalted using a Spectra/Por dialysis membrane (Spectrum
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47 laboratories Corp., Rancho Dominguez, CA). Uronic acid in each fraction was
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3 quantified by the carbazole method [22].
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8 *Purification of FM-CS/DS* The fractions, which were eluted with buffers containing 1.0
9 and 2.0 M NaCl from an Accell QMA Plus cartridge, presumably contained
10 GAG-peptides from FMs, and were digested with hyaluronidase from *Streptomyces*
11 *hyalurolyticus* to eliminate HA as described previously [13]. The resultant HA
12 fragments were removed by Centricon YM-10 (cut off molecular weight: 10,000)
13 (Millipore, Bedford, MA). The 1.0 and 2.0 M NaCl-eluted fractions were then subjected
14 to nitrous acid treatment to eliminate HS as reported previously [13], and the resultant
15 HS fragments were removed by Centricon YM-10. The 1.0 and 2.0 M NaCl-eluted
16 fractions were then digested with keratanase II in a 10 mM sodium acetate buffer, pH
17 6.5, at 37 °C for 1 h, and the resultant KS fragments were removed by Centricon YM-10.
18 Finally, the purified fractions were treated with 1.0 M NaBH₄/0.5 M NaOH at room
19 temperature for 2 h to liberate peptides from GAG polysaccharides. The samples were
20 acidified with acetic acid to decompose excess NaBH₄, neutralized with 1 M sodium
21 carbonate, and passed through a Sep-PakC₁₈ (Waters) cartridge to remove peptide
22 fragments. The purified 1.0 and 2.0 M NaCl-eluted FM-CS/DS fractions were used for
23 the structural and functional analyses.
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52 *Enzymatic digestion* The GAG-peptide fractions from FMs, which were eluted using 1.0
53 and 2.0 M NaCl from an Accell QMA Plus cartridge, were digested with a mixture of
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3 CSases ABC and AC-II, CSase B, a mixture of CSases AC-I and AC-II [13], or a
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5 mixture of Hepases I and III [23]. Unsaturated di- and oligosaccharides produced were
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7 derivatized with 2-aminobenzamide (2AB), and then analyzed by anion-exchange
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9 HPLC on an amine-bound silica PA-03 column (YMC Co., Kyoto, Japan) [24]. The
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11 GAG-peptide fractions from FMs were also digested with keratanase II as described
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13 above. The digests were derivatized with 2AB, and then analyzed by anion-exchange
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15 HPLC on an amine-bound silica PA-03 column [24].
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25 *Gel-filtration chromatography of the FM-CS/DS preparations* The FM-CS/DS
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27 preparations (20 µg each as CS/DS) were subjected to gel-filtration chromatography on
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29 a column of Sephacryl S-400 HR16/60 (GE healthcare, Uppsala, Sweden) using 0.2
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31 M ammonium acetate as an effluent with a flow rate of 0.4 ml/min. Fractions were
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33 collected at 1min intervals, lyophilized, and digested with CSase ABC. The digests were
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35 derivatized with 2AB, and then analyzed by HPLC on an amine-bound silica column
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47 *Analysis of the distribution of IdoA residues in the FM-CS/DS preparations* The 1.0 and
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49 2.0 M NaCl-eluted FM-CS/DS preparations (1 µg each as GAG) were digested with
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51 CSase AC-I. Each digest was labeled with 2AB, and analyzed by gel-filtration HPLC on
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53 a column (10 X 300 mm) of SuperdexTM Peptide HR10/330 (Amersham Biosciences,
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55 Uppsala, Sweden), which was eluted with 0.2 M ammonium bicarbonate at a flow rate
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3 of 0.4 ml/min. The percentage of galactosaminidic bonds cleaved by CSase AC-I was
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6 calculated based on the fluorescent intensity across peaks relative to the total amount of
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8 the disaccharides generated by digestion with CSase ABC [12]. The molar ratio of IdoA
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10 to GlcA (IdoA/GlcA) was calculated based on the peak area as follows: (the amount of
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12 total disaccharides released by CSase ABC minus the amount of oligo- and
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14 disaccharides released by CSase AC-I)/(the amount of oligo- and disaccharides released
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16 by CSase AC-I).

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25 *Assays for NOP activity* Cultures of hippocampal cells were established from E16 mice
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27 as previously described [11, 25]. Briefly, 2 µg/well of a FM-CS/DS preparation was
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29 coated onto coverslips precoated with P-ORN at 4 °C overnight. The hippocampal cells
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31 were freshly isolated from E16 mouse embryos, suspended in Eagle's minimum
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33 essential medium containing N2 supplements [26], seeded on coverslips at a density of
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35 20,000 cells/cm², and allowed to grow in a humidified atmosphere for 24 h at 37 °C
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37 with 5 % CO₂.

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44 For the inhibition assay using antibodies, polyclonal anti-PTN (10 µg/ml),
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46 anti-MK (10 µg/ml), anti-FGF-2 (10 µg/ml), or anti-HGF (10 µg/ml) antibodies were
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48 added to the medium, 2 h after the seeding of the hippocampal cells. After incubation
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50 overnight, the cells were fixed using 4% (w/v) paraformaldehyde for 1 h at room
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52 temperature, and the neurites were visualized by immunochemical staining using
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54 anti-microtubule associated protein 2 (Lieco Technomogies Inc., St. Louis, MO) and
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3 anti-neurofilament (Sigma) antibodies as described [11]. The antibodies were then
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5 detected using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA) with
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7 3,3'-diaminobenzidine as a chromogen. NOP activity was evaluated by determining the
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9 length of the longest neurite and the number of primary neurites of 100 randomly
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11 selected neurons using FLVFS-LS software (Olympus, Tokyo, Japan).
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19 *Surface plasmon resonance analysis* The interaction of various growth/neurotrophic
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21 factors with the FM-CS/DS preparations was examined using a BIAcore X system
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23 (BIAcore AB, Uppsala, Sweden) as reported [12]. For the kinetic analysis, various
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25 concentrations of each growth/neurotrophic factor were injected onto the surface of the
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27 FM-CS/DS-immobilized sensor chip in the running buffer, pH 7.4 (HBS-EP, BIAcore
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29 AB), containing 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% (w/v) Tween
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31 20 at a flow rate of 30 μ l/min [12]. Before each injection, base-line stabilization was
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33 achieved by injecting 1.0 M NaCl for 2 min. The kinetic parameters were evaluated
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35 with BIAevaluation software 4.1 (BIAcore AB) using a 1:1 binding model with mass
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37 transfer, and the association and dissociation rate constants (k_a and k_d) as well as the
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39 dissociation equilibrium constants (K_d) were determined.
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52 **Results**

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54 *Characterization of GAGs in porcine FMs* To characterize GAGs in porcine FMs, a
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56 GAG-peptide fraction was prepared by digestion with actinase E, followed by
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3 trichloroacetic acid treatment and ethanol precipitation. This GAG-peptide fraction was
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5 further fractionated by anion-exchange chromatography using an Accell™ Plus QMA
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7 cartridge, which was eluted stepwise with buffers containing 0.15, 1.0, and 2.0 M NaCl.
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9 Only trace amounts of GAGs were detected in the fraction eluted with the buffer
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11 containing 0.15 M NaCl. The fractions eluted with 1.0 and 2.0 M NaCl were digested
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13 with a mixture of CSases ABC and AC-II, a mixture of Hepases I and III, or keratanse II.
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15 The digests were labeled with a fluorophore 2AB and analyzed by anion-exchange
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17 HPLC, and the GAG disaccharide composition in each fraction was quantified based on
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19 the fluorescent intensity of the derivatives (Table 1).
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28 Representative chromatograms for the CS/DS disaccharide analysis are shown
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30 in Fig. 1, and the data are summarized in Table 2. The FM-CS/DS chains in the 1.0 M
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32 NaCl-eluted fraction were low sulfated as evidenced by a significant proportion of
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34 nonsulfated disaccharide unit, Δ HexA-GalNAc (40.5%), with a sulfate/disaccharide unit
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36 ratio of 0.6, whereas the FM-CS/DS chains in the 2.0 M NaCl-eluted fraction were
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38 enriched with monosulfated disaccharide units, Δ HexA-GalNAc(4-*O*-sulfate) (61.0%),
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40 and Δ HexA-GalNAc(6-*O*-sulfate) (27.6%), with a sulfate/disaccharide unit ratio of 0.9
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47 (Table 2).
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50 To examine whether the FM-GAG fractions contain DS, an anion-exchange
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52 HPLC analysis was carried out after digestion with a mixture of CSases AC-I and AC-II
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54 or CSase B alone, which specifically cleaves the *N*-acetylgalactosaminidic linkages
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56 flanked by GlcA or IdoA residues, respectively (Fig. 1 and Table 2). The GAG-peptide
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3 fractions were sensitive to the action of both types of enzymes, suggesting that the
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6 CS/DS chains have a hybrid structure.
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9 Disaccharides derived from HA were also detected in the digest with a
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11 mixture of CSases ABC and AC-II. The total amounts of HA disaccharides produced by
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13 digestion of the 1.0 and 2.0 M NaCl-eluted GAG-peptide fractions with CSases were
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15 1,085 and 104 pmol per mg of porcine FM (wet weight), respectively (Table 1). The
16
17 GAG-peptide fractions were also subjected to a compositional analysis of HS
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19 disaccharides after digestion with a mixture of Hepases I and III. The total amounts of
20
21 HS disaccharides produced were only 57 and 2 pmol per mg of porcine FM (wet
22
23 weight), respectively. When the digest of the GAG-peptide fractions with keratanase II
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25 was analyzed by anion-exchange HPLC, KS disaccharides were detected only in the 2.0
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27 M NaCl-eluted GAG-peptide fraction and the amount was 11 pmol per mg of porcine
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29 FM (wet weight).
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38 The molecular size of CS/DS chains in the 1.0 and 2.0 M NaCl-eluted
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40 fractions was analyzed by gel-filtration chromatography. An aliquot of each eluate was
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42 subjected to digestion with CSase ABC and the digests were labeled with 2AB and
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44 analyzed by anion-exchange HPLC. The amount of the 2AB-derivatives of unsaturated
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46 CS/DS disaccharides was calculated based on fluorescent intensity. Using a calibration
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48 plot generated with data obtained with polysaccharides of known sizes (Fig. 2, *inset*),
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50 the average molecular size of the major CS/DS chains in the 1.0 and 2.0 M NaCl-eluted
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52 fractions was estimated to be 301 and 249 kDa, respectively (Fig. 2).
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3 To further characterize the structural properties of the IdoA-containing
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5 FM-CS/DS preparations, the distribution of IdoA residues along the chains was
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7 compared between the 1.0 and 2.0 M FM-CS/DS preparations. Each FM-CS/DS
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9 preparation was treated with CSase AC-I, and each digest was labeled with 2AB and
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11 analyzed by gel-filtration HPLC on a column of Superdex peptide. As shown in Fig. 3,
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13 fluorescent peaks derived from each digest were detected at the elution of positions
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15 corresponding to hexa- and tetrasaccharides, in addition to di-, mono-, and nonsulfated
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17 disaccharides. Considering the substrate specificity of CSase AC-I (27), the
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19 oligosaccharide resistant to CSase AC-I is most likely derived from the sequences
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21 containing IdoA residues. Upon digestion of the 1.0 M NaCl-eluted FM-CS/DS fraction,
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23 disaccharides were predominantly detected as shown in Fig. 3A. On the other hand, the
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25 digestion of the 2.0 M NaCl-eluted FM-CS/DS fraction with CSase AC-I gave
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27 oligosaccharides ranging in size from disaccharides to polysaccharides (Fig. 3B). The
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29 oligosaccharides in the digest obtained with CSase AC-I are composed of contiguous
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31 IdoA-containing disaccharide units. The molar ratio of IdoA to GlcA plus IdoA was
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33 calculated based on the peak area, and in the 1.0 and 2.0 M NaCl-eluted FM-CS/DS
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35 fractions was 0.23 and 0.62, respectively. These results suggest that the proportion of
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37 the characteristic DS domain constituted of IdoA-containing disaccharide units is
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39 approximately 3 times higher in the 2.0 M than 1.0 M NaCl-eluted FM-CS/DS fraction.
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57 *NOP activity of the FM-CS/DS preparations* To evaluate the biological activity of the
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3 CS/DS chains in FM, they were purified from the GAG-peptide fractions of porcine FM.
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5 HA, HS and KS were removed from the GAG-peptide fractions by digestion with
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7 hyaluronidase, treatment with nitrous acid, and digestion with keratanase II, respectively.
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10 The purified fractions were treated with 1.0 M NaBH₄/0.5 M NaOH at room
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12 temperature for 2 h to liberate CS/DS chains from the core peptides, and the products
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14 were passed through a Sep-PakC₁₈ cartridge to remove peptide fragments.
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19 The purified 1.0 and 2.0 M NaCl-eluted FM-CS/DS fractions were assessed
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21 for NOP activity. Mouse E16 hippocampal cells were cultured on plastic coverslips
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23 coated with P-ORN and then with the 1.0 or 2.0 M NaCl-eluted FM-CS/DS fraction
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25 (Figs. 4A and 4B) or CS-E (Fig. 4C) as a positive control. After incubation for 24 h, the
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27 cells were fixed and immunostained with anti-microtubule-associated protein 2 and
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29 anti-neurofilament antibodies. In the negative control experiment, cells were cultured on
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31 coverslips coated with P-ORN alone (Fig. 4D). The mean length of the longest neurite
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33 and the number of primary neurites per cell of 100 randomly selected neurons cultured
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35 on various substrates were measured.
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44 The mean length of the longest neurites was significantly greater for the
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46 neurons cultured on the FM-CS/DS preparations than for the cells cultured on
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48 coverslips coated with P-ORN alone (Fig. 5). The number of primary neurites was also
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50 larger for the neurons cultured on the FM-CS/DS preparations than those cultured on
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52 P-ORN alone (Fig. 5). Although only a single long neurite per neuron was usually
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54 observed for neurons cultured on P-ORN alone, neurons grown on the plates coated
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3 with the 1.0 and 2.0 M NaCl-eluted FM-CS/DS fractions projected 1.5 and 2.6 neurites
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5 per cell, respectively (Fig. 5B). The NOP activity level of the 2.0 M NaCl-eluted
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7 fraction was significantly higher than that of the 1.0 M NaCl-eluted fraction. The NOP
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9 activity of the FM-CS/DS fractions was eliminated by digestion with CSase ABC (Fig.
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11 5), suggesting the polymeric structure of CS/DS chains but not contaminants to indeed
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13 be required for the expression of the NOP activity.
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19 Although both the FM-CS/DS preparations and CS-E displayed marked NOP
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21 effects (Fig. 4), the morphology of the neurons cultured on these substrates was
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23 different. The neurons cultured on the CS-E-coated substrate exhibited a round-shaped
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25 morphology (Fig. 4C), being consistent with previous observations [11]. On the other
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27 hand, the neurons cultured on the FM-CS/DS preparations extended multiple neurites
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29 and showed a flattened dendrite-like morphology (Figs. 4A and B) as observed for the
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31 neurons cultured on a CS-D-coated substrate [25].
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38 CS/DS specifically interacts with PTN, MK, FGF-2, and HGF, which have
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40 neurotrophic activity and are widely expressed as autocrine and paracrine pleiotropic
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42 factors in distinct neuronal cell populations of the developing and adult brain, including
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44 hippocampal neuronal cells [28-30]. Hence, these endogenous heparin-binding growth
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46 factors may be involved in the mechanism of the NOP activity of FM-CS/DS. To verify
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48 this possibility, antibodies against HGF, PTN, MK, and FGF-2 were individually added
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50 and it was examined whether the NOP activity of FM-CS/DS chains toward
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52 hippocampal neurons was neutralized. The addition of anti-MK antibody markedly
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3 suppressed the NOP activity of 1.0 M NaCl-eluted FM-CS/DS chains to the background
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5 level of P-ORN, suggesting that the NOP activity of the 1.0 M NaCl-eluted FM-CS/DS
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7 chains is expressed mainly through the signaling pathway of MK (Fig. 6A). On the
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9 other hand, the NOP activity of the 2.0 M NaCl-eluted FM-CS/DS chains was
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11 suppressed by the antibodies against PTN and FGF-2 by 54 and 51%, respectively,
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13 suggesting the involvement of the signaling pathways of PTN and FGF-2 in the
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15 expression of the NOP activity of the 2.0 M NaCl-eluted FM-CS/DS chains.
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25 *Kinetic analysis of the binding of growth factors to FM-CS/DS* The NOP activity of the
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27 FM-CS/DS chains was suppressed by the antibodies against PTN, MK, and FGF-2 (Fig.
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29 6). Since CS/DS chains have been demonstrated to act as co-receptors for various
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31 growth factors [7], interactions with these heparin-binding growth factors may be
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33 involved in the promotion of neurite outgrowth by the FM-CS/DS chains. To examine
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35 this possibility, a kinetic analysis of the binding of these growth factors to immobilized
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37 FM-CS/DS chains was carried out using the BIAcore system. The FM-CS/DS
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39 preparations were biotinylated and immobilized on the streptavidin-precoated sensor
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41 chip. The heparin-binding growth factors (PTN, MK, FGF-2, and HGF) were injected at
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43 different concentrations individually onto the surface of the sensor chip. Overlaid
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45 sensorgrams are shown in Fig. 7. These sensorgrams were analyzed with BIAevaluation
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47 4.1 software using the "1:1 binding model with mass transfer" to calculate the kinetic
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49 parameters (Table 3).
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3 Although MK and FGF-2 bound to the 1.0 M NaCl-eluted FM-CS/DS chains,
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5 PTN and HGF did not (data not shown). MK displayed quick binding and slow
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7 dissociation, giving a K_d value of 27.1 nM and signifying their strong affinity for the 1.0
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9 M NaCl-eluted FM-CS/DS chains. On the other hand, all the growth factors tested
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11 bound to the 2.0 M NaCl-eluted FM-CS/DS chains. PTN and MK showed high affinity,
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13 displaying K_d values of 463.4 and 53.3 nM, respectively. The kinetic parameters for the
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15 binding of FGF-2 and HGF are not shown in Table 3, because good fitting models were
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17 not obtained for their sensorgrams (data not shown). However, the data on the binding
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19 of FGF-2 and HGF to the FM-CS/DS chains can be used qualitatively.
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30 **Discussion**

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32 In this study, to elucidate the biological activities of CS/DS chains in AM, the structure
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34 and bioactivity of FM-CS/DS chains were investigated. The disaccharide compositional
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36 analysis showed that the major components of the FM-CS/DS chains are GlcA-GalNAc,
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38 GlcA-GalNAc(4-*O*-sulfate), and GlcA-GalNAc(6-*O*-sulfate) disaccharide units, and
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40 that IdoA-containing disaccharide units are clustered in the polysaccharide chains.
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42 Because of the flexibility of its sugar ring, IdoA shows a conformational diversity [31,
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44 32], which may provide IdoA-containing GAGs with a range of finely tuned affinities
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46 for different bioactive proteins [33]. DS, which mainly consists of
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48 IdoA-GalNAc(4-*O*-sulfate) disaccharide units, promotes the binding of FGF-7 to a FGF
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50 receptor and the cell proliferation of keratinocytes [6], and also acts as a co-receptor
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3 required for the formation of an active HGF signaling complex [34, 35]. The DS-like
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5 structure in *Xenopus* CS/DS was reported to be involved in its binding to FGF-1 [36].
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8 The unusually flexible ring conformation of IdoA residues in FM-CS/DS may be
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10 involved in the biological activities of AM such as promotion of the proliferation of
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12 corneal epithelium [18] and regeneration of esophagus and skin [37, 38] mediated by
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14 growth factors' signaling.
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19 Based on the analysis of the molecular sizes of the 1.0 and 2.0 M NaCl-eluted
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21 FM-CS/DS preparations, the average molecular sizes of the 1.0 and 2.0 M NaCl-eluted
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23 FM-CS/DS chains were estimated to be 301 and 249 kDa, respectively. These chains are
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25 linear polymers consisting of approximately 500 - 600 repeating disaccharide units and
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27 unusually larger than the CS/DS chains from other sources including embryonic pig
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29 brain (40 kDa) [12], shark skin (70 kDa) [13], shark liver (76 kDa) [16], and hagfish
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31 notochord (18 kDa) [14]. The markedly large CS chains (350 kDa) attach to type IX
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33 collagen in the chick embryo vitreous humor [39], which are presumed to distribute in a
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35 regular periodic arrangement along the collagen fibrils. AM has been utilized for the
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37 treatment of several corneal diseases such as corneal opacity and damaged corneas [18].
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39 Since normal cornea abundantly contains collagen fibrils, the markedly large size of
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41 FM-CS/DS may contribute to the treatment of corneal diseases.
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52 The NOP activity of the FM-CS/DS preparations was analyzed in this study to
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54 investigate potential applications to nerve regeneration. The 1.0 M NaCl-eluted
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56 FM-CS/DS exhibited NOP activity with a dendrite-like morphology, and this activity
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3 was completely inhibited by the antibody against a neurotrophic factor MK, which
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5 showed a strong affinity for the 1.0 M NaCl-eluted FM-CS/DS chains displaying a K_d
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7 value of 53.3 nM. Several studies have demonstrated that endogenous
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9 growth/neurotrophic factors such as PTN, HGF, MK, and brain-derived neurotrophic
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11 factor are recruited by CS/DS chains and then presented to neuronal cells to promote
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13 neuritogenesis [4, 15, 16, 40]. These findings suggest that endogenous MK appears to
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15 be recruited by the 1.0 M NaCl-eluted FM-CS/DS chains and involved in the induction
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17 of the NOP activity. MK is a heparin-binding neurotrophic factor that promotes the
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19 growth, survival, and migration of various cells [41]. A cluster of
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21 GlcA-GalNAc(4,6-*O*-disulfate) units in CS-E from squid cartilage exhibits strong
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23 binding to MK ($K_d = 62$) [42, 43]. However, the FM-CS/DS chains contain little
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25 GlcA-GalNAc(4,6-*O*-disulfate) unit (0.03 %), indicating that the functional domains in
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27 the 1.0 M NaCl-eluted FM-CS/DS chains for the MK-binding do not appear to contain
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29 GlcA-GalNAc(4S,6S) units, but rather a cluster of IdoA-containing disaccharides,
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31 which interacts with MK [12] and influences the biological activity of MK, may be
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33 involved [44].
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46 The 2.0 M NaCl-eluted FM-CS/DS preparation also displayed marked NOP
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48 activity with a dendrite-like morphology, and its activity level was significantly higher
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50 than that of the 1.0 M NaCl-eluted FM-CS/DS chains. A contribution of
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52 IdoA-containing disaccharide units to the neuritogenic activity of CS/DS chains has
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54 been demonstrated using CS/DS preparations derived from embryonic pig brain [12],
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3 shark skin [13], and hagfish notochord [14]. Consecutive IdoA residues appear to be
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5 essential for the NOP activity of the FM-CS/DS chains, being consistent with the
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7 observation that the 2.0 M NaCl-eluted FM-CS/DS chains contain a higher proportion
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9 of IdoA-containing disaccharide units than the 1.0 M NaCl-eluted FM-CS/DS chains.
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11 The NOP activity of the 2.0 M NaCl-eluted FM-CS/DS chains was diminished by the
12
13 antibodies against PTN and FGF-2 by 54 and 51%, respectively, suggesting the
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15 involvement of the signaling pathways of PTN and FGF-2 in the expression of the NOP
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17 activity of the 2.0 M NaCl-eluted FM-CS/DS chains. However, the NOP activity of the
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19 2.0 M NaCl-eluted FM-CS/DS chains was not completely eliminated by the addition of
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21 the mixture of these antibodies (data not shown). Recently, Mikami *et al.* reported that
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23 CS-E can act as an effective ligand for the neuronal cell surface receptor contactin-1
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25 that stimulates the intracellular signaling pathway involved in neuritogenesis [45]. Cell
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27 adhesion molecules such as contactin-1 may be involved in the induction of the NOP
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29 activity of the 2.0 M NaCl-eluted FM-CS/DS chains to some extent.
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41 The matrix components of human AM can convert embryonic stem cells into
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43 neural tissues selectively [19]. Since CS/DS is a major component of the extracellular
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45 matrix, the NOP activity of the FM-CS/DS chains demonstrated in this study may be
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47 involved in the neurogenerative activity of AM. AM has also been applied to several
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49 ocular disorders, because it can promote epithelialization, inhibit fibrosis, and reduce
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51 ocular surface inflammation [17]. In addition, it has antimicrobial and antiviral
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53 properties and high hydraulic conductivity [17]. Basement membrane components in
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3 AM can improve the morphogenesis of epidermis and basement membranes in
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5 constructing a human living skin-equivalent [38]. The *in situ* tissue-engineered
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7 esophagus, which is prepared by cultivating oral keratinocytes and fibroblasts on human
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9 AM, can successfully replace the intrathoracic esophagus [37]. Since CS/DS is involved
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11 in biological events such as cell proliferation [3, 6], viral infection [46], inflammation
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13 [47], anti-coagulation [33], and wound healing [5, 6, 48], it may contribute to some of
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15 the biological activities of AM described above. The information obtained in this study
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17 facilitates deeper understanding of the biological activities of AM, and suggests
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19 potential applications of FM-CS/DS chains.
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3 **Footnotes**
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3 **Figure legends**
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5 **Fig. 1** Anion-exchange HPLC of CSase digests of the 2.0 M NaCl-eluted FM-CS/DS
6 fraction. The 2.0 M NaCl-eluted FM-CS/DS fraction was digested with a mixture of
7 CSases ABC and AC-II (A), a mixture of CSases AC-I and AC-II (B), or CSase B (C).
8 Each digest was labeled with 2AB, and analyzed by anion-exchange HPLC on an
9 amine-bound silica PA-03 column using a linear NaH₂PO₄ gradient (indicated by the
10 *dashed line*). The elution positions of authentic 2AB-labeled unsaturated disaccharides
11 are indicated by *bars*. 1, ΔHexA-GlcNAc; 2, ΔHexA-GalNAc; 3, ΔHexA
12 -GalNAc(6-*O*-sulfate); 4, ΔHexA-GalNAc(4-*O*-sulfate); 5, ΔHexA(2-*O*-sulfate)
13 -GalNAc(6-*O*-sulfate); 6, ΔHexA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate); 7, ΔHexA
14 -GalNAc(4,6-*O*-disulfate). The peaks marked by *asterisks* are derived from the
15 2AB-labeling reagents. The insets in (A) and (C) show enlarged views (100-fold and
16 20-fold, respectively).
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41 **Fig. 2** Determination of the molecular size of the FM-CS/DS chains by gel-filtration
42 chromatography. The FM-CS/DS preparations eluted with buffers containing 1.0 or 2.0
43 M NaCl (20 μg of each as GAG) were fractionated on a column of Sephacryl S-400
44 using 0.2 M ammonium acetate as an effluent with a flow rate of 1 ml/min. Fractions
45 were collected at 2 min intervals. Each fraction was subjected to digestion with CSase
46 ABC and labeled with 2AB. The 2AB-labeled digest was analyzed by anion-exchange
47 HPLC on an amine-bound silica PA-03 column. The amount of the 2AB-derivatives of
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3 unsaturated CS/DS disaccharides was calculated based on fluorescent intensity. The
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5 *inset* shows a calibration curve giving a linear relationship between $\log M_r$ and elution
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7 time, which was prepared using size-defined commercial polysaccharides; dextran
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9 (average M_r : 200,000, 70,000, 18,100). V_0 and V_t represent the void volume and the
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11 total volume, respectively.
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19 **Fig. 3** Analysis of the distribution of IdoA residues in the FM-CS/DS preparations.
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22 The 1.0 M (A) and 2.0 M NaCl-eluted (B) FM-CS/DS preparations (1 μg of each as
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24 GAG) were digested with CSase AC-I, and the digests were labeled with 2AB and
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26 analyzed by gel-filtration HPLC on a Superdex peptide column. V_0 , void volume; V_t ,
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28 total volume. The elution positions of authentic unsaturated CS oligosaccharides labeled
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30 with 2AB are indicated by *bars*: 1, hexasaccharides; 2, tetrasaccharides; 3, trisulfated
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32 disaccharide; 4, disulfated disaccharides; 5, monosulfated disaccharides; 6, nonsulfated
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34 disaccharide.
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44 **Fig. 4** Morphology of E16 mouse hippocampal neurons cultured on coverslips coated
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46 with the FM-CS/DS preparations. E16 mouse hippocampal cells (20,000 cells/cm²)
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48 were cultured on plastic coverslips coated with P-ORN and then with the 1.0 M (A) or
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50 2.0 M (B) NaCl-eluted FM-CS/DS preparation, or CS-E (C) as a positive control (2 μg
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52 of each as GAG). The cells were fixed and stained with anti-microtubule-associated
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54 protein 2 and anti-neurofilament antibodies. In the control experiments, cells were
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3 cultured on coverslips coated with P-ORN alone (D). *Scale bar*, 50 μm .
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8 **Fig. 5** NOP activity of the FM-CS/DS preparations. E16 mouse hippocampal cells
9 (20,000 cells/cm²) were cultured for 24 h on plastic coverslips coated with P-ORN and
10 then with the 1.0 or 2.0 M NaCl-eluted FM-CS/DS preparation, or with their CSase
11 ABC digests (2 μg of each as GAG). The cells were fixed and stained with
12 anti-microtubule-associated protein 2 and anti-neurofilament antibodies. In the control
13 experiments, cells were cultured on coverslips coated with P-ORN alone. The mean
14 length of the longest neurite per cell (A) and the number of primary neurites per cell (B)
15 of 100 randomly selected neurons cultured on various substrates were measured (see
16 "Materials and methods"). The statistical analysis was performed using
17 Mann-Whitney's *U* test (*n.s.*, not significant; *, $p < 0.01$; **, $p < 0.001$).
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38 **Fig. 6** Effects of antibodies against growth factors and neurotrophic factors on the NOP
39 activity of the FM-CS/DS preparations. E16 mouse hippocampal cells (20,000
40 cells/cm²) were cultured on plastic coverslips coated with P-ORN and then with the 1.0
41 or 2.0 M NaCl-eluted FM-CS/DS preparation (2 μg of each as GAG). Two hours after
42 seeding, anti-PTN (10 $\mu\text{g}/\text{ml}$), anti-MK (10 $\mu\text{g}/\text{ml}$), anti-FGF-2 (10 $\mu\text{g}/\text{ml}$), or anti-HGF
43 (10 $\mu\text{g}/\text{ml}$) antibodies were added to the culture, and incubated for 24 h. The mean
44 length of the longest neurite was evaluated as described in the legend to Fig. 5. The
45 same amount of an IgG from the corresponding animal serum (mouse or goat) was used
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3 as a negative control. Values represent the means \pm S.D. obtained from three
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5 independent experiments.
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10 **Fig. 7** Binding of various growth factors to immobilized FM-CS/DS chains. Various
11 concentrations of MK (A, D), FGF-2 (B, E), PTN (C), or HGF (F) were injected onto
12 the surface of a sensor chip immobilized with 1.0 M (A, B) or 2.0 M NaCl-eluted
13 FM-CS/DS chains (C-F). Sensograms obtained with various concentrations of each
14 growth/neurotrophic factor were overlaid using BIAevaluation software (version 4. 1).
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Figure1
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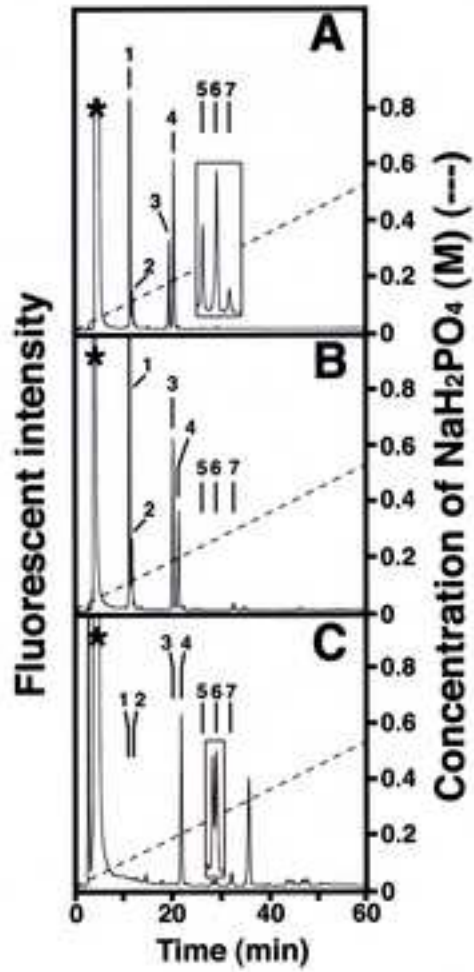
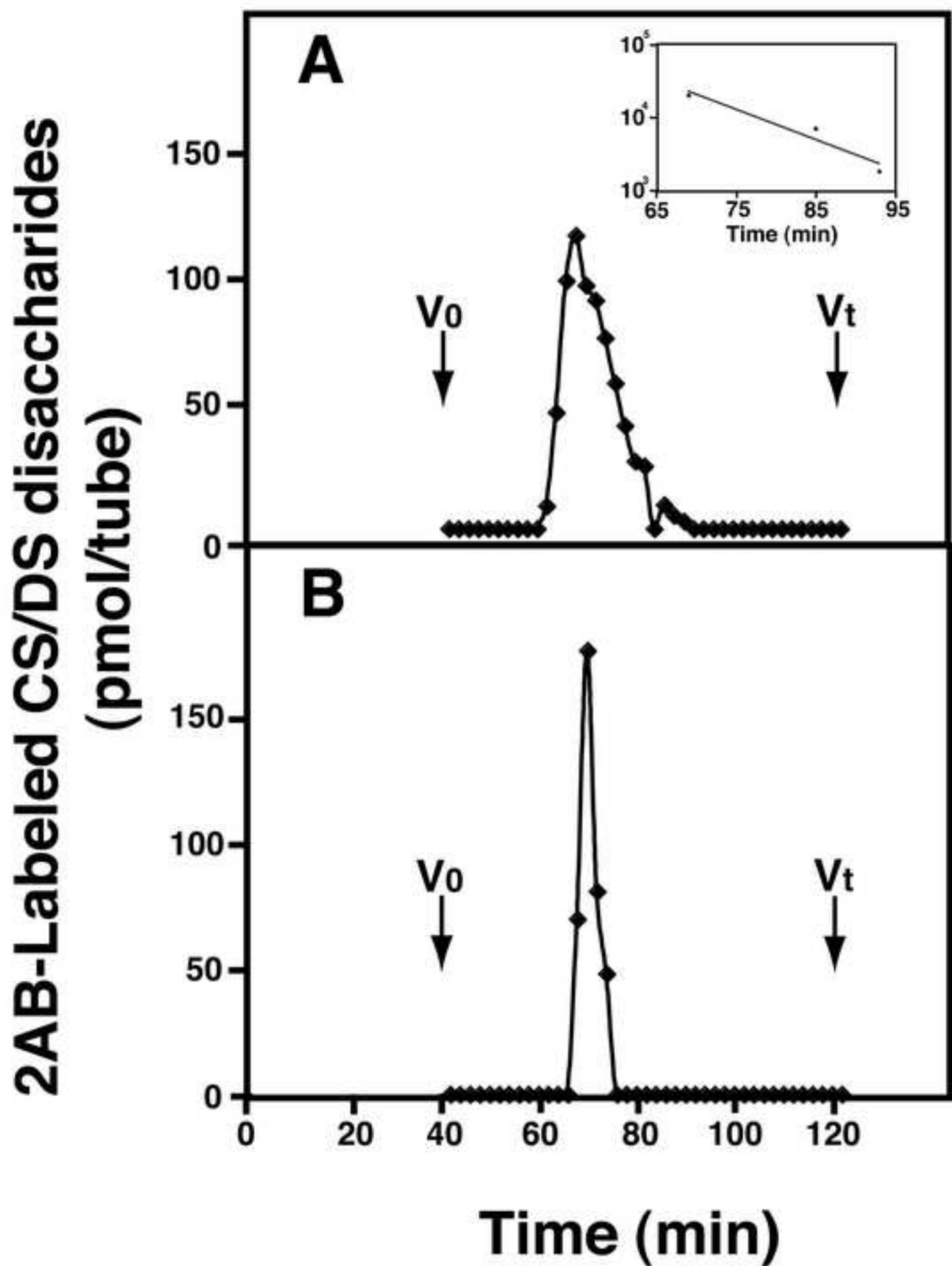


Figure2
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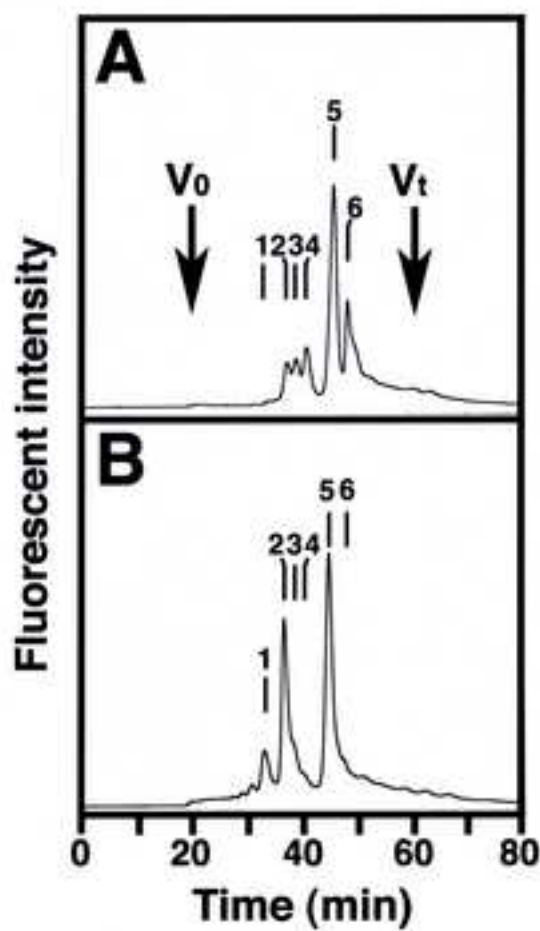


Figure 4

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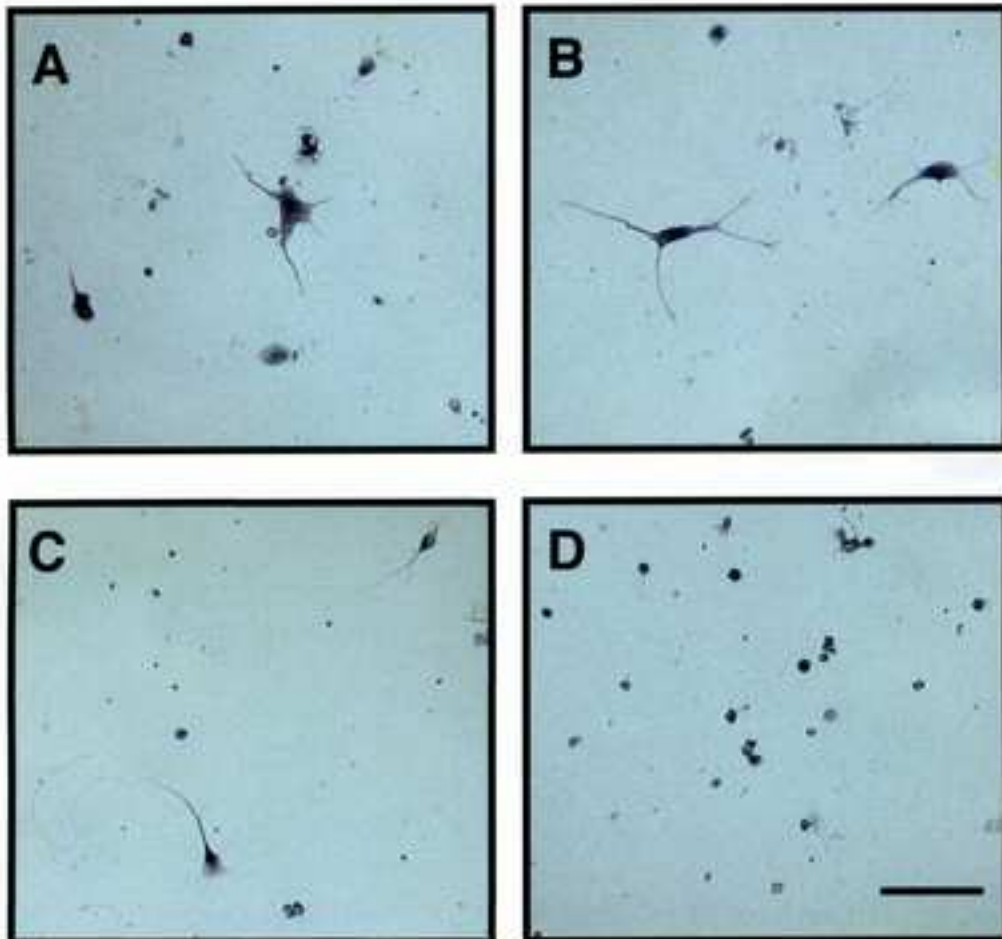


Figure5

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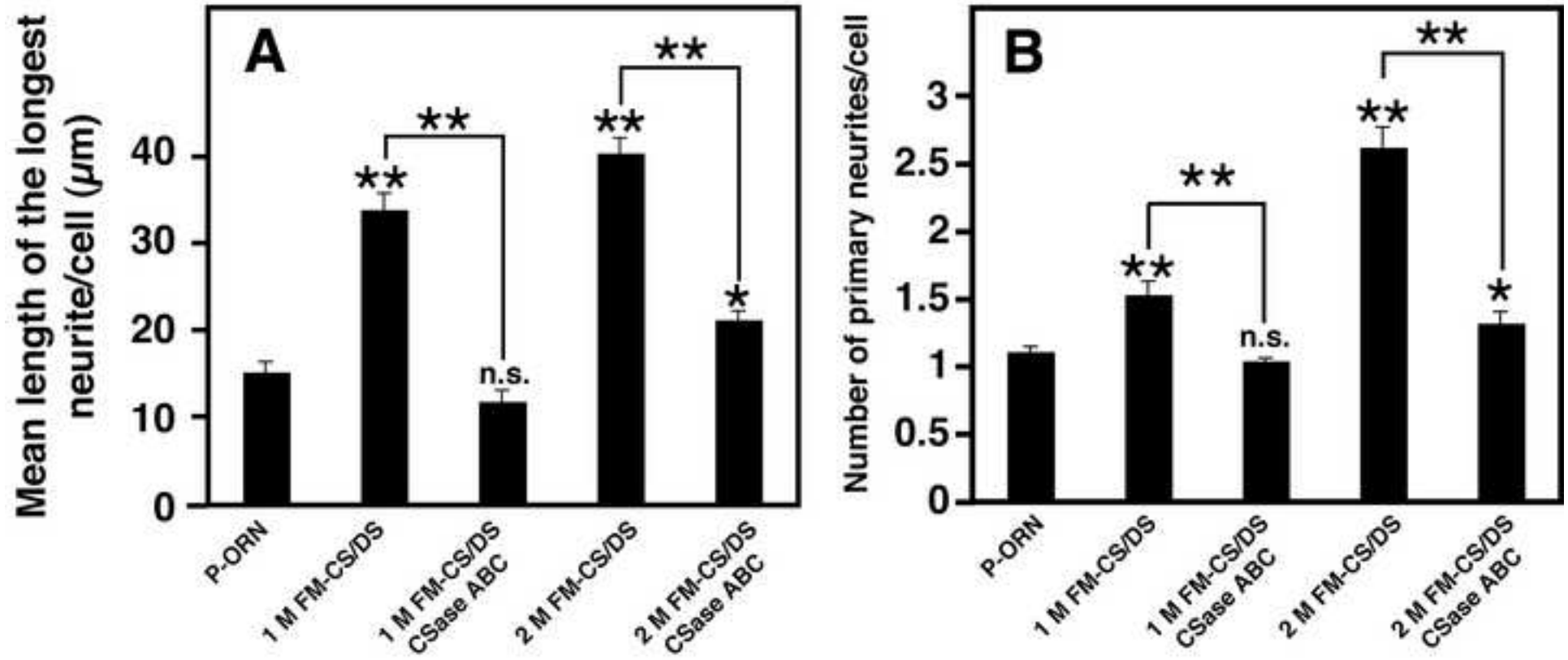


Figure6
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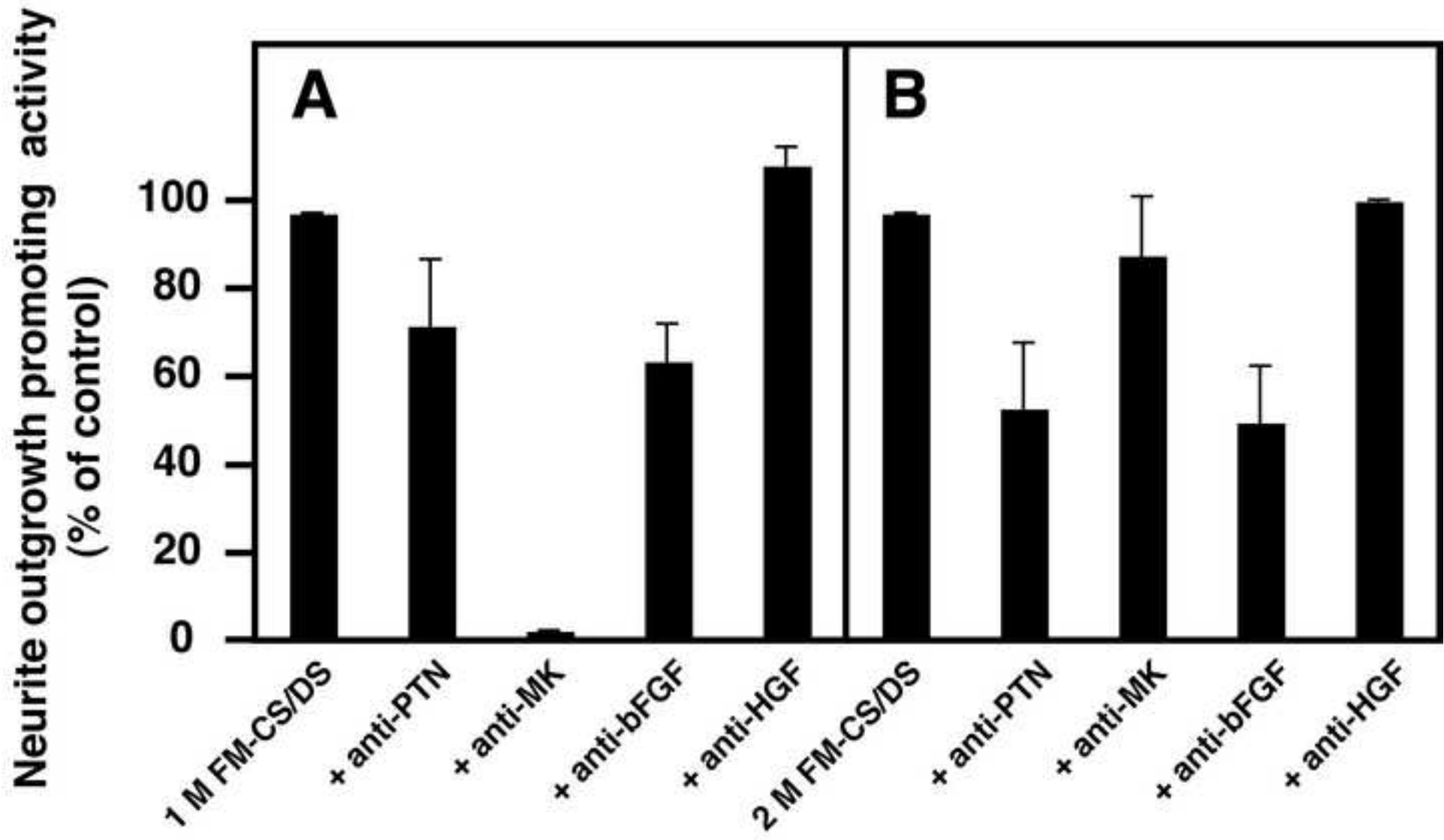


Figure7

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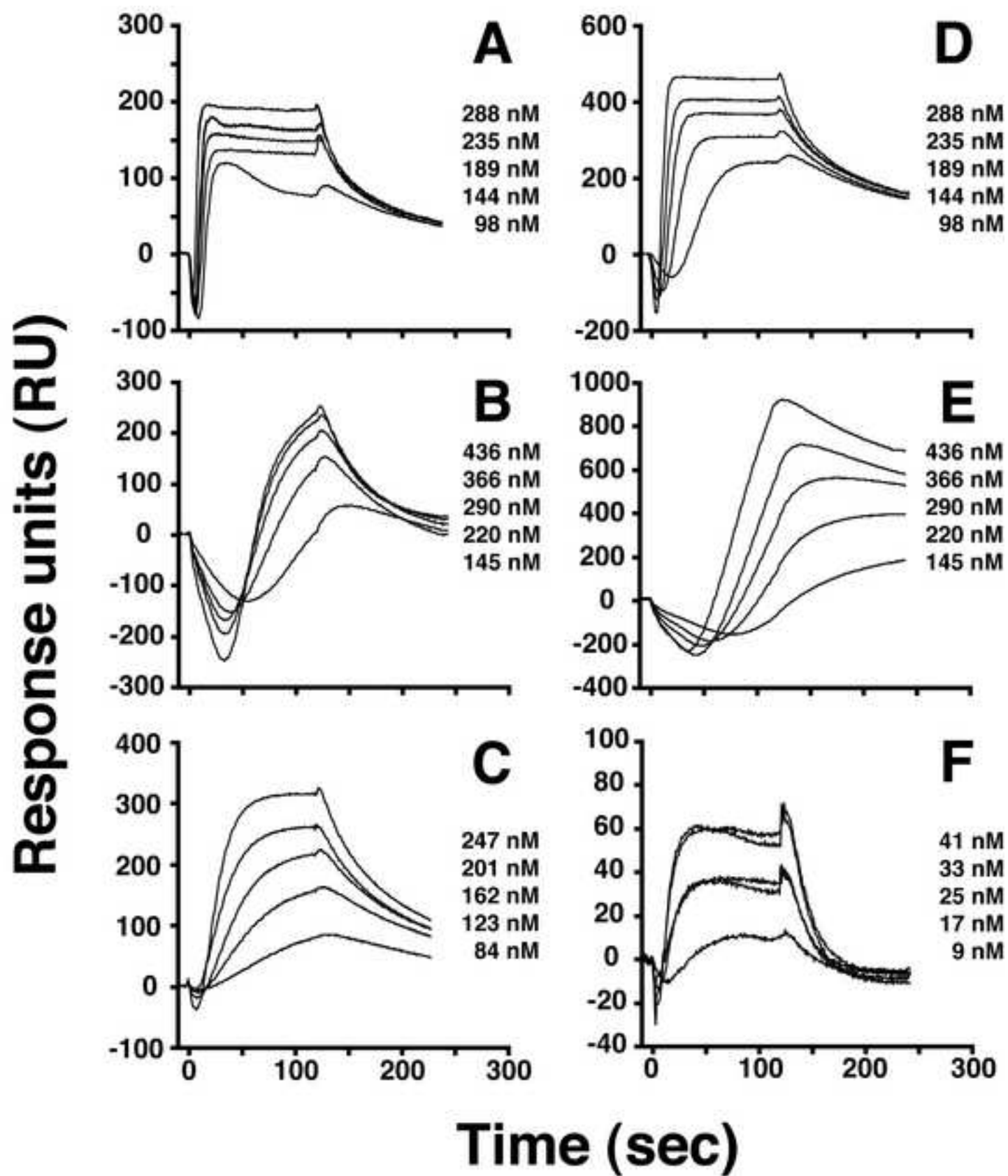


Table 1 Analysis of GAG composition of the fractions isolated from the FM-GAG fraction^a

	1.0 M NaCl-eluted FM-GAG fraction	2.0 M NaCl-eluted FM-GAG fraction
	pmol as disaccharides/mg tissue	
CS/DS	684 (37.5) ^b	149 (56.0)
HA	1,085 (59.4)	104 (39.1)
KS	ND ^c	11 (4.1)
HS	57 (3.1)	2 (0.8)
Total GAG	1,826 (100)	266 (100)

^aFM-GAG fractions were digested with a mixture of CSases ABC and AC-II, a mixture of Hepases I and III, or keratanase II. Each digest was analyzed by anion-exchange HPLC after 2AB-derivatization, and the GAG composition of each fraction was quantified based on the fluorescent intensity of the derivatives (see "Materials and methods").

^bThe values in parentheses give the proportions of the respective GAGs.

^cND, not detected.

Table 2 Disaccharide composition of the FM-CS/DS preparations^a

CS/DS disaccharides	1.0 M NaCl-eluted FM-CS/DS			2.0 M NaCl-eluted FM-CS/DS		
	CSases ABC and AC-II	CSases AC-I and AC-II	CSase B	CSases ABC and AC-II	CSases AC-I and AC-II	CSase B
	pmol as disaccharides/mg tissue			pmol as disaccharides/mg tissue		
ΔHexA-GalNAc	277.1 (40.5) ^b	250.6 (45.9)	ND	14.8 (9.9)	12.2 (16.9)	ND
ΔHexA-GalNAc(6S)	178.9 (26.2)	166.1 (30.3)	ND	41.1 (27.6)	35.1 (48.2)	ND
ΔHexA-GalNAc(4S)	223.5 (32.7)	130.3 (23.8)	17.3 (97.4)	90.9 (60.9)	25.3 (34.8)	12.2 (94.8)
ΔHexA(2S)-GalNAc(6S)	2.6 (0.4)	ND ^c	ND	0.7 (0.5)	ND	ND
ΔHexA(2S)-GalNAc(4S)	1.4 (0.2)	ND	0.5 (2.6)	1.4 (0.9)	ND	0.7 (5.2)
ΔHexA-GalNAc(4S,6S)	0.2 (0.03)	ND	ND	0.2 (0.2)	0.1 (0.1)	ND
Total	683.7 (100)	546.9 (100)	17.7 (100)	149.1 (100)	72.7 (100)	12.9 (100)
S/unit ^d	0.6	0.5	1	0.9	0.8	1.1

^aFM-CS/DS preparations were digested with a mixture of CSases ABC and AC-II, a mixture of CSases AC-I and AC-II, or CSase B, and individually analyzed by anion-exchange HPLC after 2AB-labeling (see "Materials and methods").

^bThe values in parentheses give the molar proportions of the respective disaccharides.

^cND, not detected

^dS/unit, the number of sulfate groups per disaccharide unit.

Table 3 Kinetic parameters for the interactions of neurotrophic factors with immobilized FM-CS/DS preparations^a

Neurotrophic factors	1.0 M NaCl-eluted FM-CS/DS			2.0 M NaCl-eluted FM-CS/DS		
	k_a	k_d	K_d	k_a	k_d	K_d
	$M^{-1}s^{-1}$	s^{-1}	nM	$M^{-1}s^{-1}$	s^{-1}	nM
PTN	- ^b	-	-	$(1.9 \pm 0.8) \times 10^4$	$(8.5 \pm 4.2) \times 10^{-3}$	463.4 ± 245.1
MK	$(2.1 \pm 0.8) \times 10^6$	$(3.8 \pm 2.7) \times 10^{-2}$	27.1 ± 1.0	$(1.2 \pm 0.5) \times 10^6$	$(6.0 \pm 2.4) \times 10^{-2}$	53.3 ± 16.1

^a The k_a , k_d , and K_d values were determined using a 1:1 binding model with mass transfer as described under "Materials and methods".

The values for each neurotrophic factor are expressed as the mean \pm S.E. obtained with five different concentrations.

^b-, no significant interaction with the 1.0 M NaCl-eluted FM-CS/DS preparation.