SUPPLEMENTAL INFORMATION

METHODS

Disaccharide composition analysis of the CS, DS, and HS chains isolated from the LLC and B16 melanoma cells—The disaccharide composition of the GAG preparations from LLC and B16 mouse melanoma cells was determined as described previously (20). Briefly, the extracted GAG-peptides were dissolved in PBS, and an aliquot was individually digested with chondroitinase ABC, a mixture of chondroitinase AC-I and AC-II, chondroitinase B, and a mixture of heparinase-I and -III, each digest was labeled with 2AB (33), and excess 2AB was removed by extraction with chloroform (43). The 2AB-labeled digest was analyzed by anion-exchange HPLC on a PA-03 silica column (YMC Co., Kyoto, Japan). Identification and quantification of the resulting disaccharides were achieved by comparison with the elution positions of CS- or HS-derived authentic unsaturated disaccharides (Ref. 33 and supplemental Tables S1, S2).

Relative Quantification of Gene Expression of RAGE—The primer sequences used were as follows: *RAGE* (260 bp), F 5'-TTC AGC TGT TGG TTG AGC CTG AA-3' and R 5'-TCG CCG GTT TCT GTG ACC CTG AT-3'; glyceraldehyde-3-phosphate dehydrogenase (*g3pdh*) (205 bp), F 5'-CAT CTG AGG GCC CAC TG-3' and R 5'-GAG GCC ATG TAG GCC ATG A-3'.

Quantitative real-time PCR was performed using a BrilliantII SYBER Green QPCR master mix in Mx3005P Real Time QPCR (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocols. The expression level of RAGE mRNA was normalized to that of the *g3pdh* transcript. The ΔΔCt method was utilized to determine the relative transcript levels for *RAGE* (Livak, K. J., and Schmittgen, T. D. (2001) *Methods* **25**, 402–408).

Assay of Binding of RAGE-derived Mutant Peptides to GAG—To further identify the residues in the peptides from RAGE and show electrostatic interaction, mutant peptides, CKGAPAAPPQQLEWKLNTGRTEA and GTFRCRATNAAGKEVKSNYRVRVY (Hokudo Co., Sapporo, Japan), were chemically synthesized and examined their interactions with CS-E. RAGE-derived mutant peptides (0.2 or 2 μg) was immobilized on MaxSorp® 96-well microtiter plates (Nunc, Roskilde, Denmark) at room temperature. The wells were blocked with 3% bovine serum albumin (BSA) and/or 3% blocking reagent (Roche, Mannheim, Germany) in PBS for 1 h at room temperature. After washing with PBS containing 0.005% Tween-20 (PBS-T), biotinylated GAGs (0.5 or 2 μg) were added, and the plate was incubated for 1 h at room temperature. Bound GAGs were detected using a streptavidin-conjugated alkaline phosphatase (0.1 or 1 μg/ml) (Thermo Scientific, Rockford, IL), followed by the phosphatase activity was detected using *p*-nitrophenyl phosphate as a substrate, and absorbance was measured at 415 nm.

Table S1. Disaccharide composition of CS and DS chains in LLC and B16 melanoma cells.

The GAG preparation from each cell line was individually digested with three kinds of chondroitinases and analyzed by anion-exchange HPLC after labeling with a fluorophore 2AB as detailed under "supplemental methods."

LLC	CS/DS[a]	CS[a]	DS[a]
	pmol/mg protein (mol%)[b]		
ΔO[c]: ΔHexUA-GalNAc[d]	45 (2.7)	38 (3.1)	N.D.
ΔC: ΔHexUA-GalNAc(6S)	N.D.[e]	N.D.	N.D.
ΔA: ΔHexUA-GalNAc(4S)	1,534 (92.6)	1,130 (92.7)	196 (91.0)
ΔD: ΔHexUA(2S)-GalNAc(6S)	N.D.	N.D.	N.D.
ΔB: ΔHexUA(2S)-GalNAc(4S)	6 (0.4)	N.D.	19 (9.0)
ΔE: ΔHexUA-GalNAc(4S,6S)	73 (4.4)	52 (4.2)	N.D.
Total	1,658 (100)	1,219 (100)	215 (100)

B16 melanoma	CS/DS[a]	CS[a]	DS[a]
	pmol/mg protein (mol%)[b]		
ΔO[c]: ΔHexUA-GalNAc[d]	1,441 (42.7)	1,202 (56.2)	N.D.
ΔC: ΔHexUA-GalNAc(6S)	132 (3.9)	105 (4.9)	N.D.
ΔA: ΔHexUA-GalNAc(4S)	1,802 (53.3)	833 (39.0)	333 (100)
ΔD: ΔHexUA(2S)-GalNAc(6S)	N.D.	N.D.	N.D.
ΔB: ΔHexUA(2S)-GalNAc(4S)	3 (0.1)	N.D.	N.D.
ΔE: ΔHexUA-GalNAc(4S,6S)	N.D.	N.D.	N.D.
Total	3,378 (100)	2,140 (100)	333 (100)

a, The total amount and disaccharide composition of CS/DS, CS, and DS chains in the cell lines were calculated based on the peak area in the chromatograms of digests obtained with chondroitinases ABC, AC, and B, respectively.
b, Values are expressed as pmol of disaccharide per mg of protein in the cells, and calculated based on the peak areas of the disaccharides detected by anion-exchange HPLC (data not shown).
c, For abbreviations of disaccharides, see Ref. 35
d, ΔHexUA represents 4,5-unsaturated hexuronic acid.
e, N.D., not detectable (<1 pmol/mg protein).

Table S2. Disaccharide composition of HS chains in LLC and B16 melanoma cells.

The GAG preparation from each cell line was digested with a mixture of heparinase-I and -III and analyzed by anion-exchange HPLC after labeling with a fluorophore 2AB as detailed in "supplemental methods."

	LLC	B16 melanoma
	HS[a] pmol/mg protein (mol%)[b]	
ΔHexUA-GlcNAc	76 (40.3)	126 (43.3)
ΔHexUA-GlcNAc(6S)	2 (0.8)	46 (16.0)
ΔHexUA-GlcNS	53 (28.3)	97 (33.4)
ΔHexUA-GlcNS(6S)	5 (2.9)	N.D.[c]
ΔHexUA(2S)-GlcNS	25 (13.5)	N.D.
ΔHexUA(2S)-GlcNS(6S)	27 (14.2)	21 (7.3)
Total	188 (100)	290 (100)

a, The total amount and disaccharide composition of HS chains in the cell lines were calculated based on the peak area in the chromatograms of the digests obtained with a mixture of heparinase-I and -III.
b, The values are expressed as pmol of disaccharide per mg of protein of the cells, and calculated based on the peak areas of the disaccharides detected by anion-exchange HPLC (data not shown).
c, N.D., not detectable (<1 pmol/mg protein).

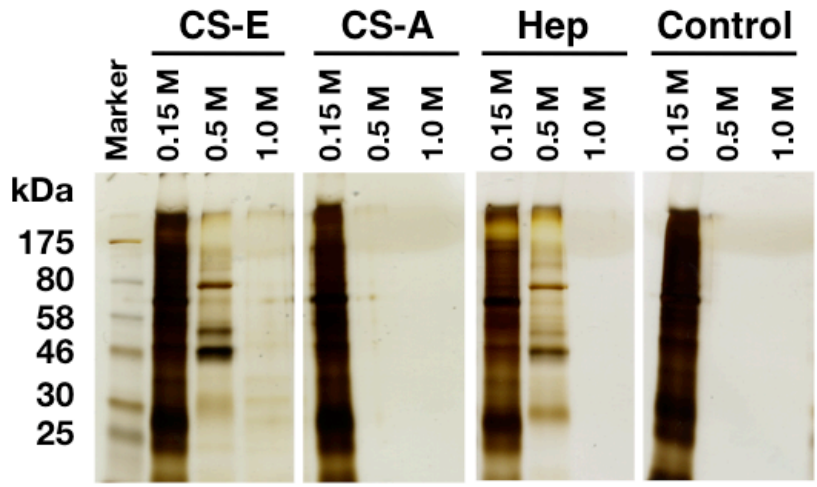


Fig. S1. Silver staining of the proteins recovered from the columns immobilized with CS-E, CS-A, and heparin.

A mouse lung homogenate was fractionated on an affinity column immobilized with CS-E, CS-A, or heparin, or a control column containing no GAGs. Proteins in each fraction eluted from the column were separated by SDS-PAGE, and visualized by silver staining. Significant bands at 46, 52, and 80 kDa were detected in the 0.5 M NaCl-eluted fraction from the CS-E and heparin columns but not in the CS-A column or the control column. The positions of standard protein markers are shown on the left.

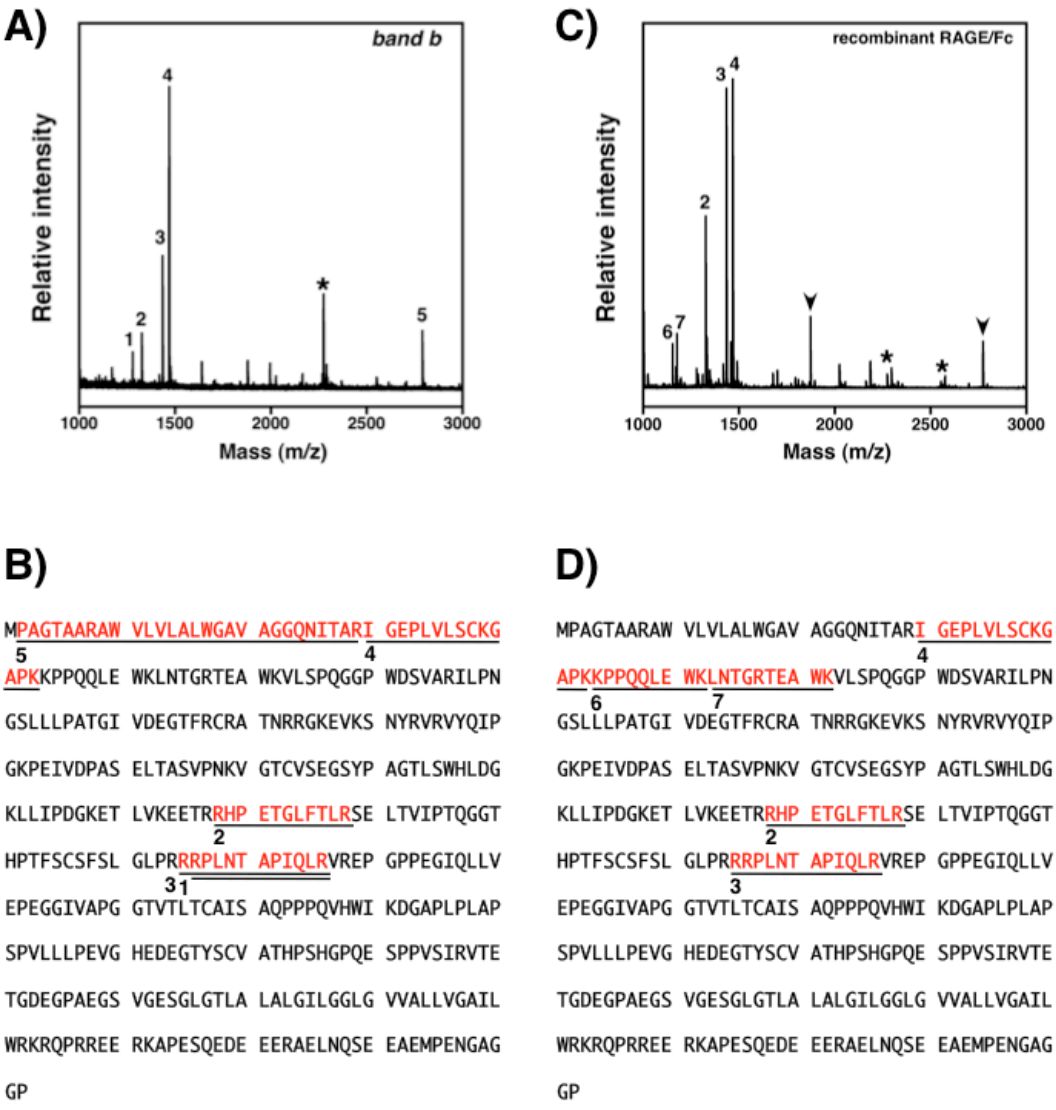


Fig. S2, Band *b* was also identical to RAGE.
DE MALDI-TOF mass spectrum of the tryptic peptides from band *b* (*A*) and recombinant RAGE (*C*). Numbered signals of the peptides from band *b* (*A*) were used for a database search with MASCOT, resulting in the matching of the peptides derived from RAGE (*B*). These results were consistent with the observations obtained using the recombinant mouse RAGE/Fc chimera (*C*, *D*). *Asterisks* indicate the peptide signals from trypsin. *Arrowheads* represent the mass spectra of the tryptic peptides from the Fc domain of human IgG_1.

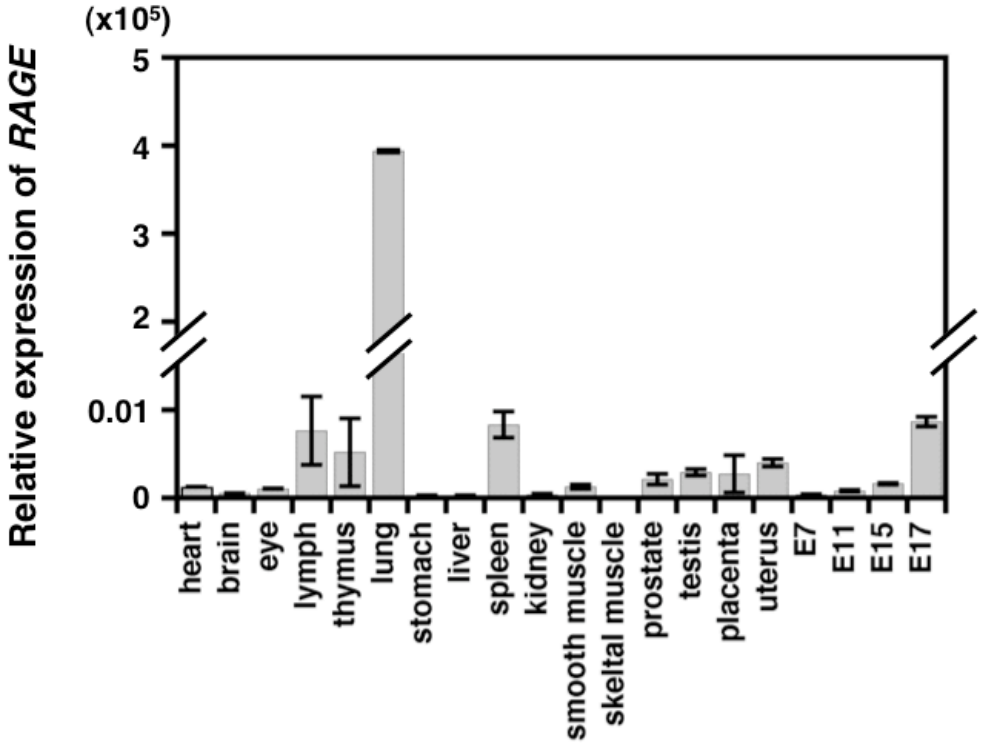
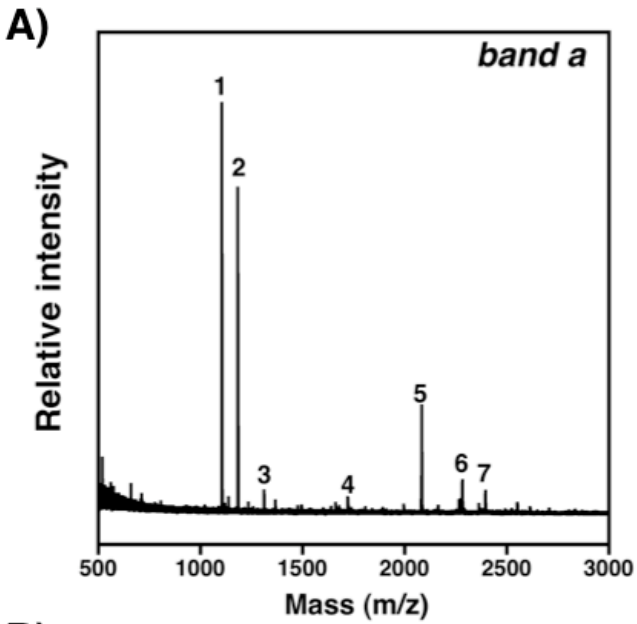


Fig. S3. Quantitative analysis of the *RAGE* transcript in mouse tissues by real-time PCR.
The expression level of the *RAGE* transcript was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (*g3pdh*) transcript, which was measured in the same cDNAs. Relative values for the expression of *RAGE* in the skeletal muscle are indicated. Data were obtained from triplicate experiments and are given as the mean ± S.D. Triplicate experiments were performed at least twice, and representative results are shown. E7-E17 represents embryonic day 7-17.



B)

MPKTISVRVT TMDAELEFAI QPNTTGKQLF DQVVKTIGLR EVWFFGLQYQ

DTKAFSTWLK LNKKVTAQDV RKESPLLFKF RAKFYPEDVS EELIQDITQR

LFFLQVKEGI LNDDIYCPPE TAVLLASYAV QSKYGDFNKE VHKSGYLAGD

KLLPQRVLEQ HKLNKDQWEE RIQVWHEEHR GMLREDAVLE YLKIAQDLEM

YGVNYFSIKN KKGSELWLGV DALGLNIYEQ NDRLTPKIGF PWSEIRNISF

NDKKFVIKPI DKKAPDFVFY APRLRINKRI LALCMGNHEL YMRRRKPDTI

EVQQMKAQAR EEKHQKQMER ALLENEKKKR ELAEKEKEKI EREKEELMEK

LKQIEEQTKK AQQELEEQTR RALELEQERK RAQSEAEKLA KERQEAEEAK

EALLQASRDQ KKTQEQLASE MAELTARISQ LEMARKKKES EAVEWQQKAQ

MVQEDLEKTR AELKTAMSTP HVAEPAENEH DEQDENGAEA SAELRADAMA

KDRSEEERTT EAEKNERVQK HLKALTSELA NARDESKKTA NDMIHAENMR

LGRDKYKTLR QIRQGNTKQR IDEFESM

Fig. S4. Band *a* was identical to moesin.

The DE MALDI-TOF mass spectrum of the tryptic peptides from band *a* (*A*) and numbered signals of the peptides from band *a* (*A*) were used for the database search with MASCOT, resulting in the matching of the peptides derived from moesin (*B*).

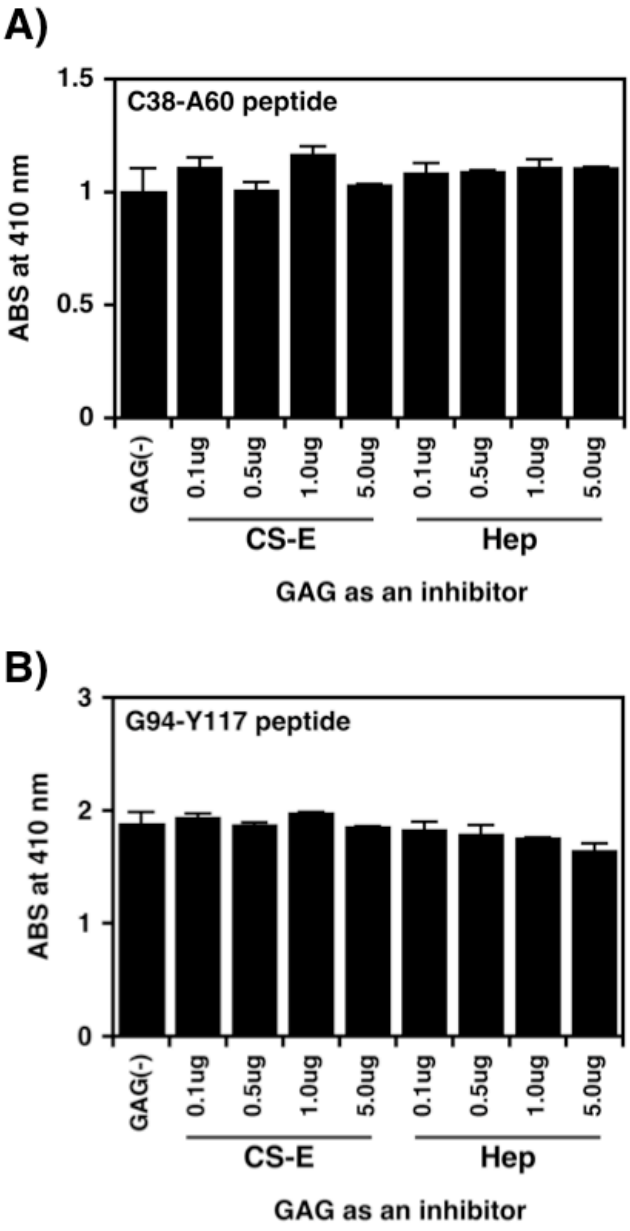


Fig. S5. Effects of the peptide-GAG complex on the recognition of the antisera.

The inhibitory activity of CS-E and heparin (Hep) against the recognition of the RAGE-peptides by the antisera was analyzed by inhibition ELISA. Briefly, the wild-type peptides, Cys38-Ala60 (*A*) and Gly94-Tyr117 (*B*), were individually immobilized to wells of a MaxiSorp® plate, and processed for subsequent incubation with CS-E or Hep, followed by the respective antisera. Bound antisera were visualized by incubation with alkaline phosphatase-linked goat anti-rabbit Ig (G + M). The enzymatic activity was measured using *p*-nitrophenylphosphate as a substrate at 415 nm. Bars, mean ± S.E. (n = 3).

The bindings of antisera to the peptides were not prevented by added GAGs, indicating that the attachment site for GAG in the peptides is distinct from that for the antisera.

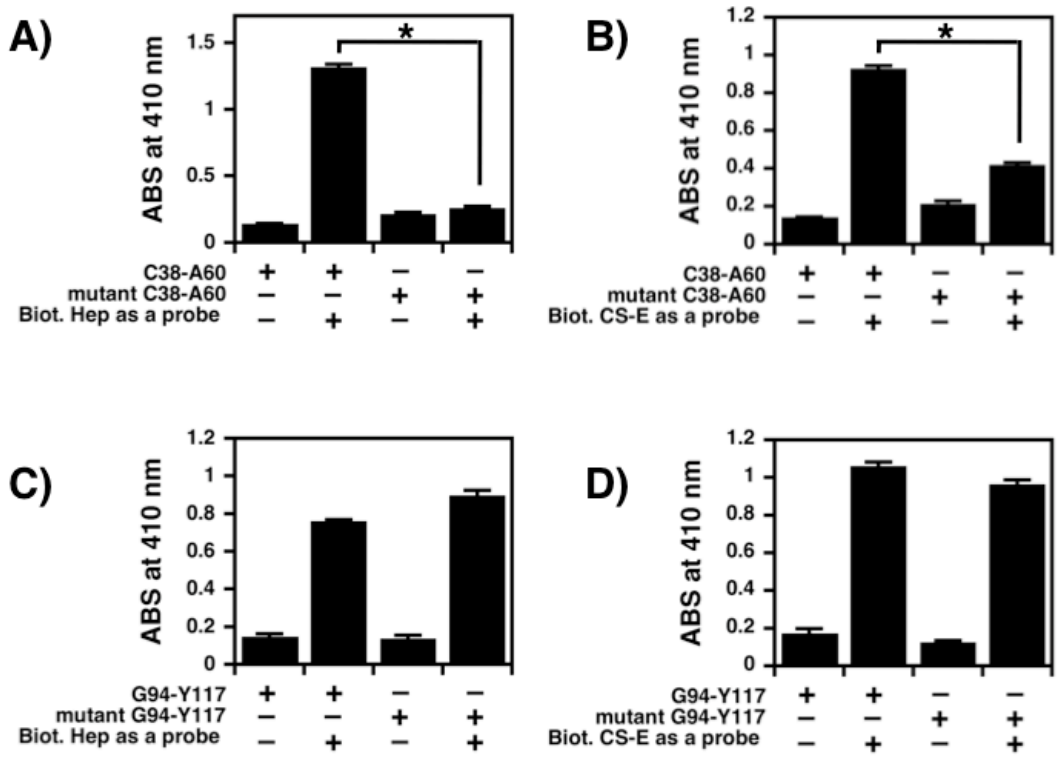


Fig. S6. Effects of substitutions of the double basic amino acid residues into alanine residues in the RAGE peptides on the interactions with GAGs.

The binding activity of wild-type and mutant peptides of Cys38-Ala60 (*A* and *B*) and Gly94-Tyr117 (*C* and *D*) from RAGE with heparin (Hep) (*A* and *C*) and CS-E (*B* and *D*) was evaluated as described in the supplemental methods. Briefly, the wild-type and mutant peptides were individually immobilized to wells of a MaxiSorp® plate, and processed for incubation with the biotinylated Hep or CS-E. Bound biotinylated GAGs were visualized by incubation with streptavidin-conjugated alkaline phosphatase. The enzymatic activity was measured using *p*-nitrophenylphosphate as a substrate at 415 nm. Bars, mean ± S.E. (n = 3). *, $p < 0.0001$ versus wild-type by Student's *t*-test.

The substitution of double basic amino acid residues in Gly94-Tyr117 has no apparent effect on the reactivity of the peptide to either Hep (*C*) or CS-E (*D*), whereas the interaction of the Cys38-Ala60 peptide with Hep (*A*) or CS-E (*B*) was abolished or significantly reduced, respectively. Note that although CS-E was not bound to the peptide, Cys38-Ala60, from the result of Fig. 3A by ELISA, CS-E was apparently interacted with Cys38-Ala60 peptide (*B*). The contrasting result may depend on the peptide antibody (Fig. 3), which maybe cannot recognize the peptide-CS-E complex.

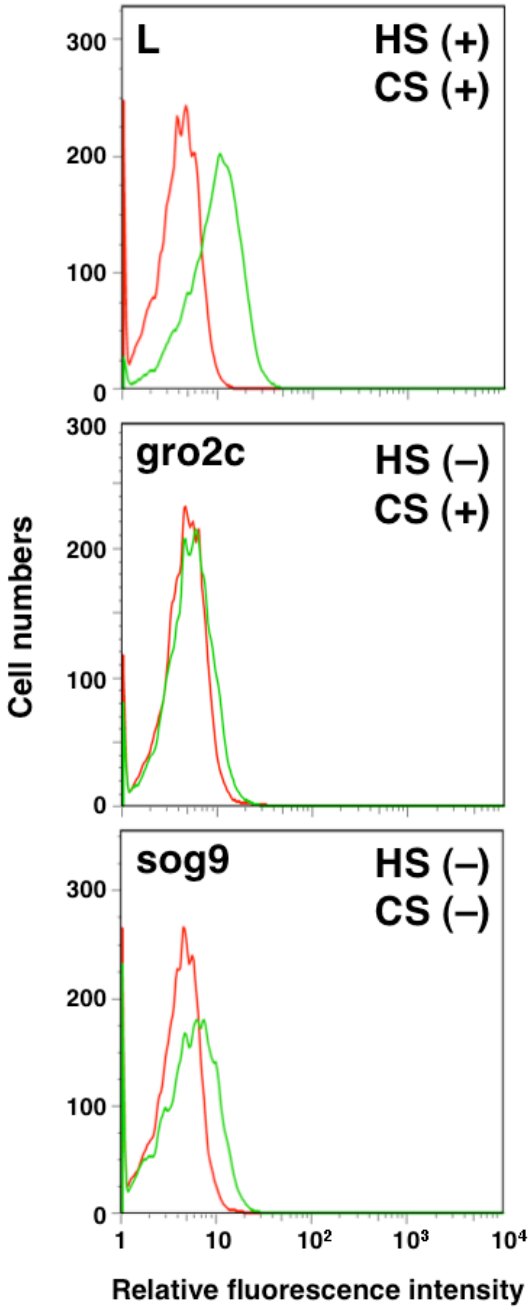


Fig. S7. Interaction of RAGE with cell-surface CS and HS using GAG-deficient cells by flow cytometry.

The binding potential of RAGE toward three mouse cell lines, wild type cells L (L-cells), *EXT1*-deficient L cells (gro2c), and *C4ST1*-deficient gro2c (sog9) cells, was assessed by immunofluorescence flow cytometry. Each cell line was treated with a RAGE/Fc chimera followed by Alexa Fluor 488-conjugated Protein G. *Green* and *red histograms* represent the RAGE-binding, and background fluorescence, respectively.

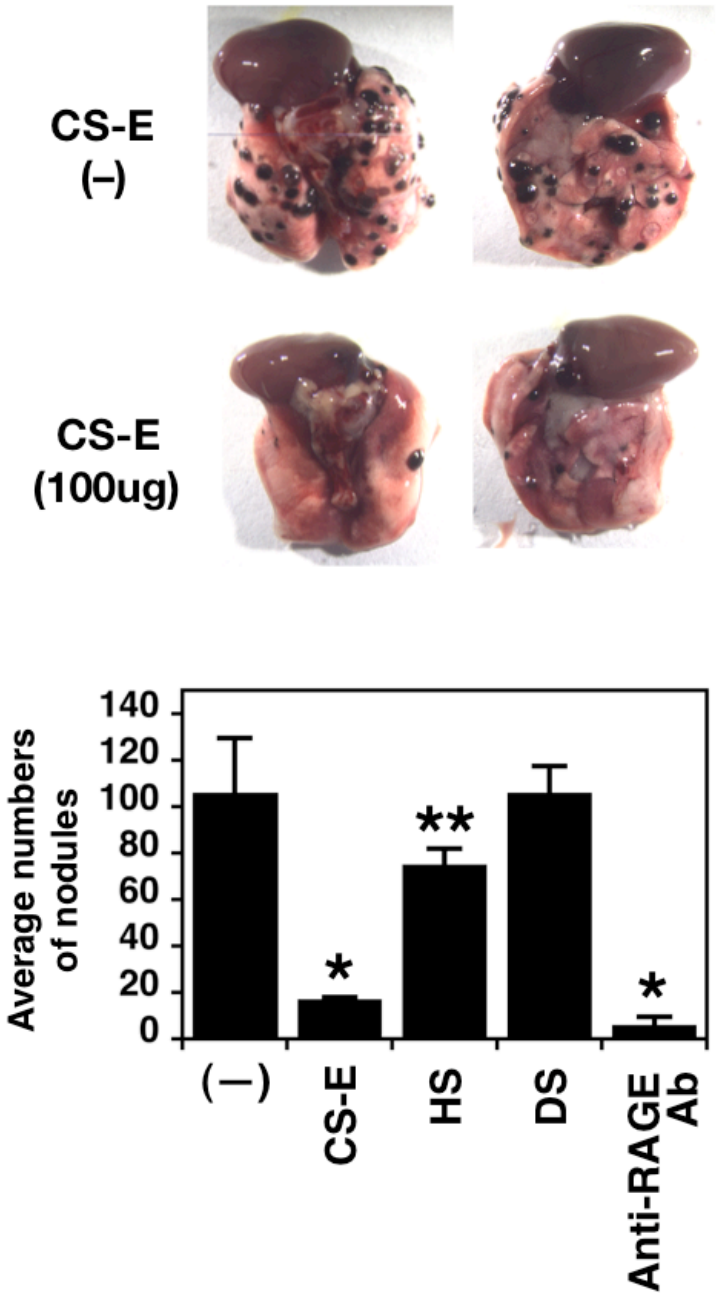


Fig. S8. Effects of GAGs and an anti-RAGE antibody on the matastasis of B16 melanoma cells.

Metastasis was analyzed as described in the legend to Fig. 5. Briefly, 1 x 10^5 cells in 200 μl of DMEM were injected into a tail vein of C57BL/6 mice, and 21 days later the number of foci in the lungs was recorded. Five mice were used per group. Pictures of representative lungs from mice pre-injected with a control buffer and CS-E (100 μg/mouse), followed by B16 cells are shown. These GAGs (100 μg each) and antibody (40 μg) in 200 μl of DMEM were injected into the tail of C57BL/6 mice 30 min prior to the injection of the B16 cells, and metastasis was analyzed. The data represent the mean ± S.E. *, $p < 0.02$; **, $p < 0.05$ versus control (–), one-way ANOVA with Dunnett's adjustment.