JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Ingestion of Proteoglycan Fraction from Shark Cartilage Increases Serum Inhibitory Activity against Matrix Metalloproteinase-9 and Suppresses Development of *N*-Nitrosobis(2-oxopropyl)amine-Induced Pancreatic Duct Carcinogenesis in Hamster

Tsukasa Kitahashi,[†] Shoko Ikawa,[†] Akika Sakamoto,[†] Yoshihiro Nomura,[‡] Toshifumi Tsujiuchi,[§] Kenji Shimizu,[∥] Shuji Sasabe,[∥] Eun Young Park,[†] Yasushi Nakamura,[†] Masahiro Tsutsumi,[⊥] and Kenji Sato*[,†]

[†]Division of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan

[‡]Applied Protein Chemistry, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8538, Japan.

[§]Laboratory of Cancer Biology and Bioinformatics, Department of Life Science, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502, Japan.

[∥]Hosokawa Micron Co. Ltd., 9-1, Shoudai Tajika, Hirakata, Osaka 573-1132, Japan

[⊥]Department of Pathology, Saiseikai Chuwa Hospital, 323 Abe, Sakurai, Nara 633-0054, Japan

ABSTRACT: A water extract of shark cartilage was fractionated into acidic and basic fractions by preparative isoelectric focusing on the basis of the amphoteric nature of samples. The acidic fraction was further fractionated into ethanol-soluble and -precipitate fractions. After the carcinogenesis treatment using *N*-nitrosobis(2-oxopropyl)amine, hamsters received a diet containing each fraction or purified chondroichin sulfate to give 0.4% (w/w) for 50 days. Only administration of the acidic ethanol-precipitate-fraction-containing diet significantly increased serum inhibitory activity against matrix metalloproteinase (MMP)-9 and reduced the number of adenocarcinomas in the pancreatic duct. The active fraction predominantly consisted of chondroichin sulfate-containing proteoglycan. However, the purified chondroichin sulfate had no significant activity. These results suggest that the protein moiety of the proteoglycan might be involved in the increase of serum inhibitory activity against MMP-9 and suppression of pancreatic carcinogenesis in hamster.

KEYWORDS: *cancer, matrix metalloproteinase, MMP, MMP-9, shark cartilage, shark, cartilage, BOP, N-nitrosobis(2-oxopropyl)amine, carcinogenesis, proteoglycan, chondroichin sulfate, hamster*

INTRODUCTION

It has been demonstrated that oral administration of shark cartilage and its extracts suppress carcinogenesis,[1−3] growth of tumor,[4,5] and also angiogenesis[6] in animal models. Angiogenesis is the formation of new capillaries from the established veins, which plays significant role in tumor growth and carcinogenesis.[7] In vitro studies also demonstrated that shark cartilage contains relatively low molecular weight proteins (smaller than 20 kDa) exerting immunostimulation,[8,9] anti-angiogensis,[10−13] and MMP-9 inhibitory activities.[10,11] These activities are closely related to suppression of carcinogenesis and tumor growth. Then, these low molecular weight proteins have been suggested to be involved in the beneficial effects by oral administration of shark-cartilage-based products. However, in vivo activities of these proteins and their bioavailability have not been fully elucidated.

Pancreatic duct adenocarcinoma is one of the most intractable malignancies in human. A rapid production model for pancreatic duct cancer in hamster based on initiation by *N*-nitrosobis(2-oxopropyl)amine (BOP) and augmentation pressure by injection of DL-ethionine under choline-deficient diet has been established.[14] By using this model, our previous studies have demonstrated that oral administration of a water extract of shark cartilage (0.4% in diet) after the carcinogenesis treatment suppresses development of carcinogenesis and also increases serum inhibitory activity against matrix metalloproteinase (MMP)-9.[1−3]

MMP is a calcium-dependent zinc proteinase and forms a family of at least 28 identified proteolytic enzymes.[15,16] Among the MMPs, MMP-9 is induced under pathological conditions and can cleave the triple helical domains of type IV and V collagens that are main constituents in the basement membrane and pericellular connective tissue, respectively.[15,16] It has been suggested that MMP-9 plays a significant role in the invasion and metastasis of various human malignancies, such as cancers of pancreas,[17,18] lung,[19] stomach,[20] breast,[21] and also tumor-induced angiogenesis.[22,23] On the basis of these facts, we have proposed a hypothesis that in vivo inhibition of MMP-9 by ingestion of the water extract of shark cartilage is involved in

Received: September 6, 2011
Revised: December 16, 2011
Accepted: December 29, 2011
Published: December 29, 2011

suppression of development of the pancreatic ductal caricinogenesis.[1−3] However, the water extract used in the previous studies was a mixture of several components, such as chondroichin sulfate, collagens, and a variety of proteins.

The objective of the present study was to identify the active component for in vivo inhibition of MMP-9 and suppression of development of pancreatic duct carcinogenesis using the same animal model. The present study demonstrates that single fraction has both activities by oral administration.

MATERIALS AND METHODS

Start Materials. Dried blue shark (*Prionace glauea*) cartilage was a kind gift from Kyodo Suisan Co. (Kesen-Numa, Japan). The blue shark was caught by long line fishing and landed at Kesen-Numa Port located in Miyagi Prefecture in Japan. The spinal column was collected from the blue shark and washed in hot water (approximately 60 °C) for a few minutes to removed adhering muscle and nerve tissues. Then the spinal column was air-dried, which was used as the dried shark cartilage in the following experiments.

Medical grade chondroichin sulfate C free from protein moiety was prepared from the dried blue shark cartilage by Maruha Nichiro Holdings Co. (Tokyo, Japan). The chonfroichin sulfate was a kind gift from Maruha Nichiro Holdings Co.

Reagents. *N*-Nitrosobis(2-oxopropyl)amine (BOP), DL-ethionine, L-methionine, Coomassie Brilliant Blue R-250 (CBB), bromophenol blue (BPB), disodium dihydrogen ethylendiamine tetraacetate (EDTA), Briji 35, Tween 20, and agarose powder were obtained from Nacalai Tesque (Kyoto, Japan). Phenylisothiocyanate (PITC) and amino acid standard type H were obtained from Wako Chemicals (Osaka, Japan). Molecular weight protein marker was obtained from TEFCO (Nagano, Japan). Triethylamine (TEA) was obtained from Pierce (Rockford, IL).

Bovine type V collagen and human fibrosarcoma MMP-9 (active form) were obtained from Yagai (Yamagata, Japan); these are now supplied from Life Laboratory (Yamagata, Japan). A formulated skim milk solution designed for blocking the nonspecific reactions of antibodies (Block Ace) was from Dainippon Pharmaceuticals (Osaka, Japan). Rabbit antibovine type V collagen IgG and goat antirabbit IgG (H + L)−horse radish peroxidase conjugate were from LSL (Tokyo, Japan) and Bio-Rad Laboratories (Hercules, CA), respectively. Other reagents were of analytical grade or better.

Fractionation of Components in Water Extract of Shark Cartilage. The dried shark cartilage was crushed into small pieces (approximately 5 mm in diameter) by a hammer mill (FM-1F, Hosokawa Micron, Osaka, Japan). The pieces were further crushed into a fine powder (60 μm in average diameter) with liquid nitrogen in a Lindex mill (Hosokawa Micron). Cold water (1.5 L) was added to 300 g of the cartilage powder. The suspension was allowed to stand for 30 min with occasional stirring. Then the suspension was centrifuged at 5000*g* for 10 min. After collection of the supernatant, another 300 mL of the water was added into the residue and stirred for 30 min. The supernatant was collected in the same manner. The supernatants were combined and used as the crude water extract of shark cartilage.

The water extract (5 L) was fractionated by the ampholyte-free preparative isoelectric focusing, which has been referred to autofocusing, by the method of Hashimoto et al.[24] using an autofocusing apparatus with 10 sample compartments (75 mm in length × 80 mm in width × 85 mm in height for each compartment). The water extract was put into all sample compartments. Autofocusing was carried out at 500 V for 18 h. The fractions showing below pH 6.0 were combined and used as an acidic fraction. The fractions showing above pH 7.0 were also combined and used as a basic fraction.

The acidic and basic fractions were mixed with 3 volumes of cold ethanol (5 °C). Precipitation occurred only in the acidic fraction. The resultant precipitate was collected by centrifugation at 5000*g* for 10 min and then freeze-dried. The soluble fractions were concentrated in a rotary evaporator to remove ethanol and then freeze-dried.

Animal Experiment. The animal experimental protocol was approved by the animal experimentation facility of Nara Medical University prior to its execution under monitoring by the committee of the facility in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105, and Japanese Government Notification on Feeding and Safekeeping of Animals Number 6.

Eighty-four female Syrian golden hamsters, 7-weeks old and weighting approximately 100 g each, were obtained from Japan SLC (Shizuoka, Japan). The hamsters were housed three to a plastic cage in an air-conditioned room at 24 °C and 60% humidity with a daily 12-h alternating cycle of light and dark and fed a basal diet (CE-2; CLEA Japan, Tokyo, Japan).

Pancreatic ductal carcinoma was induced by the rapid production method as described previously.[14] Fifty milligrams of BOP per kg body weight was injected subcutaneously for the initiation of tumor formation. Twelve days thereafter, the hamsters were subjected to the first cycle of augmentation pressure that consisted of four daily intraperitoneal injections of 500 mg of DL-ethionine per kg body weight under a choline-deficient diet (Dyets, Bethlehem, PA), followed by an intraperitoneal injection of 800 mg L-methionine per kg body weight. The animals were then returned to the basal diet and subcutaneously injected with 20 mg of BOP per kg body weight. A total of two cycles of augmentation pressures were performed. Five hamsters died in the period of the augmentation procedure. At 50 days from the first injection of BOP, hamsters were divided into five groups. One group of hamsters received the basal diet (n = 16), and others received the basal diets containing the acidic ethanol-soluble fraction (AS; n = 16), acidic ethanol-precipitate fraction (AP; n = 16), basic fraction (B; n = 16), and the purified chondroitin sulfate (CS; n=15) at 0.4% (w/w), respectively, for 50 days. All animals were killed under ether anesthesia at 100 days from the beginning of the experiment.

At the time of sacrifice, the pancreases of all animals were carefully removed and fixed in 10% neutral buffered formalin and embedded in paraffin for histological assessment. Five animals were randomly selected from the each group for blood collection. Blood was collected from the inferior vena cava and centrifuged at 3000*g* to obtain serum. The serum was stored at −80 °C until use.

Histological Assessments. Histological examination of the pancreas was carried out to evaluate the development of pancreatic duct adenocarcinoma in terms of its incidence and multiplicity that were diagnosed strictly according to the well-established criteria described earlier.[25]

MMP-9 Inhibitory Assay. The inhibitory activity against MMP-9 was estimated as described previously.[3] Ten microliters of the type V collagen solution (0.1% w/v in 10 mM HCl) was mixed with 10 μL of 100 mM Tris-HCl buffer, pH 7.5, containing 400 mM NaCl, 20 mM $CaCl_2$, 0.1% Brij 35, and 0.01% NaN_2, and 10 μL of the MMP-9 solution. Five microliters of serum that had been diluted to 5-fold with 50 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl, 10 mM $CaCl_2$, 0.05% Briji 35, and 0.01% NaN_2 was added to the reaction mixture. As a negative control (NC), the MMP-9 solution was replaced with 50 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl, 10 mM $CaCl_2$, 0.05% Briji 35, and 0.01% NaN_2. As a positive control (PC), the serum solution was replaced with the same buffer. The reaction mixtures were incubated at 37 °C for 24 h. The reaction was terminated by adding 10 μL of 500 mM EDTA sodium salt, pH 7.5, and mixing with 20 μL of 8 M urea, 5 μL of 0.1% BPB, and 5 μL of 10% sodium dodecyl sulfate (SDS). The mixture was then heated in a boiling water bath for 3 min. The type V collagen subunits α1(V) and α2(V) and their degradation products α1(V)* were resolved by SDS−polyacrylamide gel electrophoresis (PAGE) by using a 7.5% gel in Laemmli's buffer system.[26] Proteins in the gel were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Clear Blot P, ATTO, Tokyo, Japan). The membrane was immersed in the Block Ace solution for 3 h and then incubated with rabbit antibovine type V collagen IgG, which had been diluted to 1:1000 with the Block Ace solution, for 3 h at room temperature. After washing with 10% Block Ace solution containing 0.1% Tween 20 for 5 min (four times), the membrane was incubated with goat antirabbit IgG (H + L)−HRP conjugate, which had been diluted to 1:1000 in the Block Ace solution.

After washing the membrane, type V collagen and its degradation product were developed by a peroxidase substrate immunostaining kit (Wako Pure Chemical Industries, Osaka, Japan). The staining intensity of the bands for the substrate [α1(V) and α2(V)] and degradation product [α1(V)*] was determined using RFLPscan plus version 3.0 (CSPI, Billerica, MA). The ratio of degradation products (RD) was calculated by the following equation: RD (%) = 100 × [band staining intensity of α1(V)*]/[sum of band staining intensity of α1(V), α2(V), and α1(V)*]. Good linearity between RD and MMP-9 activity was observed up to RD 50%.[3] Inhibition of MMP-9 by serum was expressed as the equation inhibition (%) = $(RD_{PC} - RD_{SA})/RD_{PC} \times 100$, where RD_{PC} and RD_{SA} are the ratio of degradation products of positive control and sample, respectively.

Other Analytical Methods. Protein and its fragment contents were determined by amino acid analysis of HCl hydrolysate. Amino acid analysis was performed by the method of Bidlingmeyer et al.[27] with slight modifications.[28] Protein content was caluculated by the equation protein content (mg/mL) = $\sum[C_{AA}(MW_{AA} - 18)]$, where C_{AA} is concentration of each constituent amino acid (mM) and MW_{AA} is molecular weight of each amino acid. Tryptophan was not determined.

Chondroichin sulfate was determined by the carbazole sulfate method by using shark cartilage chondroichin sulfate C as a standard. Cellulose acetate electrophoresis was carried out as described previously.[29] Part of the sample was subjected to exhaustive proteolytic digestion to remove protein moiety before cellulose acetate electrophoresis.[29] SDS−PAGE of the sample containing chondroichin sulfate was carried out by using Laemmli's buffer system. The protein and chondroichin sulfate were double-stained with Alcian Blue and Silver by the method of Moller and Poilsen.[30]

Statistics. The difference in the number and incidence of pancreatic duct carcinomas and the serum inhibitory activity against MMPs were analyzed by one-way analysis of variance (ANOVA) by using StatView 4.11 (Abacus Concepts Inc., Berkeley, CA). Significant differences between the groups were evaluated by Fisher's protected least significant difference test.

RESULTS

Characteristics of Each Fraction. As shown in Figure 1, the water extract of shark cartilage was fractionated into acidic (no. 1−6) and basic fractions (no. 7−10) by the autofocusing. The acidic fraction consists of chondroichin sulfate and protein. On the other hand, the basic fraction (B) predominantly consists of protein. The acidic fraction was further fractionated into the acidic ethanol-precipitate (AP) and ethanol-soluble fractions (AS). The AP and AS consist of chondroichin sulfate and protein. Amino acid analysis revealed that the protein contents in the AP and AS were 21.2 and 46.2% on a dry basis, respectively. As shown in Table 1, the AS and B have the collagen-specific amino acid hydroxyproline (Hyp) in relatively high levels, being 86 and 95% of shark type II collagen, respectively.[31,32] The B has the higher amounts of basic amino acids, arginine, hydroxylysine, and lysine, and lower acidic amino acids, asparatic acid and glutamic acid, than the AS. Collagen subunit bands (100 kDa) are not observed by SDS−PAGE in either fraction (Figure 2A), which indicates that both fractions mainly consist of acidic and basic collagen fragments. On the other hand, the AP has lower hydroxyproline content (27% of type II collagen) than the other two fractions. For further characterization of the components in the AP, this fraction was subjected to cellulose acetate membrane electrophoresis. As shown in Figure 2B, the intact AP showed a broad Alcian Blue-positive band. After the exhaustive protease

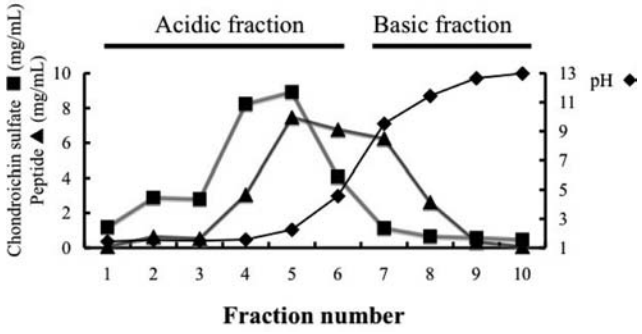

Figure 1. Fractionation of components in the water extract of shark cartilage into acidic and basic fractions by autofocusing.

Table 1. Amino Acid Composition of Acidic Ethanol-Precipitate (AP), Acidic Ethanol-Soluble (AS), and Basic Fractions (B) Compared with Those of Shark Type I and II Collagens (residues/1000 residues)

	AP	AS	B	shark type I[c]	shark type II[d]
Asp	69.7	53.8	37.6	42	48
Glu	122.3	114.3	76.7	72	89
Hyp[a]	20.7	67.1	74.3	78	82
Ser	91.6	47.7	54.3	27	36
Gly	191.0	278.2	305.8	340	332
His	15.2	6.4	7.8	6	4
Arg	47.9	41.9	58.3	50	49
Thr	47.8	43.0	26.1	21	22
Ala	81.4	57.4	85.6	128	105
Pro	105.2	140.9	102.5	113	114
Tyr	24.8	8.6	3.1	1	3
Val	39.4	20.7	32.4	21	17
Met	9.7	2.9	5.4	16	10
Ile	22.6	10.9	22.4	18	10
Leu	44.8	62.3	50.0	20	26
Hyl[b]	4.9	20.3	42.1	5	20
Phe	25.3	11.7	14.8	14	15
Lys	35.7	11.9	50.8	27	17

[a]Hydroxyproline. [b]Hydroxylysine. [c]Rama and Chandrakassen[20] [d]Kimura et al.[21]

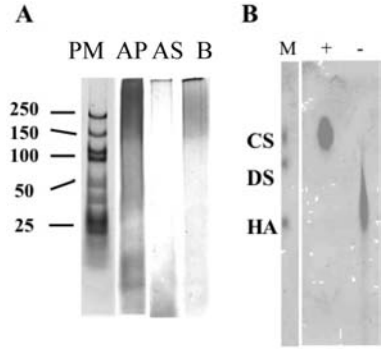

Figure 2. (A) SDS−polyacrylamide gel of acidic ethanol-precipitate fraction (lane AP), acidic ethanol-soluble fraction (lane AS), and basic fraction (lane B). Protein and chondroichin sulfate were double-stained by the method of Moller and Poilsen.[30] Lane PM represents molecular weight marker proteins (TEFCO). (B) Cellulose acetate membrane electrophoretic patterns of the intact AP (lane −) and its exhaustive protease digest (lane +) were resolved. Lane M represents glycosaminoglycan markers chondroichin sulfate C (CS), dermatan sulfate (DS), hyalnonic acid (HA).

dx.doi.org/10.1021/jf203613b | J. Agric.Food Chem. 2012, 60, 940−945

Table 2. Summary of Effects of Each Fraction on Body and Organs Weights and Development of Adenocarcinoma in Pancreas

group	effective numbers of animals	final body weight (g)	organ weight (g)		incidence of pancreatic ductal carcinoma-bearing animal (% of total animal)	number of pancreatic duct adenocaricinoma per individual
			pancreas	liver		
basal	16	180 ± 18.1	1.17 ± 0.25	12.2 ± 3.68	94	1.4 ± 0.7
AP	14	188 ± 11.1	1.12 ± 0.27	12.9 ± 3.63	33	0.5 ± 0.7[a]
AS	14	184 ± 13.9	1.19 ± 0.26	13.1 ± 3.24	57	1.0 ± 0.9
B	14	174 ± 15.6	1.34 ± 0.19[a]	14.1 ± 2.93[a]	54	1.0 ± 1.2
CS	13	188 ± 12.9	1.38 ± 0.40	16.1 ± 4.35[a]	78	1.2 ± 1.1

[a]Indicates significant difference between basal group.

digestion, a single band corresponding to chondroichin sulfate C was observed. These facts indicate that the AP consists of the chondroichin sulfate C-containing proteoglycan and collagen fragments. The contents of collagen fragments in this fraction can be estimated to be approximately 27% of the total peptide on the basis of hydroxyproline content.[32]

Effects on Carcinogenesis and Serum MMP-9 Inhibitory Activity. Numbers of survived animal and body, liver, and pancreas weights at the end of experiment are summarized in Table 2. In each group, up to 2 animals died. Macroscopic observation indicated that all causes of death were BOP-induced hepatic injury. There were no significant differences in final body weights (Table 2), diet, and water intakes among the groups (data not shown). Relative pancreas weight to body weight in the B group and liver weight in the B and CS groups were significantly higher compared to the other groups. In these groups, a few large tumors developed in the liver and pancreas, which contributed to higher organs weight.

As shown in Table 2, the incidence and number of pancreas carcinoma significantly decreased only in the AP group. However, no significant effects were detected in the CS group. As shown in Figure 3, addition of serum from the hamsters into the MMP-9 reaction mixture decreased the degradation product of type V collagen, α1(V)*, indicating that these serum have MMP-9 inhibitory activity. The AP group has significantly higher serum MMP-9 inhibitory activity than other groups.

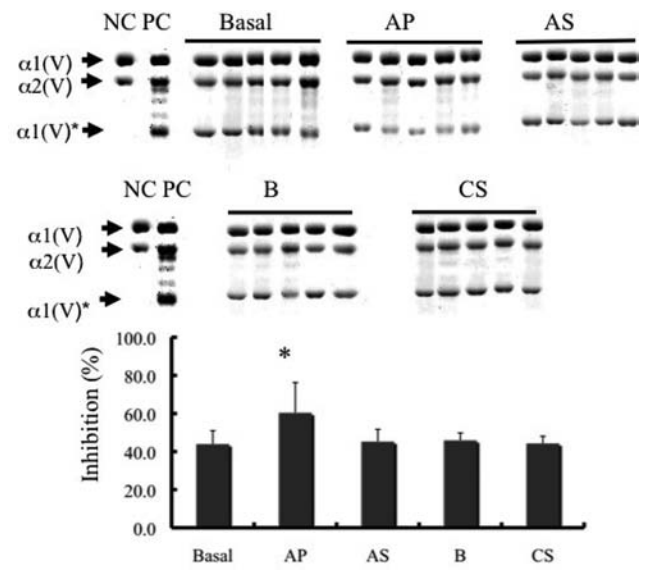

Figure 3. Inhibition of MMP-9 by serum from the hamsters in basal (basal), acidic ethanol-precipitate (AP), acidic ethanol-soluble (AS), basic (B), and purified chondoroichin sulfate (CS) groups. The serums were collected from five randomly selected animals from each group. NC and PC represent negative control and positive control as described in the Materials and Methods section.

DISCUSSION

It has been demonstrated that some food components have in vitro MMP-9 inhibitory activity.[1−3,33−35] However, only few studies have demonstrated inhibition of MMP-9 in tissue and blood by ingestion of food components.[1−3] The present in vivo activity-guided fractionation reveals that the oral administration of a single fraction, the acidic ethanol-precipitate fraction (AP), increased serum MMP-9 inhibitory activity of the cancer-bearing hamster. The same fraction also suppressed the progression of BOP-induced pancreatic carcinogenesis. These facts suggest that the increase of MMP-9 inhibitory activity in blood is involved in the suppression of pancreatic duct carcinogenesis by administration of water extract of shark cartilage.[1−3]

The AP predominantly consists of proteoglycan with a smaller amount of collagen fragments. The other inactive fractions predominantly consist of collagen fragments. On the other hand, the purified shark chondroichin sulfate free from protein moiety has no significant effect on the carcinogenesis and serum MMP-9 inhibitory activity. These facts suggest that the protein moiety of the proteoglycan is likely to be involved in the increase of serum MMP-9 inhibitory activity and suppression of carcinogenesis by ingestion. Our preliminary experiment using size exclusion chromatography (SEC) revealed that the high molecular weight fraction corresponding to 700 kDa of protein showed the highest MMP-9 inhibitory activity in the SEC fractions of hamster serum and the inhibitory activity of the high molecular weight fraction increased by ingestion of proteoglycan fraction. However, the present AP has only a negligible amount of in vitro MMP-9 inhibitory activity (data not shown). Then, the proteoglycan or its protein moiety in the AP is not likely to directly exert MMP-9 inhibitory activity in blood. Recently, occurrences of food-derived di- and tripeptides in relatively high levels (up to 60 μM) have been demonstrated in human peripheral blood after ingestion of fish, chicken, and porcine collagen peptides, of which the average molecular weight was 5 kDa.[36−39] On the basis of these facts, we propose a hypothesis that some oligopeptides, which could be generated from the protein moiety of the shark cartilage proteoglycan during digestion, might be absorbed into the blood system and induce host MMP-9 inhibitors. Unfortunately, only few antibodies against hamster serum protease inhibitors are now available. Then, it is difficult to identify the increased high molecular weight inhibitor in serum from hamster. To solve these problems,

further studies to identify the increased MMP-9 inhibitor and food-derived components in the blood after ingestion of proteoglycan fraction by using other animal models are in progress.

It has been demonstrated that ingestion of shark cartilage and its extract exerts multiple biological responses: immunostimulation, antiangiogenesis, and MMP-9 inhibition. One of or some of these responses might be related to suppression of carcinogensesis and growth of tumor by ingestion of shark-cartilage-based products. Now, some water extracts of shark cartilage have been prepared not only on a laboratory scale but also on an industrial scale.[40] The anticancer effects of these products have been examined by human trials.[41,42] Positive[41] and negative[42] results have been reported. In some cases, an ultrafiltartion technique has been involved to remove high molecular weight components from the extract, which might remove the high molecular weight proteoglycan that is a potential anticancer component as demonstrated in the present study. This process might affect the outcome. To prepare shark cartilage extract with high anticancer effect, it is, therefore, necessary to understand which response by which components is crucial for suppression of carcinogenesis and tumor growth. For this purpose, the present in-vivo-activity-guided fractionation based on large-scale fractionation and animal experiment would be useful.

AUTHOR INFORMATION

Corresponding Author

*Tel.:+81 75 703 5405. Fax:+81 75 723 3503. E-mail: k_sato@kpu.ac.jp.

Funding

This work was supported in part by a Grant-in Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan and a Grant-in-Aid for Scientific Research Expenses for Health and Welfare Programs, Third Term Comprehensive 10-Year Strategy for Cancer Control, Cancer Prevention from the Ministry of Health, Labour and Welfare of Japan.

ACKNOWLEDGMENTS

We express our appreciation to Prof. Yoichi Konishi for his consultations on the experiments and Dr. Nao Murata, Sachiko Nakai, and Megumi Inagaki for their technical assistance.

REFERENCES

(1) Sato, K.; Murata, N.; Tsutsumi, M.; Shimizu-Suganuma, M.; Shichinohe, K.; Kitahashi, T.; Nishimura, K.; Nakamura, Y.; Ohtsuki, K. Moderation of chemo-induced cancer by water extract of dried shark fin: Anti-cancer effect of shark cartilage. In *Developments in Food Science: More Efficient Utilization of Fish and Fisheries Products*; Sakaguchi, M., Ed.; Elsevier; Oxford, UK, 2004; pp 159−168.

(2) Kitahashi, T.; Sato, K.; Oka, Y.; Tsujiuchi, T.; Sakamoto, A.; Mitsui, M.; Mori, T.; Nakamura, Y.; Ohtsuki, K.; Tstsumi, M. Inhibition of pancreatic carcinogenesis by shark cartilage proteoglycan fraction in hamster. *J. Toxicol. Pathol.* **2006**, *19*, 179−184.

(3) Sato, K.; Kitahashi, T.; Itho, C.; Tstsumi, M. Shark cartilage: Potential for therapeutic application for cancer. In *Marine Nutraceuticals and Functional Foods*; Barrow, C., Shahidi, F., Eds.; CRC Press; Boca Raton, FL, 2008; pp 405−417.

(4) Barber, R.; Delahunt, B.; Grebe, S. K. G.; Davis, P. F.; Thornton, A.; Slim, G. C. Oral shark cartilage does not abolish carcinogenesis but delays tumor progression in a murine model. *Anticancer Res.* **2001**, *21*, 1065−70.

(5) Weber, M. H.; Lee, J.; Orr, F. W. The effect of neovastat (AE-941) on an experimental metastatic bone tumor model. *Int. J. Oncol.* **2002**, *20*, 299−303.

(6) Gonzalez, R. P.; Soares, F. dos S. D.; Farias, F.; Pessoa, C.; Leyva, A.; Viana, G. S.; de, B.; Moraes, M. O. Demonstration of inhibitory effect of oral shark cartilage on basic fibroblast growth factor-induced angiogenesis in the rabbit cornea. *Biol. Pharm. Bull.* **2001**, *24*, 151−154.

(7) Folkman, J. Angiogenesis. *Annu. Rev. Med.* **2006**, *57*, 1−18.

(8) Feyzia, R.; Hassan, Z. M.; Mostafaie, A. Modulation of CD^{4+} and CD^{8+} tumor infiltrating lymphocytes by a fraction isolated from shark cartilage: Shark cartilage modulates anti-tumor immunity. *Int. Immunopharm.* **2003**, *3*, 921−926.

(9) Bargahi, A.; Hassan, Z. M.; Rabbani, A.; Langroudi, L.; Noori, S. H.; Safari, E. Effect of shark cartilage derived protein on the NK cells activity. *Immunopharmacol. Immunotoxicol.* **2011**, *33*, 403−409.

(10) Oikawa, T.; Ashino-Fuse, H.; Shimamura, M.; Koide, U.; Iwaguchi, T. A novel angiogenetic inhibitor derived from Japanese shark cartilage (I). Extraction and estimation of inhibitory activities toward tumor and embryonic angiogenesis. *Cancer Lett.* **1990**, *51*, 181−186.

(11) Gingras, D.; Renaud, A.; Mousseau, N.; Beaulieu, E.; Kachra, Z.; Beliveau, R. Matrix proteinase inhibition by AE-941, a multifunctional angiogenic compound. *Anticancer Res.* **2001**, *21*, 145−156.

(12) Zheng, L.; Ling, P.; Wang, Z.; Niu, R.; Hu, C.; Zhang, T.; Lin, X. A novel polypeptide from shark cartilage with potent anti-angiogenic activity. *Cancer Biol. Therapy* **2007**, *6*, 775−780.

(13) Bargahi, A.; Rabbani-Chadegani, A. Angiogenic inhibitor protein fractions derived from shark cartilage. *Biosci Rep.* **2008**, *28*, 15−21.

(14) Iki, K.; Tsutsumi, M.; Kido, A.; Sakitani, H.; Takahama, M.; Yoshimoto, M.; Motoyama, M.; Tatsumi, K.; Tsunoda, T.; Konishi, Y. Expression of matrix metalloproteinase 2 (MMP-2), membrane-type 1 MMP and tissue inhibitor of metalloproteinase 2 and activation of proMMP-2 in pancreatic duct adenocarcinomas in hamsters treated with *N*-nitrosobis(2-oxopropyl)amine. *Carcinogenesis* **1999**, *7*, 1323−1329.

(15) Maskos, K.; Bode, W. Structural basis of matrix metalloproteinases and tissue inhibitors of metalloproteinases. *Mol. Biotechnol.* **2003**, *25*, 241−266.

(16) Nagase, H.; Visse, R.; Murphy, G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc. Res.* **2006**, *69*, 562−573.

(17) Simon, R. B.; Jhon, P. N.; Goldon, W. H. S.; Nicholas., R. L. Imbalance of expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in human pancreatic carcinoma. *J. Pathol.* **1997**, *182*, 347−355.

(18) Koshiba, T.; Hosotani, R.; Wada, M.; Fujimoto, K.; Lee, J. U.; Doi, R.; Arii, S.; Imamura, M. Detection of matrix metalloproteinase activity in human pancreatic cancer. *Surg. Today* **1997**, *27*, 302−304.

(19) Brown, P. D.; Bloxide, R.; Stuart, N. S. A.; Gatter, C.; Carmichael, J. Association between expression of activated 72-kDa gelatinase and tumor spread in non-small-cell lung carcinoma. *J. Natl. Cancer Inst.* **1993**, *85*, 574−578.

(20) Nomura, H.; Sato, H.; Seiki, M.; Mai, M.; Okada, Y. Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. *Cancer Res.* **1995**, *55*, 3263−3266.

(21) Ueno, H.; Nakamura, H.; Inoue, M.; Imai, K.; Noguchi, M.; Sato, H.; Seiki, M.; Okada, Y. Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in human invasive breast carcinomas. *Cancer Res.* **1997**, *57*, 2055−2060.

(22) Itoh, T.; Tanioka, M.; Yoshida, H.; Yoshioka, T.; Nishimoto, H.; Itohara, S. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res.* **1998**, *5*, 1048−1051.

(23) Vu, T. H.; Shipley, J. M.; Bergers, G.; Berger, J. E.; Helms, J. A.; Hanahan, D.; Shapiro, S. D.; Senior, R. M.; Werb, Z. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* **1998**, *3*, 411−422.

(24) Hashimoto, K.; Sato, K.; Nakamura, Y.; Ohtsuki, K. Development of a large-scale (50 L) apparatus for ampholyte-free isoelectric

focusing (autofocusing) of peptides in enzymatic hydrolysates of food proteins. *J. Agric. Food Chem.* **2005**, *53*, 3801−3806.
(25) Konishi, Y.; Mizumoto, K.; Kitazawa, S.; Tsujiuchi, T.; Tsutsumi, M.; Kamano, T. Early ductal lesions of pancreatic carcinogenesis in animals and humans. *Int. J. Pancreatol.* **1990**, *7*, 83−89.
(26) Laemmli, U. K. Cleavage and structural proteins during assembly of the head of the bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(27) Bidlingmeyer, B. A.; Cohen, S. A.; Tarvin, T. L. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **1984**, *336*, 93−104.
(28) Aito-Inoue, M.; Ohtsuki, K.; Nakamura, Y.; Park, E. Y.; Iwai, K.; Morimatsu, F.; Sato, K. Improvement in isolation and identification of food-derived peptides in human plasma based on precolumn derivatization of peptides with phenyl isothiocyanate. *J. Agric. Food Chem.* **2006**, *54*, 5261−5266.
(29) Kuwaba, K.; Nomura, Y.; Irie, S.; Koyama, Y. Temporal changes in disaccharide composition of derrmatan sulfate in the skin after epicutaneous application of hapten. *J. Demertol. Sci.* **1999**, *19*, 23−30.
(30) Moller, H. J.; Poilsen, J. H. Improved method for silver staining of glycoproteins in sodium dodecyl sulfate polyacrylamide gels. *Anal. Biochem.* **1995**, *226*, 371−374.
(31) Rama, S.; Chandrakasen, G. Distribution of different molecular species of collagen in the vertebral cartilage of shark (*Carcharius acutus*). *Connect. Tiss. Res.* **1984**, *12*, 111−118.
(32) Kimura, S.; Kamimura, T.; Takema, Y.; Kubota, M. Vertebrate skin type I collagen: Lower vertebrate collagen. Evidence for type I-like collagen in the skin of lamprey and shark. *Biochim. Biophys. Acta* **1981**, *669*, 251−257.
(33) Maeda-Yamamoto, M.; Kawahara, H.; Tahara, N.; Tsuji, K.; Hara, Y.; Isemura, M. Effects of tea polyphenols on the invasion and matrix metalloproteinases activities of human fibrosarcoma HT1080 cells. *J. Agric. Food Chem.* **1999**, *47*, 2350−2354.
(34) Saito, M.; Saito, K.; Kunisaki, N.; Kimura, S. Green tea polyphenols inhibit metalloproteinase activities in the skin, muscle, and blood of rainbow trout. *J. Agric. Food Chem.* **2002**, *50*, 7169−7174.
(35) Strek, M.; Gorlach, S.; Podsedek, A.; Sosnowska, D.; Koziolkiewicz, M.; Hrabec, Z.; Hrabec, E. Procyanidin oligomers from Japanese quince (*Chaenomeles japonica*) fruit inhibit activity of MMP-2 and MMP-9 metalloproteinases. *J. Agric. Food Chem.* **2007**, *55*, 6447−6452.
(36) Iwai, K.; Hasegawa, T.; Taguchi, Y.; Morimatsu, F.; Sato, K.; Nakamura, Y.; Higashi, A.; Kido, Y.; Nakabo, Y.; Ohtsuki, K. Identification of food-derived peptides in human blood after oral ingetion of gelatin hydrolysates. *J. Agric. Food Chem.* **2005**, *53*, 6531−6536.
(37) Ohara, H.; Matsumoto, H.; Itoh, K.; Iwai, K.; Sato, K. Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources. *J. Agric. Food Chem.* **2007**, *55*, 1532−1535.
(38) Ichikawa, S.; Morifuji, M.; Ohara, H.; Matsumoto, H.; Takeuchi, Y.; Sato, K. Hydroxyproline-containing dipeptides and tripeptides quantified at high concentration in human blood after oral administration of gelatin hydrolysate. *Int. J. Food Sci. Nutri.* **2010**, *61*, 52−60.
(39) Shigemura, Y.; Akaba, S.; Kawashima, E.; Park, E.-Y.; Nakamura, Y.; Sato, K. Identification of a novel food-derived collagen peptide, hydroxyprolyl-glycine, in human peripheral blood by pre-column derivatisation with phenyl isothiocyanate. *Food Chem.* **2011**, *129*, 1019−1024.
(40) Dredge, K. AE-941 (AEterna). *Curr. Opin. Invest. Drugs* **2004**, *5*, 668−677.
(41) Batist, B.; Patenaude, F.; Champagne, P.; Croteau, D.; Levinton, C.; Hariton, C.; Escudier, B.; Dupont, E. Neovastat (AE-941) in refractory renal cell carcinoma patients: Report of a phase II trial with two dose levels. *Ann. Oncol.* **2002**, *13*, 1259−1263.
(42) Lu, C.; Lee, J. J.; Komaki, R.; Herbst, R. S.; Feng, L.; Evans, W. K.; Choy, H.; Desjardins, P.; Esparaz, B. T.; Truoung, M. T.; Saxman, S.; Kelaghan, J.; Bleyer, E.; Fisch, M. J. Chemoradiotherapy with or without AE-941 in stage III non-small cell lung cancer: A randomized phase III trial. *J. Natl. Cancer Inst.* **2010**, *102*, 859−865.