Original

Inhibition of Pancreatic Carcinogenesis by Shark Cartilage Proteoglycan in Hamsters

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Abstract: Effects of shark cartilage proteoglycan (SCPG), which has heat-stable inhibitory activities against matrix metalloproteinase (MMP)-2 and -9 in vitro, on pancreatic carcinogenesis were studied in a rapid production model for pancreatic duct adenocarcinomas (PCs) in hamsters. Female Syrian golden hamsters received a diet containing 0% (group 1), 0.2% (group 2) or 0.4% (group 3) of SCPG for 50 days after initiation with N-nitrosobis(2-oxopropyl)amine followed by augmentation pressure. The number of PCs were 3.1 ± 2.0 in group 1 and 1.4 ± 0.9 in group 3, and total ductal lesions including hyperplasia, atypical hyperplasia and PCs were 9.7 ± 4.0 and 5.6 ± 2.7, respectively, the differences being significantly different (P < 0.05). These results were confirmed in a repeat experiment (P < 0.01). Gelatin zymography revealed that oral administration of SCPG did not affect the expression and activation of MMP-2 and MMP-9 in either serum or PC tissue. These results suggest that inhibition of pancreatic carcinogenesis by SCPG might involve a mechanism different from other synthetic-MMPs inhibitors. (J Toxicol Pathol 2006; 19: 179–184)

Key words: hamster, matrix metalloproteinase, N-nitrosobis(2-oxopropyl)amine, pancreatic duct adenocarcinoma, proteoglycan, shark cartilage

Introduction

Pancreatic duct carcinoma (PC) is one of the most intractable malignancies in humans. Due to its silent clinical course, at the time of diagnosis the vast majority of PC cases are incurable with a very poor prognosis. In order to control the disease, it is indispensable to detect tumors as early as possible and, in turn, to prevent their subsequent progression. An experimental model suitable for investigation of human PC development has been established in the hamster using the carcinogen, N-nitrosobis(2-oxopropyl)amine (BOP), and related compounds1–3. To facilitate studies of the underlying mechanisms, we have developed a rapid production model for PCs4–6, incorporating the principle of selection by resistance to cytotoxicity demonstrated earlier for liver carcinogenesis in rats7,8. This model has not only provided clues to molecular mechanisms for understanding ductal carcinogenesis4, but also serves as a bioassay for identification of risk factors and appropriate chemopreventive or chemotherapeutic agents5.

Matrix metalloproteinase (MMP) forms a family of 28 identified proteolytic enzymes that degrade substances within the extracellular matrix6,7. Among MMPs, the gelatinases, MMP-2 and -9, are particularly important since they can cleave triple helical domains of type IV and V collagens, which are preferentially distributed in the basement membrane and pericellular connective tissue, respectively8. These proteolytic activities of MMPs, in vivo, are known to depend on the balance between the levels of activated enzymes and specific tissue inhibitors of metalloproteinases (TIMPs)9. It has been suggested that these MMPs contribute to the invasion and metastasis of various human malignancies, such as cancers of the lung10, stomach11, breast12 and pancreas13,14. The results from experiments using the MMP knockout mouse and synthetic MMP inhibitors (MMPIs) demonstrate that MMPs play important roles in tumor-induced angiogenesis by degrading
elements of extracellular matrix and remodeling blood vessels\textsuperscript{15,16}.

Previously, we reported that overexpression of MMP-2, tissue inhibitor of metalloproteinases (TIMP) -2 and membrane-type (MT) 1-MMP, and cell surface activation of proMMP-2 by MT1-MMP are involved in the development of PCs, and that MMP-2 expression at the protein level appears in the early stage of pancreatic ductal carcinogenesis, with tumor development actually being inhibited by the synthetic MMP\textsuperscript{17}. Furthermore, we found that a high molecular proteoglycan fraction (\textgtr 450 kDa) of a water extract of shark cartilage has heat-stable inhibitory activities against MMP-2 and -9 \textit{in vitro}\textsuperscript{18}. In the present experiment, we studied the effects of oral administration of SCPG on pancreatic ductal carcinogenesis.

Materials and Methods

\textbf{Preparation of the SCPG fraction}

Dried shark fin cartilage was kindly provided by Maruha Co., Ltd. (Tokyo Japan) and the shark cartilage proteoglycan (SCPG) fraction was prepared by a method described previously\textsuperscript{18}. All preparations processes were performed at 4°C. Briefly, 5 volumes of water were added to 300 g of shark cartilage powder and stood for 30 min. The suspension filtrate was centrifuged at 3,000 rpm for 10 min and three volumes of ethanol were added to the supernatant. The resultant precipitate was harvested by centrifugation at 3,000 rpm for 10 min and the precipitate was freeze-dried and powdered. Inhibitory activities against MMP-2 and -9 of SCPG were confirmed prior to animal experiments.

\textbf{Animal experiments}

Forty-five and thirty-two female Syrian golden hamsters (Japan SLC, Shizuoka, Japan), 7 weeks old and weighing approximately 100 g each, were used in experiments 1 and 2. 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. The experimental protocol is shown in Fig. 1. Briefly, a 50 mg dose of BOP (Nacalai Tesque Inc., Kyoto, Japan) per kg body weight was injected s.c. for initiation. Twelve days thereafter, the hamsters were subjected to the first cycle of augmentation pressure, which consisted of 4 daily i.p. injections of 500 mg DL-ethionine (Nacalai Tesque Inc.) per kg body weight while being maintained on a choline-deficient diet (Dyets, Bethlehem, PA, USA), followed by an i.p. injection of 800 mg L-methionine (Nacalai Tesque Inc.) per kg body weight. The animals were then returned to the basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and injected s.c. with 20 mg BOP per kg body weight. A total of two cycles of augmentation pressure were performed. At 50 days after the beginning of initiation, hamsters were divided into 3 groups. Group 1-3 received diet containing 0%, 0.2% or 0.4% SCPG, respectively, for 50 days in experiment 1. The dose of SCPG may be applicable to human, because a diet containing 0.2% SCPG for a hamster (200 g body weight) approximates to 6–7 g per day for a 50 kg human. In experiment 2, we repeated experiment 1 with groups 1 and 3. All animals were sacrificed under ether anesthesia 100 days after the beginning of the experiment. At the time of sacrifice, blood was collected from the inferior vena cava, and centrifuged at 3,000 rpm to separate serum. Each pancreas was carefully removed and macroscopically detectable tumors were excised. A portion of each tumor was immediately frozen in liquid nitrogen for protein extraction and stored at \textdegree 80°C until use. Remaining portions and other parts of the pancreas were fixed in 10% neutral buffered formalin and embedded in paraffin for histological assessments.

A thorough histological examination for the pancreata was conducted to assess the development of hyperplasias (Hs), atypical hyperplasias (AHs) and pancreatic duct adenocarcinomas (PCs) in terms of their incidences and multiplicities. The number of pancreatic neoplasms per hamster was defined as the total number detected in all
assessed pancreas specimens of each animal, according to a method described previously19. Hyperplasias (Hs), atypical hyperplasias (AHs) and PCs were diagnosed strictly according to well-established criteria which have been described earlier20.

The animal experimental protocols were approved by the Animal Experimentation Facility of Nara Medical University prior to their execution under monitoring by a committee in accordance with the National Institute of Health Guideline for the Care and Use of Laboratory Animals, the Japanese Government Animal Protection and Management Law Number 105 and the Japanese Government Notification on Feeding and Safekeeping of Animals Number 6.

**Gelatin zymography**

Gelatin zymography was performed according to the method of Yamagata et al.21. For extraction of MMPs from normal pancreas (NP) and PCs, 10 mg portions of frozen tissue were homogenized in 500 µl of a lysis buffer containing 1% Tween-20 (Nacalai Tesque Inc.), 50 mM Tris-HCl, pH 7.4, 300 mM NaCl and 5 mM sodium EDTA. The homogenates were stored for 15 min on ice with occasional vortexing and insoluble material was removed by centrifugation. The resulting supernatants were subjected to SDS-PAGE with Laemmli's buffer system using 10% gel copolymerised with 0.06% (w/v) gelatin from porcine skin (Nacalai Tesque Inc.).

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bands were scanned and processed by RFLPscan plus ver.3.0 (Scanalytics Inc., Billerica, MA, USA) to obtain integral optical densities (IntODs).

Statistics
The statistical significances of inter-group differences of the data were assessed as follows. Fisher’s extract test was used for the incidences of pancreatic ductal neoplasms. In experiment 1, Dunnett’s T-test was conducted after one-way analysis of variance (ANOVA). In experiment 2, the two-tailed Student’s t-test was conducted because it was a comparison of two groups with no extrapolated data.

Results

Inhibition of pancreatic carcinogenesis
There were no significant differences among the groups regarding final body and organ weights in either experiment. All pancreatic lesions were of ductal origin, and no acinar or islet cell lesions were observed. Incidences and numbers of ductal lesions are summarized in Tables 1 and 2. In experiment 1, there were no significant differences among the groups regarding incidences of PCs. However, the PC number was 1.4 ± 0.9 in group 3 which was significantly lower than that of group 1 (3.1 ± 2.0) (P < 0.05). In group 2 of experiment 2, the incidence of PCs was significantly reduced, as well as PC numbers 0.2 ± 0.4 (P < 0.01) compared with group 1 (1.4 ± 0.7).

MMP levels in serum and pancreas
As shown in Fig. 2A, only latent forms of MMP-2 (72 kDa) and -9 (92 kDa) were observed in the sera of normal and PC carrying-hamsters. The serum of PC hamsters had slightly higher MMP-9 levels as compared with the controls. SCPG feeding did not significantly affect the latent MMP level (Fig. 2B).

While only low levels of latent MMP-2 and -9 existed in tissue extracts of normal pancreas, gelatinase activity bands of 69-kDa and 62-kDa in addition to latent forms were found in extracts of PC tissues (Fig. 2C). This gelatinase activity was completely suppressed by adding EDTA (data not shown). The 69-kDa and 62-kDa activities could be attributed to active forms of MMP-2 and -9, respectively, on the basis of electrophoretic mobility. Oral administration of SCPG did not affect the expression or activation of MMP-2 or -9 in either serum or PC tissue.

Discussion
In the present study, administration of diet containing 0.4% SCPG inhibited development of PCs as shown in both experiments, the efficacy being comparable with that of a synthetic MMPI, which inhibited activation of the latent forms of MMP-2 and -9 in the same animal model. However, oral administration of SCPG did not suppress expression and activation of MMPs as shown in Fig. 2B and C. Therefore, SCPG has different inhibition mechanisms from synthetic MMPI.
Crude shark cartilage has attracted much attention as a candidate cancer therapeutic or preventive agent, due to the alleged lack of nonneoplastic development in the shark. However, a recent article described that both malignant and benign neoplasms were found in sharks and their relatives. It has been reported that shark cartilage extracts can inhibit tumor vessel formation and invasion in experimental studies and its components may work as a cancer retardant, although a cartilage powder or crude extract was found to be ineffective. However, several phase III clinical trials using water extract of fresh shark cartilage containing low molecular proteinous MMPI, TIMP-like protein, on various malignant tumors are now being undertaken. It was reported that TIMP-like protein contained in shark cartilage was not stable, therefore, our materials (dried shark fin) did not contain active TIMP-like protein and the inhibitory mechanism of pancreatic carcinogenesis might have differed from other water extracts of fresh shark cartilage. Further studies are needed to elucidate the inhibitory mechanism of SCPG.

A number of low molecular weight synthetic MMPIs have been examined for their therapeutic effects on tumor progression and some were found to inhibit MMPs activities and tumor growth to some extent. However, adverse effects are obstacles to their use in cancer patients. In the present study, SCPG did not show any severe adverse effect and its efficacy was comparable with that of a synthetic MMPI, therefore, the present data suggest that SCPG has potential as a new chemopreventive agent for pancreatic carcinogenesis.

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