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Induction of Hepatocyte Nuclear Factor 3γ Gene Expression by the Extracellular Matrix Component from Fish Skin

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The skin of the halibut (*Reinhararditus hippoglossoides*) has long been discarded as a waste product by the food industry in large quantities; hence, we examined the biological activity in the skin for utilization of the waste. In the present study, we determined induction of hepatocyte nuclear factor 3γ (HNF3γ), which is involved in liver function, using human hepatoma cell line HepG2 and extracellular matrix components extracted from the fish skin. Induction of the HNF3γ was detected by reporter plasmid constructed with the promoter region of the HNF3γ gene and luciferase or green fluorescent protein genes. The alkali extract containing proteoglycan showed HNF3γ induction, but the acid-extract enriched in collagen did not. Consequently, dermatan sulfate showed the strongest activity in HNF3γ induction. These observations suggested that fish skin has some significant biological activity, and indicates its possible function in the food and drug industry.

Key words: dermatan sulfate; fish skin; human hepatoma cell 3γ; liver function; reporter assay

The halibut species *Reinhararditus hippoglossoides* is a major marine-food product processed in Tottori Prefecture, Japan. Fish skin in the marine food industry, discarded as waste like bone and entrails, amounts to more than about 10% of the whole fish, and is desired for utilization in some other products. The utilization of fish skin is expected to contribute to the development of a new local industry. Fish skin contains 34% protein and related materials, 14% oil and 52% water (Noguchi et al., unpublished data). Proteins in the fish skin are mainly composed of collagens and extracellular matrix (ECM) components such as proteoglycans. In a recent study, we showed that hepatocyte growth factor (HGF) was induced in the human fibroblast cell line by treatment with dermatan sulfate (DS) purified from fish skin, which is one of the glycosaminoglycans composed of proteoglycans (Noguchi et al., 2003). Moreover, we observed that alkali-extract containing DS exhibited inhibition of tumor cell growth using HepG2 (Noguchi et al., 2003).

On the other hand, hepatocyte nuclear factor 3γ (HNF3γ), a member of the forkhead protein family is one of the liver-enriched transcription factors for albumin and other liver-specific genes. The mouse HNF3 gene family was isolated and characterized by Kaestner et al. (1994). HNF3 genes are important in early endoderm and liver

Abbreviations: C6S, chondoitin 6-sulfate; DS, dermatan sulfate; ECM, extracellular matrix; GFP, green fluorescent protein; HGF, hepatocyte growth factor; HNF3γ, hepatocyte nuclear factor 3γ; HP, heparin; KPS, keratan polysulfate; PBS, phosphate buffered saline

development in addition to regulation of hepatic gene expression in the adult liver (Sasaki and Hogan, 1993). HNF3γ was also found in the notochord and ventral epithelium, suggesting its involvement in mesoderm and neural axis formation (Monaghan et al., 1995). However, the knockout mouse of the HNF3γ gene was reported to be fertile, to develop normally, and to show no morphological defects (Kaestner et al., 1998). Previously, we showed that the HNF3γ transduced by an adenovirus vector had a hepatoprotective effect against CCl_4-liver injury and potency for HGF induction (Nakamura et al., 1999). Therefore, taking these observations together, it is quite interesting to see whether the extracellular matrix component, especially DS, induces HNF3γ gene expression. In this study, we determined the activity for induction of HNF3γ gene expression using a reporter [the firefly luciferase and the *Aequorea* victoria green fluorescent protein (GFP)] by extraction from the skin of *Reinhararditus hippoglossoides* as industrial waste material from the viewpoint of utilization of an unused resource.

Materials and Methods

Cells

Human hepatoma cell line HepG2 was obtained from Riken Cell Bank (Tsukuba, Japan). The HepG2/GFP cell line was established by transformation with reporter (GFP) plasmid, and by lifting the fluorescent colony. All these cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Plasmid

Luciferase plasmids were constructed by insertion of the rat HNF3γ promoter segment (–1941 bp to +15 bp and –775 bp to +15 bp from the transcription initiation site) into PicaGene Basic Vector (Nippon Gene, Toyama, Japan) carrying the firefly luciferase gene (de Wet et al., 1985; Saito et al., 1999). Likewise, GFP reporter plasmid was constructed by insertion of the HNF3γ promoter segment (–775 bp to +15 bp) adjacent upstream of the jellyfish GFP coding sequence with poly A signal into pUC0CAT (Nakamura et al., 1989).

Preparation of ECM

Standard glycosaminoglycans (AMPS KIT, Seikagaku-Kogyo, Tokyo, Japan) contain bovine keratan polysulphate (KPS), heparin (HP), hog dermatan sulfate (DS) and shark chondroitin 6-sulfate (C6S), and were used as described previously (Matsumoto et al., 1993; Tsuchiya et al., 1997). GRGDSP peptide constructed from synthetic tripeptids of arginine-glycin-aspartic acid was obtained from Asahi Techno Glass, Chiba, Japan. *S*-Palmitoyl coenzyme A potassium salt was from Wako Pure Chemical, Osaka, Japan.

Fresh fish skins from *Reinhararditus hippoglossoides* were obtained as industrial waste products from a marine food processing market. Separation of ECM components was performed referring to the separation method of the collagen of Sato et al. (1986). Briefly, these skins were chopped and degreased with acetone. Lipid was obtained by removing the acetone from the extract. Alkali extract, in which proteoglycan is enriched (Noguchi et al., 2003), was prepared from the degreased skin by continuous agitation in 0.5 M NaOH at 4˚C for 16 h. Supernatant was neutralized with 0.5 M acetic acid, and low-molecular weight residues were removed by dialysis with Cellofine Film (cutting molecular weight 8000) (Seikagaku-Kogyo) against H_2O. Freeze-dried alkali extract was suspended and the concentration adjusted to 50 mg/mL with H_2O.

Acid extract enriched in collagen proteins was prepared by gentle agitation of the remnants in alkali extraction with 0.5 M acetic acid at 4˚C for 3 days, and the extract was dialyzed with Cellofine Film (cutting molecular weight 8000) against H_2O, followed by freeze-drying. The acid extract was suspended at a concentration of 50 mg/mL in 0.1 M acetic acid, and neutralized with 0.1 M NaOH.

DNA transfection and luciferase assay

HepG2 cells were plated at a density of 1×10^5 cells/mL on 24-well dishes. After incubation at 37˚C for 24 h, the cells were transfected with a reporter (luciferase) expression plasmid using calcium phosphate co-precipitation (Chen and Okayama, 1987). The cells were administered with the fish extract and further cultured at 37˚C for 48 h. The luciferase activity was quantitated with a PicaGene kit (Toyo-Inks, Tokyo) using the luminescence reader LF100 (Nissui Chemical, Tokyo) (Tamura and Noda, 1994). Intensity of luminescence was shown by relative light units (RLU) per μg protein in assay.

Quantitative analysis of GFP expression

HepG2/GFP cells were inoculated at a density of 1×10^4 cells/0.1 mL in a 96 hole microplate, and incubated with glycosaminoglycan or other compounds at 37˚C for 72 h. Fluorescence from the treated cells was determined by fluorescence microscopy and the intensity of fluorescence from individual cells quantitated by the Image Pro plus analysis system (Nikon Co., Tokyo). The differences between values from experimental samples and controls were assessed by Student's *t*-test and expressed as mean with SD. Differences with a *P* value of < 0.05 were considered statistically significant.

Results

Previously we showed that overexpression of HNF3γ gene in hepatocyte induces hepatoprotection against CCl_4-induced liver injury (Nakamura et al., 1999). It evokes our interest as to whether the extract from halibut skin is effective on induction of the HNF3γ gene. Therefore, to establish an assay system for expression of HNF3γ, we constructed a luciferase-reporter vector using the 2-kb promoter region of the HNF3γ gene. Partially differentiated from the human hepatoma cell line, HepG2 expresses the HNF3γ gene at low levels, as well as the albumin gene, a hepatic marker (Saito et al., 1999). Hence, the reporter plasmids were transfected to HepG2 cells, and determined by luciferase assay. As shown in Fig. 1A, luciferase plasmid containing

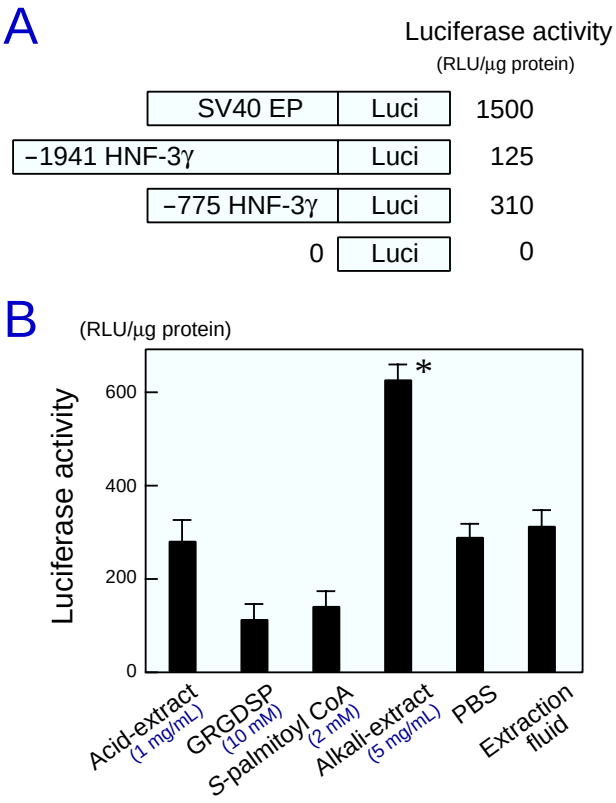

Fig. 1. Transient expression of luciferase gene (Luci).
A: Luciferase reporter plasmids are constructed with simian virus early promoter (SV40 EP) as a positive control, –1941 bp to +15 bp and –775 bp to +15 bp of HNF3γ DNA fragment (–1941 HNF3γ and –775 HNF3γ, respectively) and no DNA fragments (0) as negative controls. Luciferase activity is shown in the right panel in relative light units (RLU)/μg protein.
B: Luciferase activity was determined in the presence of acid extract (1 mg/mL), synthetic hexapeptides GRGDSP (10 mM), *S*-palmitoyle CoA (2 mM), alkali-extract (5 mg/mL), phosphate-buffered saline (PBS) and extraction fluid as controls. Concentrations of these samples were used as described by Tsuchiya et al. (1997). Values are means with standard deviations from at least 3 independent experiments. $*P < 0.01$ (significant against PBS). HNF, hepatocyte nuclear factor; CoA, coenzyme A.

the promoter region (–775 bp to +15 bp from transcription initiation site) showed a significant level of transcriptional activity, rather than the reporter carrying a longer promoter (–1941 bp to +15 bp).

Agents or chemical substances that are responsible for induction of HNF3γ gene expression could have predictable hepatoprotective actions (Nakamura et al., 1999). For screening of inducer substances for HNF3γ expression, we applied various extracts from the fish skin into the HepG2 cell culture transfected with luciferase plasmid carrying –775 to +15 bp of the HNF3γ gene. As shown in Fig. 1B, alkali extract from the fish skin exhibited a strong inducing activity for reporter plasmid shown by a 2-fold increase in luciferase activity. Interestingly, synthetic hexapeptids of GRGDSP constructed with a core sequence of arginine-glycin-aspartic acid, which binds to integrins and antagonizes intracellular signal transmission, considerably inhibited the gene expression as well as *S*-Palmitoyl coenzyme A which is a HNF4 ligand. Meanwhile, administration of acid extract enriched in collagen scarcely increased luciferase activity compared with phosphate-buffered saline (PBS) and extraction fluid as controls. These observations suggested that proteoglycans abundantly contained in alkali extract may be involved in induction of HNF3γ.

Furthermore, to improve the assay system for extension of HNF3γ gene expression, we established the HepG2 cell line, which was transformed with the green fluorescent protein (GFP) gene linked with the –775 bp promoter region of the HNF3γ gene. This cell line should express GFP with green fluorescence responding to stimuli for HNF3γ gene expression. This supposition was confirmed by supplementation of alkali extract of the fish skin to the cell culture. As shown in Fig. 2A, PBS-treated cells were slightly fluorescent at the base level; in contrast, administration of alkali extract to the cells generated strong fluorescence. Intensity of fluorescence from the individual cells (more than 50 cells) was determined by an Image Pro plus analysis system, and alkali extract showed a 2.5-fold induction of GFP in the transformed cell line compared with control expression, suggesting utilization of the cell line carrying a HNF3γ-promoted GFP gene as a screening system for hepatoprotective agents.

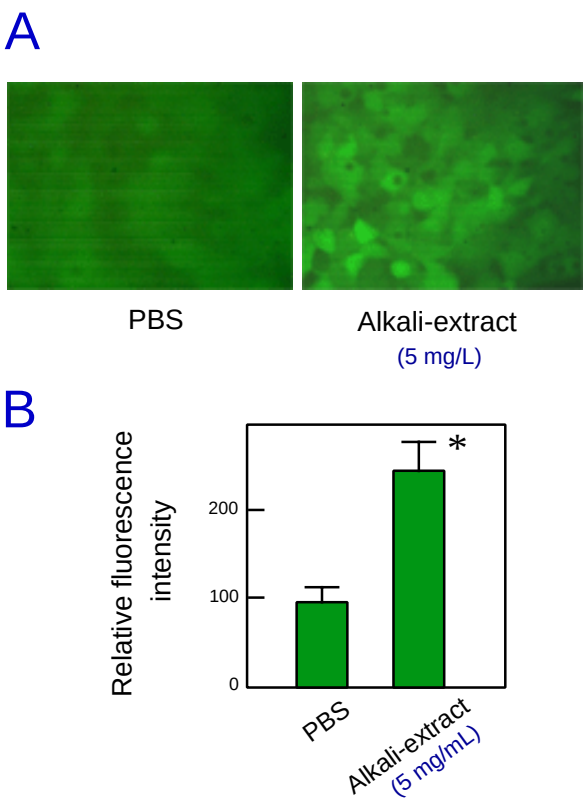

Fig. 2. Fluorescence from HepG2/GFP cells treated with alkali extract.

A: HepG2/GFP cells were administered with 5 mg/mL alkali extract from fish skin for 3 days, and determined by fluorescent microscopy (original magnification, × 200).

B: Intensity of fluorescence from individual cells quantitated by the Image Pro plus analysis system, and relative fluorescence intensity is shown by counting the intensity from PBS-treated cells as 100. Values are means with SDs from at least 50 independent cells. $*P < 0.01$ (against PBS). GFP, green fluorescent protein; PBS, phosphate-buffered saline.

In a recent study, we showed what compound included in alkali extract of the fish skin operates as an inducer of HNF3γ gene expression: we fractionated the extract by ion-exchange chromatography, and identified dermatan sulfate (DS) as a main component in the skin glycosaminoglycan (Noguchi et al., 2003). Hence, we assessed inducing activity for

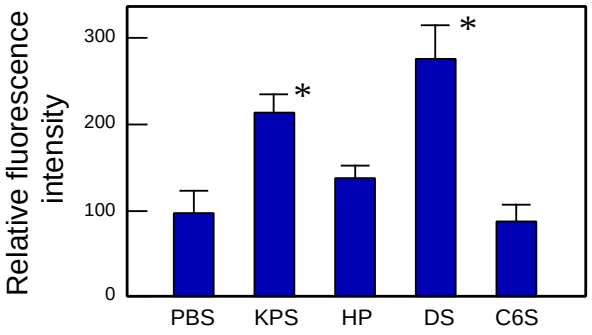

Fig. 3. Fluorescence from HepG2/GFP cells treated with glycosaminoglycans. HepG2/GFP cells were treated with 50 μg/mL keratan polysulphate (KPS), heparin (HP), dermatan sulfate (DS) and condoroitin 6 sulfate (C6S) for 3 days. Relative intensity of fluorescence is exhibited as in Fig. 2. Values are means with SDs from at least 50 independent cells. $*P < 0.01$ (significant against PBS). GFP, green fluorescent protein; PBS, phosphate-buffered saline.

the HNF3γ gene by expression of GFP reporter using purified DS and other glycosaminoglycans. As shown in Fig. 3, DS showed strong activity for induction of expression of GFP, as well as KPS which was also expressed at a considerably high level. In heparin, it increased a little. In comparison to these glycosaminoglycans, C6S hardly induced GFP expression. These results indicated that DS and KPS had an inducing activity to HNF3γ gene expression.

Discussion

Reinhararditus hippoglossoides skin, which is usually discarded as industrial waste, contains abundant collagens and proteoglycans, and its utilization as a biofunctional material can be expected. In this study, we determined the biological function of these extracellular matrix components by induction of the HNF3γ gene using GFP and luciferase reporter assays. DS showed a significant amount of activity for the induction of HNF3γ in the HepG2 human hepatoma cell culture. Previously, we showed that overexpression of HNF3γ mediated by adenovirus vector revealed some hepatoprotective ability to the injured liver (Nakamura et al., 1999). This hepatoprotection by HNF3γ turned out to be due to superinduction of hepatocyte growth factor HGF. Similarly, forced expression of HNF3γ in primary hepatocyte culture by adenovirus vector showed conservation effects on liver function such as albumin gene expression, as well as overexpression of HNF3γ-induced proliferation arrest in the cells (Nakamura et al., 1999). These observations indicate that induction of HNF3γ gene expression is involved in a pivotal role in the maintenance of hepatocyte differentiation.

Previously, we reported that DS dose-dependently increased the HGF concentration in the culture medium of MRC-5 human fibroblast cells (Noguchi et al., 2003). This stimulatory effect of DS on HGF production was evident in various types of cells, such as MRC-9, IMR-90, WI-38 and HL-60 (Matsumoto et al., 1995). Furthermore, we observed that alkali extract from fish skin has a growth inhibitory function that is possibly mediated by HGF induction in various cell types, such as HepG2, human hepatoma, HL60 and U937, histiocytic lymphoma cells (Noguchi et al., 2003).

Taken together, these observations suggested that DS from the fish skin should be involved in induction of HNF3γ expression mediated by intracellular signal transduction, followed by enhancing of HGF expression, and that HNF3γ also induced liver-specific gene expression and HGF-encouraged liver function or hepatoprotective function.

In this study, we feel we have shown that proteoglycan or glycosaminoglycan, especially DS derived from fish skin, can be used successfully in medications and functional foods for the maintenance of human liver function.

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