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The Effect of Silver Carp Skin-Derived Peptides on the Activities of VEGF and Hsp70.1 Gene Transcription Promoters

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Abstract

Background. Studies conducted on human cell culture models have demonstrated that collagen-derived peptides can exert a beneficial effect in medicine. However, all these studies were conducted using animal collagen samples, most often originating from bovine or porcine skin. Currently attempts are being made to replace animal collagen with fish collagen.

Objectives. The aim of the study was to compare the effect of silver carp skin-derived peptide extract on the transcriptional activities of human VEGF and hsp70.1 gene promoters inserted into the plasmids with secreted alkaline phosphatase as a reporter gene.

Material and Methods. Changes in the activity of the promoters were investigated using a HEK293FT cell line transfected with pVEGF-SEAP or pHsp70-SEAP. The cells were cultured in dishes containing peptides separated using reverse-phase high performance liquid chromatography.

Results. The study demonstrated that the silver carp skin-derived peptide extract exerts both an inhibitory effect on the VEGF gene promoter and activating effect on the hsp70.1 gene promoter. Higher biological activity was recorded in the case of a freshly prepared peptide extract compared to one stored at 4°C for three months.

Conclusions. The silver carp skin-derived collagen peptides influence VEGF and hsp70.1 gene promoters' transcriptional activity (Adv Clin Exp Med 2016, 25, 3, 415–423).

Key words: collagen, fish collagen, skin-derived peptide, gene promoters.

For over 20 years collagen of animal origin has been one of the hopes for elaborating novel therapeutic strategies. Studies conducted on animal models have demonstrated that collagen-derived peptides can be beneficial in fighting cancer, accelerating wound healing, and repairing damaged neurons, among other applications. However, all these studies were conducted using animal collagen samples, most often originating from bovine or porcine skin. Currently, due to reported cases of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD), scientists are try-

ing to replace animal collagen with another type, e.g. fish collagen [1].

Studies on extracellular matrix proteins, including collagen, elastin, laminin and proteoglycans (among others), have revealed that they participate in the regulation of key cellular processes such as cell migration, adhesion and differentiation, organogenesis, mitogenesis and apoptosis [2].

This regulation is mediated by signal peptides, so-called matrikines, produced after a partial proteolysis of the matrix components; this term was first proposed by Maquart to define peptides that

activate specific signaling pathways by binding to specific receptors located on the cell surface [3]. The signals reach numerous cells such as fibroblasts, smooth muscle cells, epithelial cells, macrophages, neutrophils, leukocytes, monocytes and lymphocytes [4]. They stimulate fibroblasts, both in cell cultures and *in vivo*, to increase their production of collagen [5]. Signal peptides produced as a result of metalloproteinase action can be up to about 20 kDa in size. These peptides are recognized by specific receptors located on the cell surface [6].

The number of collagen applications in medicine has increased thanks to a better understanding of the biological properties of peptides. The small size of peptides allows them to penetrate natural integuments, which results in a better therapeutic effect. They are applied to stimulate wound healing, to stop bleeding [7], and to reconstruct nerve fibers [8]. The use of collagen peptides for drug delivery is under investigation [9]. Collagen peptides demonstrate biological activity both in physiological and pathological processes, which makes them a good tool for therapies for various kinds of pathophysiological diseases, including vascular diseases, rheumatoid arthritis and all types of cancers.

This study focused on two system regulators which were expected to be regulated by skin-derived matrikines, namely vascular endothelial growth factor (VEGF) and heat shock protein hsp70.1. In a previous study the authors used mass spectrometry to demonstrate that fish skin contains small peptides (containing 7–29 amino acid residues) which could be potential matrikines [10]. The most common peptides were derived from decorin, lumican and $\alpha 1$, $\alpha 2$ and $\alpha 3$ chains of type I collagen, among others. One of the purposes of the present study is to investigate the suitability of the peptide extracts obtained to produce preparations that can accelerate wound healing.

VEGF is one of the most important regulators of angiogenesis. It binds to three types of receptors located on the surface of endothelial cells: VEGFR-1 (flt-1), VEGFR-2 (KDR), and neuropilin. Some studies also report VEGFR-3 (FLT-4) [11]. These receptors stimulate cell migration, proliferation, permeability and activity [12]. Expression of the VEGF-A gene is regulated in different tissues by a number of factors, including cytokines (e.g. EGF, TGF- β) [13], growth factors, prostaglandins, nitric oxide [14] and oncogenes (e.g. Ras) [15]. VEGF also plays an important role in the tissue regeneration process, in organ mass adjustment [16] and neurogenesis, including orientation of axonal growth [17]. Currently, angiogenesis inhibitors which could be applied in the treatment of vascular diseases and cancer are under investigation. One

of the therapeutic strategies involves collagen-derived matrikines as a factor inhibiting the growth of cancer cells [18]. These matrikines include endostatin, restin and vastatin, as well as non-collagenous domains of collagen IV. Endostatin specifically blocks the development of new blood vessels, causing latency and regression of the tumor [18].

Hsp70.1 is the main representative of the heat shock proteins, and also regulates their expression. These proteins protect the cell against the harmful consequences of environmental and physiological stress, i.e. heat, mechanical damage of tissue, UV radiation, hypoxia, heavy metals, reactive oxygen species and cell differentiation [19]. They prevent protein aggregation and the formation of abnormal connections between them. They have the ability to fold unfolded proteins, and thus they can be applied to slow down such diseases as Parkinson's, Alzheimer's and Huntington's diseases. They also initiate the immune response, including the production of pro-inflammatory cytokines, expression of MHC class II, and the release of nitric acid. They play an important role in antigen presentation and tumor immunity [20]. Heat shock protein activity also occurs during tissue damage and its regeneration, as the damaged skin barrier no longer protects against any adverse impact of the external environment [21]. A high level of hsp70 protein is recorded in the case of well-healing wounds, while in the case of chronic wounds its expression is low or does not occur [22]. Both increased production of heat shock proteins and their active participation in the process of wound healing have been demonstrated over a period of approximately two to ten hours after the damage [23, 24]. It is known that increased production of hsp70 protein may occur in the case of tissue damage that is caused both by internal tissue factors, such as PHSRN peptide derived from fibronectin [25], and bacterial lipopolysaccharides [26], where hsp70 expression inhibits cytokine excretion induced by lipopolysaccharides; this increases the survival rate of (for example) rats injected with lipopolysaccharides [27].

To date, no research concerning the impact of fish collagen peptides on the hsp protein family has been carried out. All the studies of collagen peptides in the available literature have focused on animal-derived collagen. Thus, knowledge of the biological activity and possible applications of such fish peptides in new therapeutic strategies is scarce. One of the advantages of the use of fish collagen is that it does not transmit viruses to humans, which makes fish collagen peptides potentially safer than animal-derived ones.

Therefore, this study aims at verifying whether the peptides of fish collagen exhibit pro- or anti-angiogenic characteristics, and whether they

activate the heat shock response. The study concentrated on the effect of peptides on the key regulator of angiogenesis: VEGF, whose activity contributes, among other things, to the growth and metastasis of cancer cells. Moreover, the biological activity of fish peptides was checked for response to thermal shock, using a plasmid with the hsp70.1 gene promoter sequence. The activation of this gene may also have a therapeutic relevance.

Material and Methods

Plasmids

pVEGF-SEAP is the plasmid containing cDNA of the VEGF human gene promoter with a reporter gene of the secreted alkaline phosphatase based on the pSEAP2-control plasmid [28, 29].

pHsp70-SEAP is the plasmid containing cDNA of the hsp70.1 human gene promoter with a reporter gene of the secreted alkaline phosphatase based on the pSEAP2-control plasmid.

The hsp70.1 gene promoters were cut from the pHBCAT plasmid (received from Prof. R. Morimoto at Northwestern University, USA) using *Bam*HI enzyme. They were then cloned, using the blunt-end approach, into the SEAP2 control plasmid (Clontech Laboratories Inc., Mountain View, USA) in place of the SV40 promoter that was cut out using *Bgl*III and *Nru*I enzymes. *Bam*HI and *Bgl*III restriction sites were blunted using mung bean nuclease. The correctness of the construct was confirmed by sequencing.

Cell Line Culture

The HEK293FT cell line was grown on the complete culture medium DMEM/F-12 (Sigma-Aldrich, St. Louis, USA) with the addition of 7% FBS (Sigma-Aldrich). Transfection was performed using TransFast reagent (Promega, Fitchburg, USA). The culture media were supplemented with penicillin and streptomycin, as well as antibiotic-antimycotic solution (Sigma-Aldrich).

Measurement of the Protein Activity of the Secreted Alkaline Phosphatase (SEAP)

The quantity of SEAP protein in the culture medium was investigated using a GREAT EscAPE SEAP Detection Kit (BD Clontech, Takara Bio Inc., Shiga, Japan). In the case of both VEGF and the hsp70 promoter, 24 h after the transfected cells had been seeded into 24-well plates, 50 μ L of the culture media was collected into Eppendorf tubes

and centrifuged. The supernatant was subjected to the procedure specified by the manufacturer, and finally the level of chemiluminescence was measured with a Victor device (PerkinElmer Inc., Waltham, USA) for 10 s. During each measurement of the activity of the transcription promoter, the survival rate of the cell culture, which was tested with a standard MTT assay, was taken into account. The percentage of activity change was calculated for successive fractions relative to the transfected cells cultured into an empty well.

Preparation of the Collagen Extract

Skins of silver carp, cleaned of muscle tissue and fat, were sanitized with 1% hydrogen peroxide. Then 100 mL of sterile deionized water was added to the 5 g of skin, which was incubated in a water bath at 60°C (or 80°C to examine thermal resistance) for two hours, and stirred frequently. The obtained gel was filtrated on 10 kDa centrifugal filters (Centricon, Millipore Corporation, Billerica, USA) at 20°C. The extract was concentrated *in vacuo* to 10–40-fold and stored at 4°C.

Separation of the Extract of Collagen Peptides on Reverse Phase High Performance Chromatography (RP-HPLC)

A column Ultrasphere C-18 (Hichrom Limited, Reading, UK) was used. In different experiments, from 75 μ L to 250 μ L of the extract was applied. The peptides were eluted with a gradient of 0.01% acetic acid/acetonitrile, 0–80% acetonitrile, 1 mL/min. To avoid too much dilution of the sample, the separation was performed under conditions of a considerable slope gradient of acetonitrile, so the separation was far from optimal. Eleven successive fractions containing peptides were collected in 24-well plates. The twelfth well was filled with a control sample without peptides. Two separations were performed on each plate. The plates were dried at 37°C, then the HEK293FT cells transfected with a suitable plasmid were seeded on them and grown on the DMEM/F-12 culture medium with 7% FBS and antibiotics.

Results

An example separation of peptides on the RP-HPLC bed (with the absorbance value measured at 280 nm), is shown in Fig. 1. The first hydrophilic peptides that exhibited a weak affinity for the li-

Fig. 1. Example separation chart of silver carp skin-derived collagen peptides on an RP-HPLC, using an ultrasphere C18 column

Gradient elution, flow rate 1mL/min, gradient: 0–1 min; 100%; 0.01% acetic acid; 1–8 min; 0–40% acetonitrile, 8–14 min; 80% acetonitrile and 20%; 0.01% acetic acid; 15–24 min; 0.01% acetic acid. Fractions were collected from the 4th to the 14th min onto a 24-well plate.

gand were eluted. The extracts of collagen peptides from different samples showed significant differences when observed in their chromatograms. Hydrophilic peptides (4–10 min of separation) were especially variable, and therefore they were not included in certain result sets.

The Effect of Collagen Peptides on the VEGF-A Human Gene Promoter

The pVEGF-SEAP plasmid containing cDNA of the VEGF human gene promoter with a reporter gene of the secreted alkaline phosphatase based on the pSEAP2-control plasmid was constructed by one of the authors of this paper (JGW) in the Department of Biotechnology of the Medical University of Gdańsk, Poland. The effectiveness of the measurement method activating the VEGF promoter in HEK293 cells was checked using hydrogen peroxide, which is known to stimulate the VEGF promoter. Stimulation of the promoter was 2.6-fold of the control sample at a concentration of 200 μ M of H_2O_2 , and a concentration of 500 μ M was lethal for HEK293FT cells.

Whether silver carp skin-derived collagen peptides can affect the activity of the VEGF-A promoter and thereby participate in the regulation of angiogenesis was investigated. For this purpose HEK293FT cells transfected with pVEGF-

SEAP plasmid were cultured on 24-well plates filled with the separated and evaporated extract of collagen peptides. Samples were collected 24 h after the seeding of the transfected cells on the collagen peptides, and the transcription activity of the VEGF promoter was measured using the aforementioned SEAP detection kit (BD Clontech). Ten samples of silver carp skin-derived collagen peptides were measured. The concentration of peptides before chromatographic separation was 11 mg/mL, and the amount applied to the RP-HPLC was 50–250 μ L. The results were divided by the value of the survival rate of the cell culture that was ascertained by the MTT assay. Subsequently, the percentage of change in VEGF promoter activity level relative to the control sample (cells transfected with the pVEGF-SEAP plasmid and not cultured on collagen peptides) was calculated.

The series of experiments showed that the fish-derived collagen peptides generally inhibit the activity of the VEGF promoter (Fig. 2). In some cases (the fractions containing hydrophilic peptides) activation of only the VEGF promoter was observed, which indicated that the overall effect is a result of inhibitory and activating peptides. In hydrophobic fractions, a stronger inhibitory effect on the activity of the VEGF promoter occurred, but generally all the fractions influenced the VEGF promoter.

Due to the fact that commercial collagen extracts contain lactic acid, which is difficult to sepa-

Fig. 2. The level of activation of human VEGF gene promoter induced by silver carp skin-derived collagen peptides

The successive bars represent the mean of 10 experiments for each successive fraction of the collagen peptide extracts separated by RP-HPLC. The fractions correspond to one-minute separation times, with fraction 1 starting from the fourth minute and fraction 11 corresponding to a retention time of 14 min. The vertical axis represents the activity of the VEGF promoter relative to the control, expressed as a percentage. Standard error is indicated in each column. Most of the fractions exhibited inhibition of the VEGF promoter.

rate from biologically active peptides present in the extract, a more drastic method was used: extraction in deionized water at 60°C (and separately at 80°C to check the stability of the peptides in the extraction process), which does not introduce any additional chemical substances. Figure 3 presents the inhibiting impact of the individual fractions produced in the chromatographic separation of the extracts at a concentration of 11.5 mg/mL and at temperatures of 60°C and 80°C, respectively. Four repetitions for each temperature were performed.

The series of experiments showed that the extracts of collagen peptides prepared at 60°C and 80°C differ in their activity on the VEGF promoter by approximately 15% (Fig. 3). Taking into consideration that, according to van't Hoff's rule, for each 10°C rise in reaction temperature the rate of reaction (including decomposition) increases 2–4 fold, the preparations prepared at 60°C can be regarded as fully active.

The activity of collagen peptides in the extract was also tested after three months of storage at 4°C. Four assays were performed on a 3-month-old extract of collagen peptide, and the results were compared with 4 assays made shortly after the preparation of the extract. The results are presented in Fig. 4. The figure shows an approximately two-fold decrease in activity after 3 months of storage at 4°C.

In further tests the effect of the peptide concentration on the level of inhibition of the VEGF gene promoter was examined. Four concentrations of peptides obtained after the HPLC columns

Fig. 3. A comparison of the inhibition level of the VEGF promoter by successive fractions of collagen peptide extracts, separated by RP-HPLC, prepared at two temperatures: 60°C and 80°C

The dark bars represent peptide extraction at 60°C and the light ones represent extraction at 80°C. On the Y axis the inhibition of the VEGF promoter is expressed as a percentage relative to the activity of cells that were not treated with the collagen peptides. Each bar represents the mean of 4 preparations.

Fig. 4. The influence of the 3-month storage of the concentrated solution of peptides on their activity on VEGF

The dark bars represent the inhibition activity of freshly prepared concentrated peptides; the light bars show the inhibition activity of concentrated peptides stored in a refrigerator for three months at 4°C. Each bar shows the mean results of four experiments. VEGF promoter inhibition activity is given in percentages relative to the activity of HEK293 cells which were not treated with peptides.

were compared (applying 75 µL, 150 µL, 200 µL or 250 µL of the sample to the RP-HPLC at a concentration of 11 mg/mL). Four assays for each quantity were performed; the results were averaged and are presented in Fig. 5.

Fig. 5. A comparison of VEGF promoter inhibition levels by fractions of collagen peptides at different concentrations

The different concentrations of peptides resulted from differences in the amount of concentrated extract applied to the RP-HPLC column. The bars in each group correspond to 75 µL, 150 µL, 200 µL and 250 µL, respectively; the average results of 4 tests at each concentration are presented. The inhibition is given in percentages relative to the activity of HEK293 cells that were not treated with peptides.

This experiment showed that an increase in the concentration of the collagen peptides does not increase the inhibition of the VEGF promoter's transcriptional activity. It is probable that at these concentrations the promoter has already been saturated. However, it should be considered that once the amount of the extract applied to the chromatographic column is increased, the resolving power decreases.

The Effect of Collagen Peptides on the hsp70.1 Human Gene Promoter

The effects of collagen peptides on the hsp70.1 human gene promoter were investigated using a pHsp70-SEAP plasmid constructed by one of the authors of this study (DW) in the Department of Biotechnology of the Medical University of Gdańsk, Poland. It contains a gene-encoding secreted alkaline phosphatase under the control of the the 70.1 human gene transcriptional promoter. The transcriptional activity of the hsp70 gene was measured with the same SEAP detection kit mentioned above, using secreted alkaline phosphatase as the reporter gene.

In order to verify whether the function of the hsp70.1 human gene promoter situated on the pHsp70-SEAP plasmid was correct, the HEK-293FT cells transfected with this plasmid were subjected to heat shock in a shaking water bath. Samples of the culture medium were collected every 2 h; each collection was followed by a change of the medium, so that the level of protein production over time was known. The change in transcriptional activity was investigated with the SEAP detection kit previously described. Six hours after the heat shock, the activity of the hsp70.1 promoter in HEK293 cells subjected to a temperature of 43.5°C had increased 4-fold.

In order to investigate the biological activity of the collagen extracts, HEK293FT cells transfected

with the pHsp70-SEAP plasmid were cultured on 24-well plates filled with the separated extract of collagen peptides, prepared as described above. Samples of the medium were collected 24 h after the seeding of the transfected cells on the collagen peptides. The hsp70.1 promoter's transcriptional activity was measured in relation to the survival rate of the cell culture. The results were processed as in the case of the VEGF gene. Ten assays were performed using silver carp skin-derived collagen peptides at a concentration of 11 mg/mL before the chromatographic separation. The results are presented in Fig. 6.

This series of experiments showed that collagen peptides activate the hsp70.1 human gene promoter at a 1.5-fold level. In the fractions showing relatively high hsp70.1 promoter activity, the peptides are hydrophobic, and they are eluted last in chromatographic separation.

As in the case of the VEGF promoter, experiments were also conducted to check whether the extraction temperature affects the level of the peptides' biological activity on the hsp70.1 promoter. The activities of collagen peptides extracts whose concentration before the chromatographic separation equaled 11 mg/mL and which were prepared at 60°C and 80°C were compared. For each temperature 4 assays were performed, comparing the fractions of the extract separated on RP-HPLC. The results are shown in Fig. 7.

Contrary to expectations, a comparison of the effect of collagen peptides prepared at 2 different temperatures, 60°C and 80°C, on the activity of the hsp70.1 promoter revealed that in fractions 7, 8 and 10 the peptides prepared at 80°C evoked a considerably stronger activation.

In the next stage of the study a test was performed to find out whether different concentrations of peptides affect the level of activation of the hsp70.1 gene promoter. In this experiment 3 different amounts of peptide extract were ap-

Fig. 6. The level of activation of the hsp70.1 human gene promoter induced by silver carp skin-derived collagen peptides

The successive bars represent the mean of 10 experiments for successive fractions of collagen peptide extracts separated by RP-HPLC. The fractions are analogous to the chromatographic separation shown in Fig. 2. The vertical axis represents the activity of the hsp70.1 promoter relative to the control sample, expressed as percentages. Standard error is indicated in the columns. The majority of fractions showed activation of the hsp70.1 promoter.

Fig. 7. A comparison of the activation level of the hsp70.1 promoter by fractions of collagen peptide extracts prepared at 60°C and 80°C

The dark bars represent extraction of the peptides at 60°C, and the light ones represent extraction at 80°C. The change in activity of the hsp70.1 promoter is expressed as a percentage relative to the activity of cells which were not treated with collagen peptides. Each bar represents the average of four preparations.

plied to the RP-HPLC column at a concentration of 11.5 mg/mL: 100 μ L, 150 μ L and 200 μ L. Four assays for each amount were performed, and

then the results were averaged and are presented in Fig. 8.

The figure shows that there is an increase in hsp70.1 promoter activation between 100 μ L and 200 μ L.

The study has demonstrated the effect of silver carp skin-derived collagen peptides on the transcriptional activity of the VEGF and hsp70.1 genes promoters. It has been shown that VEGF activity is inhibited on average by 20–30%, while in the case of the hsp70.1 gene, transcriptional activity increased by 30–50%. As a result of extraction of the peptides with water at 60–80°C, a biologically active preparation was obtained without the presence of any organic acids or salts that would contaminate the preparation in the case of acid or alkaline hydrolysis. However, storage of the preparation for 3 months at 4°C reduced the biological activity of the extract to approximately one half in terms of VEGF gene transcription. This probably translates into similar changes in industrial silver carp skin-derived collagen extract obtained with the use of lactic acid.

The results obtained show that fish skin-derived peptides may be applied as an anti-angiogenic factor and as a factor stimulating the repair response by activation of the heat shock protein re-

Fig. 8. A comparison of the level of stimulation of the hsp70.1 promoter activity by collagen peptide fractions of different concentrations

The different concentrations of peptides resulted from differences in the amount of concentrated extract applied to the RP-HPLC column. The bars in each column represent 100 μ L, 150 μ L and 200 μ L, respectively. Each bar represents the average of 4 tests at each concentration.

sponse. The study was a preliminary one, and was performed on HEK293 cell line only, and thus it requires confirmation with the use of other cell lines. This includes fibroblasts, especially in terms of wound healing, for which silver carp skin-derived collagen extracts have already shown their effectiveness. However, the inhibitory effect on VEGF observed in this study is ambiguous. As some animal collagen peptides are already being clinically tested for use as angiogenic inhibitors to inhibit tumor cell growth, fish peptides might also be applied in a similar way. Due to the origin of the skin extract, stimulation of the hsp70.1 gene transcription caused by bacterial LPS should be excluded. However, the literature data show that the hsp70.1 protein occurs in well-healing wounds, so stimu-

lation of hsp70.1 expression was expected. The effect of fish-derived collagen peptides on VEGF and heat shock proteins has not yet been examined.

This paper demonstrated that hydrophobic peptides are characterized by the greatest biological activity. This is favorable in the sense that silver carp skin-derived peptide separation using extraction with organic solvents, e.g. chloroform, leads to active peptides with some degree of hydrophobicity [10], which in turn allows the peptides to be concentrated in a simple manner for use in cosmetic and medicinal products. Another important characteristic of small peptides is their effective penetration into the dermis, which has been confirmed for fish peptides obtained by extraction with enzymes [30].

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