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ORIGINAL ARTICLE

Collagen-derived dipeptide, proline-hydroxyproline, stimulates cell proliferation and hyaluronic acid synthesis in cultured human dermal fibroblasts

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ABSTRACT

Orally ingested collagen undergoes degradation to small di- or tripeptides, which are detected in circulating blood 2 h after ingestion. The influence of collagen-derived peptides on dermal extracellular matrix components and cell proliferation was studied using cultured human dermal fibroblasts. Of the various collagenous peptides tested here, the dipeptide proline-hydroxyproline (Pro-Hyp) enhanced cell proliferation (1.5-fold) and hyaluronic acid synthesis (3.8-fold) at a dose of 200 nmol/mL. This was concomitant with a 2.3-fold elevation of hyaluronan synthase 2 (*HAS2*) mRNA levels. Small interfering RNA (siRNA)-mediated knockdown of the *HAS2* gene in human dermal fibroblasts inhibited Pro-Hyp-induced *HAS2* mRNA transcription and cell mitotic activity. Addition of genistein or H7, a protein kinase inhibitor, abolished the Pro-Hyp-induced *HAS2* mRNA stimulation. Pro-Hyp elevated phosphorylation of signal transducer and activator of transcription 3 (STAT3) within a short time period (60 min). These results suggest that Pro-Hyp stimulates both cell mitotic activity and hyaluronic acid synthesis, which is mediated by activation of *HAS2* transcription.

Key words: collagen peptide, fibroblast, hyaluronan, hyaluronan synthases 2, signal transducer and activator of transcription.

INTRODUCTION

Collagen is a major constituent of connective tissues of animals, birds and fish. Gelatin, a denatured form of collagen, prepared on an industrial scale from these materials,[1] is used as a folk medicine to improve joint condition by reducing pain.[2] Some animal experiments and preclinical human trials have also suggested that oral ingestion of gelatin hydrolysate might have beneficial effects. We previously reported that daily ingestion of a type I collagen hydrolysate mixture, including 5 g of fish type I collagen hydrolysate, improved skin moisture content and elasticity.[3] Therefore, we hypothesized that supplementation with collagen hydrolysate potentially changes extracellular matrix (ECM) metabolism in the skin.

It is difficult to understand how collagen, a high molecular weight protein, could be absorbed in the intestine and transported to the dermis. Osser *et al.*[4] determined the bioavailability of collagen hydrolysate after p.o. administration in mice using ^{14}C-labeled hydrolysate. The distribution of labeled amino acids in skin was confirmed and 58% of the peak value of labeled amino acids remained 192 h after administration. Iwai *et al.*[5] identified a small dipeptide, proline-hydroxyproline (Pro-Hyp), in the blood of healthy human volunteers who had ingested porcine skin collagen hydrolysate. Although there are no previous reports describing absorption of collagen hydrolysate

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through the intestinal epithelium, it was found that some small peptides, such as carnosine (beta-Ala-L-His), were absorbed from the small intestine.[6] It is well known that the abundance of the oligopeptide transporter (PEPT-1) in the brush-border membranes of intestinal epithelium is the principal regulatory mechanism for transport of protein digestion products (dipeptides and tripeptides).[7] It therefore may be possible for Hyp-containing di- or tripeptides to be absorbed transcellularly, at least partly, through this peptide transporter. We have previously reported that several collagen-derived peptides were detected in human blood 2 h after oral ingestion of fish collagen hydrolysate.[8] The major constituent of collagen-derived peptides that remained in blood was identified as Pro-Hyp (39% of total Hyp-containing peptides), while the minor components were Ala-Hyp (15%), Ala-Hyp-Gly (16%), Pro-Hyp-Gly (5%), Leu-Hyp (2%), Ile-Hyp (2%) and Phe-Hyp (3%).[5,8]

In this report, we studied eight collagen-derived Hyp-containing peptides on cell motility and modulation of ECM proteins using *in vitro* cultured dermal fibroblasts, which are the major ECM-producing cells in skin.

METHODS

Reagents

Hydroxyproline (Hyp) and proline (Pro) were purchased from Nacalai Tesque (Kyoto, Japan). Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Pro-Hyp-Gly, Ile-Hyp, Leu-Hyp and Phe-Hyp were purchased from Kokusan Chemical (Tokyo, Japan), and Pro-Hyp was from Bachem (Bubendort, Germany). Genistein, sodium orthovanadate and basic fibroblast growth factor (bFGF) were purchased from Wako Pure Chemical Industries (Osaka, Japan). H7 dihydrochloride (H7) was from Sigma (St Louis, MO, USA). (6-^{3}H) Glucosamine/HCl (1,3 TBq/mmol) was purchased from Amersham Bioscience (Piscataway, NJ, USA).

Cell culture

Human dermal fibroblast cells were explanted from a skin biopsy sample from a normal individual. Cells were grown at 37°C in 35-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) in a humidified atmosphere of 5% CO_2/95% air and were subcultured every 7 days. Cells in passage 5–7 were used.

Cell growth kinetics

Cells were plated at a density of 3.6×10^4 in each well of a 6-well plate and grown for 1 day in 10% FBS-DMEM. On day 1, culture medium was replaced with 1% FBS-DMEM with collagen-derived Hyp-containing peptides (200 nmol/mL). Cells were incubated for a further 6 days. On day 7, cells were trypsinized (0.25%) and cell numbers were counted with a hemocytometer. For a dose-dependent analysis of the Pro-Hyp peptide on cell proliferation, fibroblasts were again plated at a density of 3.6×10^4 in 6-well plates and incubated for 1 day in 10% FBS-DMEM. After a further incubation of 6 days in 1% FBS-DMEM containing Pro-Hyp (0, 50, 100, 200 and 400 nmol/mL), cells were trypsinized and cell numbers were counted. bFGF (50 ng/mL) treatment was used as a positive control.

Glycosaminoglycan synthesis

Cells were grown to confluency in 10% FBS-DMEM and then placed in serum-free DMEM for 24 h. Cells were treated with Pro-Hyp (200 nmol/mL) and (6-^{3}H) glucosamine/HCl for the last 24 h of treatment. Glycosaminoglycans were isolated from the medium and cell layer separately as previously described.[9] Cells were trypsinized and an aliquot was taken for cell counting. (^{3}H)-labeled hyaluronate was electrophoretically resolved on a cellulose acetate membrane. For quantitative determinations, each spot was cut out and dissolved in 1 mL dioxane. Radioactivity was determined with a Beckman LS 9000 liquid scintillation system (Beckman Instruments, Fullerton, CA, USA).

Total RNA isolation, cDNA synthesis and quantitative real-time reverse transcription polymerase chain reaction (RT–PCR) analysis

Total RNA was isolated from human dermal fibroblasts using the RNeasy mini-kit (QIAGEN, Hilden, Germany). Extracted RNA was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at a wavelength of 260 nm. RT was performed using a cDNA synthesis kit (Fermentas, Burlington, ON, Canada). The cDNA was stored at −80°C until subsequent analysis. Real-time PCR was performed using the Applied Biosystems 7900 HT

Real-Time PCR system according to the supplier's recommendations. Primers and probes (TaqMan Assays-on-Demand Gene Expression Products) of the genes for collagen type I (*COL1A1*), type III (*COL3A1*), type IV (*COL4A1*), elastin (*ELN*), hyaluronan synthases 1, 2 and 3 (*HAS1*, *HAS2*, *HAS3*), and β-glucuronidase (*GUS*) were designed by Applied Biosystems from gene sequences obtained from GenBank (accession no.: NM_00008.3 for *COL1A1*, NM_00009.3 for *COL3A1*, NM_001845.3 for *COL4A1*, NM_000501.1 for *ELN*, NM_001523.1 for *HAS1*, NM_005328.1 for *HAS2*, NM_005329.2 for *HAS3* and NM_000181.3 for *GUS*). Relative mRNA amounts of selected genes were calculated using the standard curve method. mRNA quantity was normalized to the amount of *GUS* mRNA in each cDNA sample. TaqMan quantitative RT–PCR results are presented as the mean ± standard error of the mean (SEM).

Small interfering RNA (siRNA)-mediated silencing of *HAS2* expression

Four siRNA duplexes, designed with symmetric 3′TT overhangs to target different nucleotide sequences (no. 1, cat no. SI00075810; no. 2, cat no. SI00075817; no. 3, cat no. SI00075824; no. 4, cat no. SI00075831) of the human *HAS2* gene, and ''allstars'' negative control siRNA were obtained from QIAGEN. Human dermal fibroblast cells were grown to subconfluency in 10% FBS-DMEM (6-well plates). Subconfluent cells were placed in serum-free DMEM and transfected separately with one of four siRNA duplexes or with control siRNA at a final concentration of 5 nmol/L using HiPerFect Transfection Reagent (QIAGEN) according to the manufacturer's instructions. Furthermore, cells were treated with Pro-Hyp (200 nmol/mL) in some of the transfection experiments. The suspension efficiency of each siRNA duplex was examined by measuring *HAS2* mRNA expression levels using real-time RT–PCR 24 h after transfection.

Effect of cell growth kinetics by *HAS2* siRNA transfection

Cells were plated at a density of 3.6×10^4 in each well of a 6-well plate and grown for 1 day in 10% FBS-DMEM. On day 1, cells were transfected with control siRNA or *HAS2* siRNA (no. 4) as described above. Differences in the cell growth kinetics between *HAS2* siRNA and control siRNA transfected cells were examined in the absence or presence of Pro-Hyp (200 nmol/mL) by counting cell numbers on day 7. Cell numbers were determined using the cell counting kit-8 (Dojindo, Kumamoto, Japan).

Effect of protein kinase inhibitors on Pro-Hyp-induced *HAS2* mRNA expression

Confluent cultures of human dermal fibroblasts were washed twice with phosphate buffered saline and placed in serum-free DMEM. Twenty-four hours later, the cells were pre-incubated for 2 h with or without the following inhibitors at optimal concentrations: tyrosine kinase inhibitor (50 μg/mL genistein),[10] serine/threonine kinase inhibitor (80 μmol/L H7)[11,12] and tyrosine phosphatase inhibitor (100 μmol/L sodium orthovanadate).[10] Sodium orthovanadate was used as a control. Cells were then incubated for a further 24 h with or without 200 nmol/mL Pro-Hyp.

Direct monitoring of signal transducer and activator of transcription 3 (STAT3) phosphorylation

Human dermal fibroblasts were plated at a density of 2.0×10^4 in each well of a 96-well plate and grown for 1 day in 10% FBS-DMEM. On day 1, the culture medium was replaced by serum-free DMEM. Twenty-four hours later, cells were treated with 200 nmol/mL Pro-Hyp for 0, 30 or 60 min. The amount of activated (phosphorylated) STAT3 protein was analyzed using the cellular activation of signaling enzyme-linked immunosorbent assay (ELISA) kit for STAT3 Y705 (CASE; SuperArray Bioscience, Frederick, MD, USA) according to the manufacturer's instructions.[13]

Statistics

Statistical analyses were conducted using SPSS ver. 10.02 for Windows with data expressed as mean ± SEM. Data were analyzed by Dunnett's multiple comparison test or an unpaired Student's *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS

Effects of collagen-derived Hyp containing peptides on cultured human dermal fibroblast proliferation

Human dermal fibroblasts were cultured in the presence of collagen-derived Hyp containing peptides

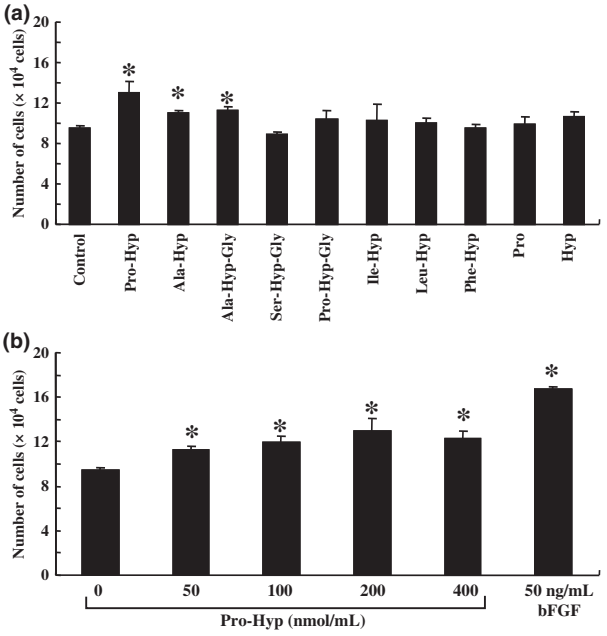

Figure 1. Effects of hydroxyproline (Hyp)-containing peptides on fibroblast proliferation. (a) Fibroblasts were treated with 1% fetal bovine serum Dulbecco's modified Eagle's medium (FBS-DMEM) containing various species of collagen-derived Hyp containing peptides (200 nmol/mL) for 6 days. On day 7, cells were harvested and the number of cells in four different fields was counted with a hemocytometer. Values are mean ± standard error of the mean (SEM) obtained from three independent experiments. *Statistical significance at $P < 0.05$. (b) Fibroblasts were treated with 1% FBS-DMEM containing proline-hydroxyproline (Pro-Hyp) (0, 50, 100, 200 and 400 nmol/mL). On day 7, cell numbers were counted. Values are mean ± SEM from three independent assays. Basic fibroblast growth factor (bFGF) (50 ng/mL) was used as a positive control. *Statistical significance at $P < 0.05$.

(Pro-Hyp, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Pro-Hyp-Gly, Ile-Hyp, Leu-Hyp, Phe-Hyp, Pro and Hyp) for 6 days, each at a concentration of 200 nmol/mL, and cell numbers were counted (Fig. 1a). The peptides, Pro-Hyp, Ala-Hyp and Ala-Hyp-Gly, stimulated fibroblast proliferation slightly but significantly ($P < 0.05$). Maximal stimulation of cell proliferation (1.5-fold) was observed by Pro-Hyp treatment. On the basis of these cell kinetic results, further studies were focused on the peptide Pro-Hyp. Cell numbers were increased dose-dependently by Pro-Hyp at concentrations ranging 0–200 nmol/mL, but declined at a dose of 400 nmol/mL. A positive control using 50 ng/mL bFGF stimulated cell proliferation 1.8-fold as previously described (Fig. 1b).[14]

Effects of collagen-derived Hyp containing peptides on hyaluronan synthase (*HAS*) mRNA levels

No significant changes in *COL1A1, COL3A1, COL4A1, ELN*, *HAS1* and *HAS3* mRNA levels were

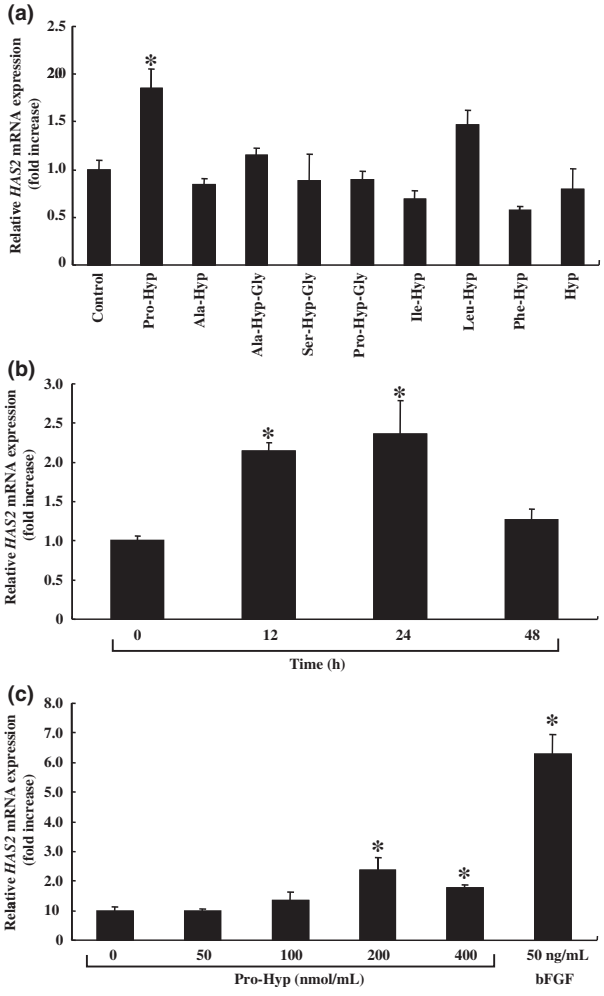

Figure 2. Hyaluronan synthase 2 (*HAS2*) mRNA levels in cultured fibroblasts treated with various hydroxyproline (Hyp)-containing peptides. (a) Fibroblasts were treated with various species of collagen-derived Hyp containing peptides (200 nmol/mL) for 24 h in serum-free Dulbecco's modified Eagle's medium (DMEM). (b) Fibroblasts were treated with proline hydroxyproline (Pro-Hyp) (200 nmol/mL) for 0, 12, 24 and 48 h in serum-free DMEM. (c) Fibroblasts were treated with Pro-Hyp for 24 h at a dose of 0, 50, 100, 200 and 400 nmol/mL in serum-free DMEM. RNA was extracted from the cells and *HAS2* mRNA levels were determined by real-time polymerase chain reaction. Cells treated with basic fibroblast growth factor (bFGF) (50 ng/mL) for 24 h were used as a positive control. Values are mean ± standard error of the mean from triplicate assays. *Statistical significance at $P < 0.05$.

observed by any of the collagen-derived Hyp containing peptides examined here as measured by real-time PCR (data not shown). Relative *HAS2* mRNA content was significantly increased by Pro-Hyp treatment ($P < 0.05$), but was unchanged by other collagen-derived Hyp containing peptides (Fig. 2a). The effect of Pro-Hyp on mRNA expression of *HAS2* was determined by real-time PCR. To accomplish this, 0, 12, 24 and 48 h after addition of Pro-Hyp, cells were harvested, total mRNA isolated and quantitative real-time PCR for *HAS2* was performed. Maximum mRNA levels were reached 24 h after addition of Pro-Hyp (Fig. 2b). The average induction of *HAS2* mRNA at 24 h was 2.3-fold above controls. Based on these results, in subsequent experiments, we determined *HAS2* mRNA levels at 24 h. These results led us to study the effects of Pro-Hyp in detail using various doses of Pro-Hyp. The increase of *HAS2* mRNA levels by Pro-Hyp was dose-dependent up to a concentration of 200 nmol/mL, but declined at 400 nmol/mL (Fig. 2c).

Effects of collagen-derived Hyp containing peptides on hyaluronic acid synthesis

Treatment of cultured human dermal fibroblasts with 200 nmol/mL Pro-Hyp for 24 h increased total hyaluronic acid synthesis (medium + cell) approximately 3.8-fold (Fig. 3), which is comparable to the increase of *HAS2* mRNA levels (Fig. 2c). Treatment with bFGF (50 ng/mL) also increased *HAS2* mRNA levels by approximately 4-fold as previously reported.[15]

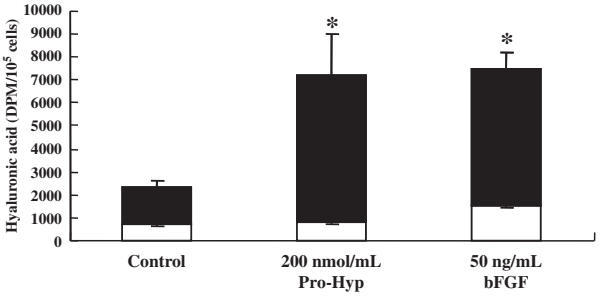

Figure 3. Hyaluronic acid synthesis is increased by proline-hydroxyproline (Pro-Hyp). Fibroblasts were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) containing Pro-Hyp (200 nmol/mL) and (^{3}H) glucosamine for 24 h. Glycosaminoglycans were isolated from medium (closed) and cells (open column) as described in Methods. Total hyaluronic acid synthesis was calculated from the culture medium and cells. Cells treated with basic fibroblast growth factor (bFGF) (50 ng/mL) for 24 h were used as a positive control. Values are mean ± standard error of the mean from five independent experiments. *Statistical significance at $P < 0.05$.

Effect of *HAS2* mRNA expression and cell growth kinetics by *HAS2* siRNA transfection

To further examine the role of *HAS2* in Pro-Hyp-induced cell proliferation, siRNA experiments were performed. We investigated the consequences of suppression of the *HAS2* gene by designing specific *HAS2* siRNA duplexes. Transfection of cells with 5 nmol/L of siRNA duplexes targeted to four different sites of *HAS2* cDNA led to 0.1–0.3-fold *HAS2* transcript suppression irrespective of the species of

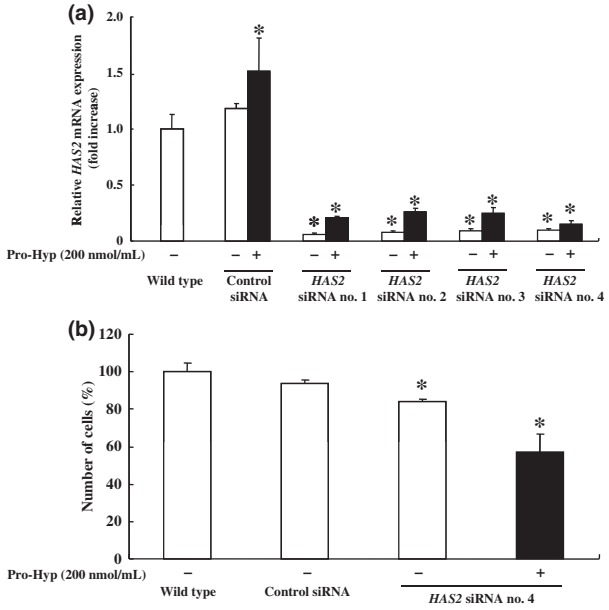

Figure 4. Effects of small interfering RNA (siRNA)-mediated knockdown of hyaluronan synthase 2 (*HAS2*) on the expression level of *HAS2* mRNA and cell growth kinetics. (a) Fibroblasts were transfected with 5 nmol/L of control siRNA or four different *HAS2* siRNA duplexes (nos. 1, 2, 3 or 4) in the absence or presence of proline-hydroxyproline (Pro-Hyp) (200 nmol/mL). The expression levels of *HAS2* mRNA were quantified by real-time reverse transcription polymerase chain reaction 24 h after transfection in serum-free Dulbecco's modified Eagle's medium. Values are mean ± standard error of the mean (SEM) from triplicate assays. *Statistical significance at $P \leq 0.05$. (b) Fibroblasts were transfected with 5 nmol/L of control siRNA or *HAS2* siRNA (no. 4) in the absence or presence of Pro-Hyp (200 nmol/mL). On day 7, cell numbers were counted. Values are mean ± SEM from triplicate assays. *Statistical significance at $P \leq 0.05$.

HAS siRNA (numbers 1–4) compared to wild type (Fig. 4a). We chose *HAS2* siRNA (no. 4) for further experiments examining the effects of *HAS2* siRNA transfection on cell growth. The results showed that *HAS2* siRNA (no. 4) transfection, both in the absence or presence of Pro-Hyp, significantly inhibited cell proliferation but the effect was greater in the presence of Pro-Hyp (Fig. 4b).

HAS2 mRNA induction by Pro-Hyp is dependent on protein kinases

To identify the signaling pathway induced by Pro-Hyp, we examined the effects of inhibitors of kinases on Pro-Hyp-induced *HAS2* mRNA expression in human dermal fibroblasts. Both genistein, a tyrosine kinase inhibitor, and H7, a serine/threonine kinase inhibitor, reduced basal *HAS2* mRNA levels. Threshold cycle (Ct) values of *GUS*, used as housekeeping gene, in genistein (23.97 ± 0.08) and H7 groups (24.01 ± 0.05) were not significantly different than that of the control group (24.03 ± 0.03). This result indicated that cell cytotoxicity is not affected by each inhibitor. These compounds were found to be effective antagonists of Pro-Hyp-induced stimulation. A control using sodium orthovanadate, a tyrosine phosphatase inhibitor, increased basal *HAS2* mRNA levels by approximately 2.4-fold. However, this showed no inhibiting effects on Pro-Hyp-stimulated *HAS2* mRNA expression (Table 1). On the other hand, inhibition of the *HAS2* mRNA expression by genistein and H7 does not depend on restraint of the transcription activity in this experiment and does not confirm it whether it is the thing by reduction of mRNA stability. However, we think that both these are possible.

Table 1. Effects of various kinase inhibitors on proline-hydroxyproline (Pro-Hyp)-induced hyaluronan synthase 2 (*HAS2*) mRNA expression

	HAS2 mRNA level	
Inhibitor	Pro-Hyp$^-$	Pro-Hyp$^+$
None	1 ± 0.05	2.36 ± 0.43*
Genistein	0.32 ± 0.01*	0.33 ± 0.01*
H7	0.02 ± 0.01*	0.02 ± 0.01*
Sodium orthovanadate	2.48 ± 0.78*	6.04 ± 2.14*†

Cells were pre-incubated for 2 h with genistein (50 μg/mL), H7 (80 μmol/L) or sodium orthovanadate (100 μmol/L), then incubated a further 24 h with Pro-Hyp (200 nmol/mL) in the presence or absence of these inhibitors. RNA was extracted from the cells and *HAS2* mRNA levels were determined by real-time polymerase chain reaction. Values are mean ± standard error of the mean from triplicate assays. *Significantly different from control levels (minus inhibitors, minus Pro-Hyp; $P < 0.05$). †Values are significantly different from cells treated with Pro-Hyp alone ($P < 0.05$).

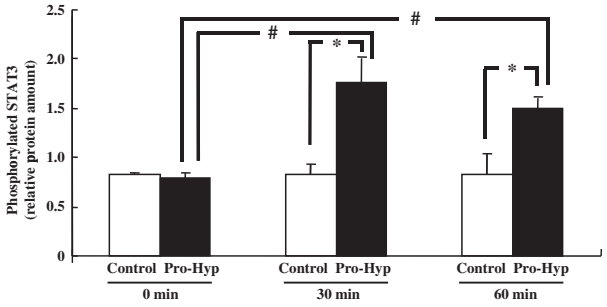

Figure 5. Effect of proline-hydroxyproline (Pro-Hyp) on the levels of phosphorylated signal transducer and activator of transcription 3 (STAT3) in human dermal fibroblasts. Cells were treated with 200 nmol/mL Pro-Hyp for 0, 30 or 60 min. The amount of phosphorylated STAT3 was determined by enzyme-linked immunosorbent assay. Values are mean ± standard error of the mean from triplicate assays. *Significant increase as compared with untreated cells ($P < 0.05$). #Significant increase as compared with 0 min ($P < 0.05$).

Effect of Pro-Hyp on phosphorylated STAT3 levels in human dermal fibroblasts

Recent reports indicate that phosphorylated STAT3 enhanced *HAS2* transcription.[16] Therefore, we examined phosphorylation of STAT3. Pro-Hyp (200 nmol/mL) markedly induced phosphorylation of STAT3 after 30 and 60 min of incubation, while phosphorylation of STAT3 was constant in control (untreated) cells (Fig. 5).

DISCUSSION

All species of collagen-derived Hyp containing peptides, except Pro-Hyp, did not change the expression of matrix-related genes including *COL1A1*, *COL3A1*, *COL4A1*, *ELN* and *HAS1–3* in cultured dermal fibroblasts. Pro-Hyp alone stimulated both cell proliferation and *HAS2* mRNA levels. In *in vitro* studies using cell culture systems, Pro-Hyp has been found to possess chemotactic activity for fibroblasts, peripheral blood neutrophils[17,18] and monocytes,[19] and is involved in collagen-platelet and collagen-cytokine interactions.[20,21] In addition, the presence of Pro-Hyp in human plasma for a relatively

longer period than other collagen peptides after ingestion of collagen hydrolysate[8] suggests its important physiological role in normal and pathological conditions such as wound healing and inflammation.

Maximal stimulation of cell proliferation and hyaluronan synthesis by Pro-Hyp in this experiment was achieved at doses of 200 nmol/mL, which is similar to physiological concentrations because the amount of collagen-derived Hyp containing peptides in plasma 2 h after oral ingestion of fish scale gelatin hydrolysate is reported to be approximately 140 nmol/mL plasma.[8]

The relationship between cell proliferation and hyaluronic acid is intriguing. Pro-Hyp-induced cell proliferation may be associated with Pro-Hyp-induced hyaluronic acid stimulation. Although there is no evidence that hyaluronic acid acts directly on fibroblast mitogenic activity, it has been shown that high levels of hyaluronic acid are present during cell mitosis and that inhibition of hyaluronic acid synthesis leads to prevention of cell mitosis and proliferation.[22,23] These previous studies suggest that hyaluronic acid does not produce mitogenic activity but instead promotes hydration of the extracellular space that aids cell proliferation.[24] In this study, we found that *HAS2* siRNA inhibited both Pro-Hyp-induced *HAS2* mRNA expression and Pro-Hyp-induced cell proliferation in human dermal fibroblasts. Increased fibroblast proliferation by Pro-Hyp in this study may be related to increased hyaluronic acid synthesis.

Hyaluronan, a linear glycosaminoglycan, consists of alternating D-glucosamine residues. Hyaluronan is found in almost all connective tissues and is thought to participate in many biological processes and hyaluronan levels are markedly elevated during embryogenesis, cell migration, wound healing, malignant transformation and tissue turnover.[25] Molecules of hyaluronan are generally of very high molecular mass, ranging approximately 10^5–10^7 Da, depending upon the tissue. Hyaluronan exhibits unusual physicochemical properties in concentrated solutions because of its capacity to interact with water molecules. A molecule of hyaluronan, therefore, has a large hydrodynamic volume and forms solutions with high viscosity and elasticity that provide space filling, lubricating and filtering functions.[26] In fact, hyaluronan injection has now proven to be the most safe and minimally complicated procedure in many injectable fillers for reconstructive and cosmetic medicine.[27] Hyaluronan synthesis is regulated by hyaluronan synthase genes (*HAS1*, *2* and *3*). *HAS1–3* are regulated independently. For instance HAS3 produces lower molecular mass hyaluronan than *HAS2*. HAS2, but not HAS1 or HAS3, is responsible for hyaluronan synthesis in cultured skin fibroblasts.[28] These unique properties of *HAS1–3* support our results that *HAS1* and *3* mRNA were unchanged by all species of collagen-derived Hyp containing peptides examined here, but *HAS2* mRNA alone was increased by Pro-Hyp. This was accompanied by a comparable increase of hyaluronan synthesis in cultured dermal fibroblasts. Our results also suggest that oral ingestion of collagen hydrolysate may lead to more viscous and elastic skin resulting in improvement of skin appearance.

A previous report investigated the effect of bFGF, insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β1 on the expression of *HAS2* mRNA in dermal fibroblast cells.[15,29] Greenwel *et al.*[12] indicated that tyrosine dephosphorylation of nuclear proteins mimics the stimulation of *COL1A2* transcription by the TGF-β1-activated signaling pathway using genistein and H7. In the case of genistein, *COL1A2* mRNA expression did not change, while treatment with H7 increased *COL1A2* mRNA expression. It was suggested that TGF-β1 used a different signaling pathway than Pro-Hyp.

Recent reports have provided evidence for enhanced *HAS2* transcriptional activation by STAT3.[16] Epidermal growth factor (EGF) initiates a signaling pathway where activated EGF receptors (EGFR) induce phosphorylation of STAT proteins 1, 3 and 5 through the tyrosine kinase Src[30,31] and phosphorylated STAT leads to increased transcriptional initiation of *HAS2* gene expression.[32] Therefore, Pro-Hyp-induced *HAS2* mRNA upregulation may be mediated by the Src kinase pathway and phosphorylation of STAT3. In this study, we found that a tyrosine kinase inhibitor and a serine/threonine kinase inhibitor reduced Pro-Hyp-induced *HAS2* mRNA upregulation, but a tyrosine phosphatase inhibitor showed no such effects on Pro-Hyp-stimulated *HAS2* mRNA. The results were, therefore, consistent with the notion that preventing phosphorylation specifically and negatively affects *HAS2* transcription. Conversely, blocking kinase activity downregulates production of *HAS2* transcripts. Furthermore, this counteracts the

stimulation of *HAS2* by Pro-Hyp. Thus, like EGF signaling, treatment with Pro-Hyp significantly elevated phosphorylation of STAT3, while kinase inhibition was able to counteract *HAS2* upregulation by Pro-Hyp. Although more detailed studies are necessary, this evidence suggests that phosphorylation of STAT3 proteins by activated kinase will be involved, at least in part, in the Pro-Hyp signaling pathway. The possibility of the presence of a specific receptor for Pro-Hyp still remains. A recent report indicated that Pro-Hyp may be transported into small intestinal epithelial cells through the H^+-coupled transporter, PEPT-1.[33] Moreover, Pro-Hyp may be transported into osteogenic cells by PEPT-1, where Pro-Hyp then becomes a direct signal. Therefore, in the case of fibroblast cells, Pro-Hyp may be transported into the cells where it is able to signal directly.

In conclusion, we found that collagen-derived Hyp-containing peptides stimulated cell proliferation, *HAS2* mRNA expression and hyaluronan production in human dermal fibroblasts. These results suggest that cells and ECM in the skin are modulated by oral ingestion of collagen hydrolysate. The underlying mechanism and the effects of various collagen-derived Hyp-containing peptides on cell motility and modulation of ECM proteins using *in vitro* cultured epidermal keratinocytes require further investigation.

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