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Effect of Prolyl-hydroxyproline (Pro-Hyp), a Food-Derived Collagen Peptide in Human Blood, on Growth of Fibroblasts from Mouse Skin

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We examined the effect of prolyl-hydroxyproline (Pro-Hyp), which occurs in human peripheral blood after ingestion of collagen peptide, on the migration and growth of mouse skin fibroblasts. Mouse skin discs were cultured on a 24-well plastic plate in a fetal bovine serum (FBS)-free medium. Addition of Pro-Hyp (200 nmol/mL) significantly increased the number of fibroblasts migrating from the skin to the plate after incubation for 72 h. This effect of Pro-Hyp was abolished by the addition of mitomycin C. The fibroblasts that had migrated from the mouse skin were collected and cultured on collagen gel. The growth of fibroblasts on the collagen gel was suppressed even in the presence of FBS, while rapid fibroblast growth was observed on the plastic plate. Addition of Pro-Hyp (0−1000 nmol/mL) to the medium containing 10% FBS enhanced the growth of fibroblasts on the collagen gel in a dose-dependent manner. These results suggest that Pro-Hyp might stimulate the growth of fibroblasts in the skin and consequently increase the number of fibroblasts migrating from the skin.

KEYWORDS: Collagen; collagen peptide; Pro-Hyp; collagen gel; skin; fibroblast; growth; gelatin; wound healing.

INTRODUCTION

Collagen is one of the major constituents of the extracellular matrix. The primary biochemical and structural features of collagen include the presence of hydroxyprolyl and hydroxylysyl residues and a triple helical structure. The triple helical structure is lost by heat treatment, and the denatured form of collagen is referred to as gelatin. Gelatin is commonly used in foods, pharmaceuticals, photographic film, cosmetics, etc. Gelatin has also been used in folk medicine in Asia to improve blood circulation and arrest bleeding (*1*). In Western countries, gelatin consumption has been believed to improve joint condition by reducing pain. In order to increase the solubility of gelatin, partially hydrolyzed gelatin products have been prepared and have been referred to as collagen peptides. A recent preclinical trial suggested that daily ingestion of collagen peptides improves the skin properties of women in winter (*2*). Some animal experiments and preclinical trials have supported the beneficial effects of collagen peptides (*3*−*6*). However, the mechanism behind the beneficial effects of collagen peptides has not been entirely elucidated.

Iwai et al. found the occurrence of food-derived peptides in human peripheral blood after ingestion of collagen peptide preparations (*7*). Pro-Hyp, Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp, and Phe-Hyp have been identified to be food-derived collagen peptides (*7, 8*). Among these peptides, Pro-Hyp is a major food-derived collagen peptide and remains in circulation for a few hours (*7, 8*). In previous studies, the maximum plasma concentration of food-derived collagen peptides in some individuals reached up to approximately 200 μM (*8*). It has been demonstrated that millimolar concentrations of some collagen-derived oligopeptides such as Pro-Hyp-Gly and Pro-Hyp exert a chemotactic action on fibroblasts, peripheral blood neutrophils (*9, 10*), and monocytes (*11*) in Boyden chemotaxis chambers. These cells play a significant role in wound healing and inflammation (*12*−*14*). On the basis of these facts, it has been speculated that food-derived collagen peptides such as Pro-Hyp might enhance

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wound healing by stimulating the migration of fibroblasts and other cells to the injured tissue (*7*). However, the millimolar concentrations of collagen peptides required for in vitro chemotactic activity were shown to be significantly higher than the micromolar concentrations of food-derived collagen peptides in human peripheral blood (50−200 μM). Furthermore, it has also been demonstrated that fibroblasts stop their growth in the extracellular matrix and on collagen gel; however, they grow rapidly in plastic wells and reach confluence within a few days (*15*). Therefore, there exists a possibility that the fibroblasts in the Boyden chemotaxis chambers might not reflect the in vivo response of fibroblasts.

The objective of the present study was to develop an in vitro model by using the extracellular matrix, which can simulate the in vivo wound healing step, and to examine the response of fibroblasts to the plasma level of Pro-Hyp.

MATERIALS AND METHODS

Chemicals. The amino acid standard mixture (Type H) and acetonitrile (HPLC-grade) were purchased from Wako Chemicals (Osaka, Japan). Hydroxyproline (Hyp), proline (Pro), 0.25% trypsin-EDTA solution, and mitomycin C were purchased from Nacali Tesque (Kyoto, Japan). Pro-Hyp was purchased from Bachem (Bubendorf, Switzerland). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Chemicals (St. Louis, MO). Dulbecco's phosphate-buffered saline (D-PBS) and gentamicin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum BWT S-1820 (FBS) was purchased from Biowest (Nuaillé, France). Calf acid-soluble type I collagen solution (0.5%) was purchased from Koken (Tokyo, Japan). Cell Counting Kit-8 was purchased from Dojin Glocal (Kumamoto, Japan). All other reagents were of analytical grade or better.

Estimation of Migration of Fibroblasts from Mouse Skin. Five-weeks-old male Balb/c mice were purchased from SLC Japan (Shizuoka, Japan). The mice were killed by cervical dislocation, and their skin was sterilized with 70% ethanol. The abdominal skin was stripped immediately after slaughter. The skin was rinsed with D-PBS to remove ethanol and was punched out into discs measuring 3 mm in diameter using a Dermalpunch (Nipro, Tokyo, Japan) in DMEM. The skin discs prepared from seven animals were used for each group. The punched skin was placed into a 24-well plastic plate (Falcon BD, Franklin Lakes, NJ), and 300 μL of DMEM containing 584 mg/L L-glutamine, 0.01 mg/mL gentamicin, the test components, and 0, 2, or 10% FBS were poured into the well. In order to ensure the attachment of the skin to the plastic plate, a thin plastic plate (Cell Disk; Sumitomo Bakelite, Tokyo, Japan) was mounted on the skin discs. The 24-well plastic plate was placed in a humidified incubator at 37 °C under 5% CO_2. The number of fibroblasts that migrated from the mouse skin discs up to a distance of 1 mm was directly counted every 24 h by using a phase-contrast microscope.

Abolishment of Proliferation of Mouse Skin Primary Fibroblasts by Mitomycin C. Eight pieces of the mouse skin discs were placed and cultured in a 24-well plate as described above. Mitomycin C was added in the media to give a final concentration of 10 μg/mL, and the plate was put into a CO_2 incubator for 2 h at 37 °C under 5% CO_2. After 2 h, the medium was replaced to the mitomycin C-free medium containing 0% FBS and put into the incubator for 72 h. The prolonged mitomycin C treatment showed a cytotoxic effect on the fibroblasts. The number of fibroblasts migrating from the mouse skin discs up to a distance of 1 mm was directly counted every 24 h by using a phase-contrast microscope.

Cell Culture. Fibroblasts were obtained from the skin of the Balb/c mice by the method of Rittié et al. (*16*) with slight modifications. The abdominal skin was cut into square pieces (approximately 6−7 mm in width). The 8 pieces were placed at the bottom of a culture dish (75 mm in diameter) so that they were not in contact with each other. Cultivation was carried out in 8 mL DMEM containing 584 mg/L L-glutamine, 0.01 mg/mL gentamicin, and 10% FBS in a humidified incubator at 37 °C under 5% CO_2. During cultivation, the medium was changed after every 2 days. After incubation for 2 weeks, the skin discs were removed, and fibroblasts were recovered using a 0.25% trypsin−EDTA solution.

We then prepared a fibroblast suspension containing 5×10^4 cells/mL in DMEM containing 584 mg/L L-glutamine, 0.01 mg/mL gentamicin, 0−10% FBS, and the test components. The fibroblasts were cultured on a 96-well plastic plate or on a collagen gel-coated plate. The collagen solution (0.5%) was mixed with the same volume of double-concentrated DMEM medium and the test components. The mixture (100 μL) was then poured into each well of the 96-well plastic plate and placed in the humidified incubator for 24 h at 37 °C under 5% CO_2 to allow gelation. The fibroblast suspension (100 μL) was poured on each well of collagen gel-coated and uncoated plates. The plates were placed in the humidified incubator at 37 °C under 5% CO_2. The same experiments were carried out by using an established human fibroblast cell line (MSU-2, a kind gift from Professor J. E. Trosko, Michigan State University).

To estimate fibroblasts growth, 10 μL of tetrazolium solutions (Cell Counting Kit-8) was added to each well of a 96-well plastic plate. The absorbance of the medium (70 μL) at 450 nm was measured using a Microplate Reader (Bio-Rad Laboratories, Hercules, CA) 2 h after addition of the tetrazolium solution.

Estimation of Collagen Synthesis. The fibroblasts that had been recovered from the skin of mice were cultured in a 24-well plastic plate not coated with the collagen gel and in a medium containing 10% FBS. After appropriate intervals, the cells and their products were recovered with the medium by scraping with a silicon tube and were hydrolyzed by treatment with 6 M HCl at 150 °C for 1 h. In order to estimate the collagen content in the medium, the Hyp concentration in the HCl hydrolysate was determined according to the method of Bidlingmeyer et al. (*17*) with slight modifications (*7*).

Statistical Analysis. The differences between the means were evaluated by analysis of variance followed by Fisher's PLSD method ($p < 0.05$) using StatView Version 5.0 (Abacus Concepts, Berkeley, CA).

Estimation of the Growth Factors of Mouse. The concentrations of mouse platelete-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) in the culture media were measured using a Bio-Plex 200 (Bio-Rad Laboratories). The cultured media was diluted in twice with 1% bovine serum albumin. Primary antibodies−bead conjugates and secondary antibody−fluorophore conjugate were obtained from Bio-Rad Laboratories.

RESULTS

Migration of Cells from Mouse Skin. As shown in **Figure 1** (right), spindle-shaped fibroblast-like cells were observed near the mouse skin after incubation for 96 h in the medium supplemented with 10% FBS, regardless of the presence of Pro-Hyp. The fibroblast-like cells migrating from the skin in the presence of 10% FBS were harvested and cultured on plastic plates. As shown in **Figure 2**, the Hyp content in the HCl hydrolysate of the mixture of the medium containing the cells

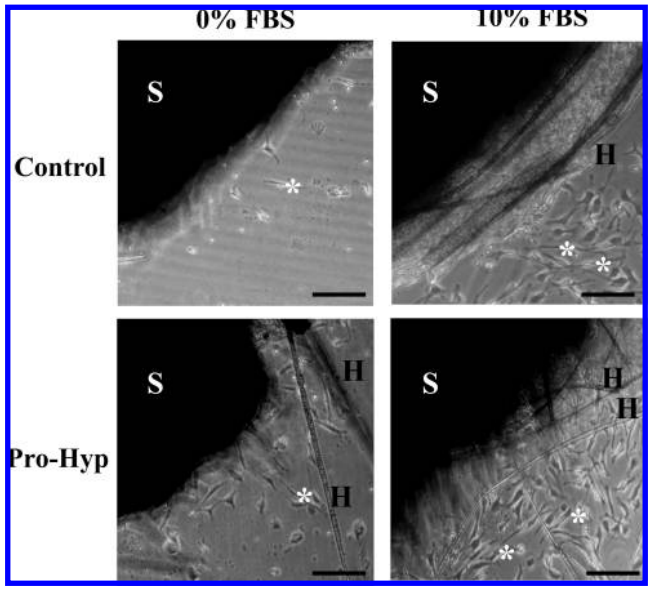

Figure 1. Migration of the fibroblast-like cells from mouse skin. The skin disc with hair was placed in a 24-well plate and incubated for 96 h in the FBS-free medium (left column) and the medium containing 10% FBS (right column). Pro-Hyp was added in the media to give 200 nmol/mL (upper row). Spindle-shaped fibroblast-like cells (∗) are observed. Bar indicates 100 μm. H, hair; S, skin.

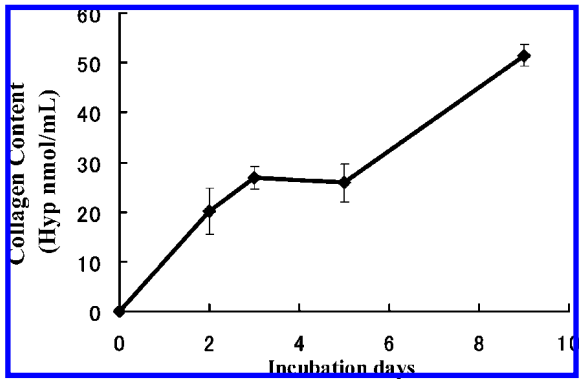

Figure 2. Production of collagen by fibroblast-like cells migrating from the mouse skin. The fibroblast-like cells from mouse skin and their products were recovered with the medium. Collagen content is represented by Hyp concentration in the HCl hydrolysates of them. The data are shown as the mean ± SD; $n = 3$.

and their products increased in a time-dependent manner, indicating that these cells can synthesize collagen: an important characteristics of fibroblast. Therefore, these cells can be classified as fibroblasts based on their morphological features and the collagen synthesis capability.

The effect of Pro-Hyp and its constituting amino acids on the number of fibroblasts migrating from the skin in media containing different concentrations of FBS has been summarized in **Figure 3**. Skin discs prepared from seven animals were used for each group. The cell number migrated from the skin increased up to 72 h. The times to reach the plateau depend on individuals. In the presence of FBS in the medium, no significant effect of Pro-Hyp (200 nmol/mL) on the number of fibroblasts migrating from the skin was observed. In the absence of FBS, the number of fibroblasts on the outside of the skin was much smaller than that in the presence of FBS. Under these conditions, the addition of Pro-Hyp significantly increased the number of fibroblasts after incubation for 72 h beyond variation between individuals, whereas the addition of free Pro and Hyp did not

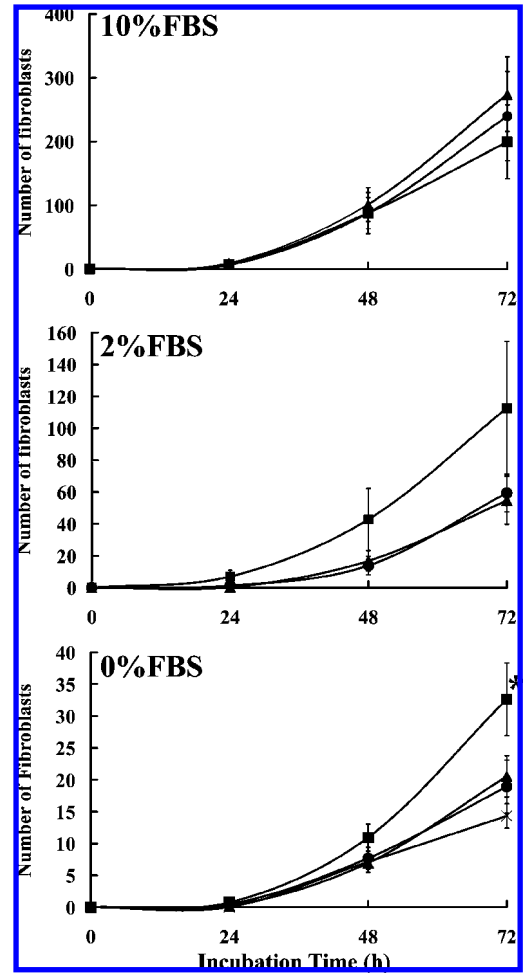

Figure 3. Effect of Pro-Hyp and constituting amino acids on the number of fibroblasts migrating from the mouse skin. Mouse skin discs were placed in 24-well plates and were incubated for 72 h in the medium containing 10, 2, and 0% FBS. Pro-Hyp (■), Hyp (▲), and Pro (×) were added into the control medium (●) to give 200 nmol/mL. The number of fibroblasts observed within reach of the mouse skin up to 1 mm distance was counted every 24 h. Approximately 24 skin discs could be prepared from one animal. 54 skin discs collected from seven animals were used for one group. The data are shown as the mean ± SE for the 54 skin discs. The asterisk indicates significant difference from the control at each point of time ($p < 0.05$).

affect the cell number (**Figure 1** left and **Figure 3**). Only negligible amounts of endogenous bFGF, PDGF, and VEGF were detected in the medium within 24 h of incubation. After incubation for 48 h, detectable amounts of these growth factors appeared in the media (38.86 pg/mL, 17.34 pg/mL, and 3.69 ng/mL in the cases of bFGF, PDGF, and VEGF, respectively), regardless of the presence of Pro-Hyp (200 nmol/mL).

As shown in **Figure 4**, the effect of Pro-Hyp on the number of fibroblasts on the outside of the mouse skin was abolished by the addition of mitomycin C.

Growth of Fibroblasts on Collagen Gel. In the presence of 10% FBS, fibroblasts rapidly grew on the plastic plate regardless of the addition of Pro-Hyp (**Figure 5**A). However, in the absence of Pro-Hyp, the growth of fibroblasts was suppressed by the collagen gel (**Figure 5**B). The addition of 200 nmol/mL Pro-Hyp significantly enhanced the growth of fibroblasts on the collagen gel, while the addition of free Hyp (200 nmol/mL) and a mixture of free Pro and Hyp (200 nmol/mL) did not show any significant effect on fibroblast growth. As shown in **Figure 6**, Pro-Hyp enhanced the growth

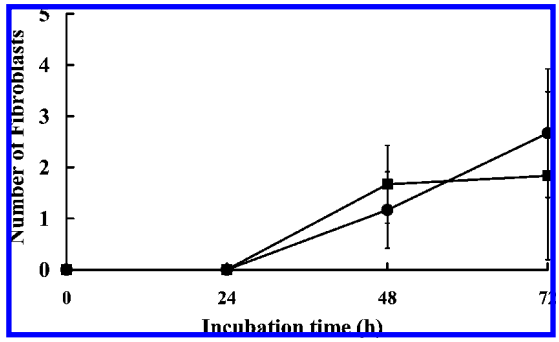

Figure 4. Abolishment of the effect of Pro-Hyp on the number of fibroblasts migrating from the mouse skin by mitomycin C. Mouse skin discs were placed in a 24-well plate and were treated for 2 h in the medium containing 0% FBS and 10 μg/mL mitomycin C. Then, the medium was replaced by mitomycin C-free medium in the absence (●) and the presence (■) of 200 nmol/mL Pro-Hyp. The number of fibroblasts observed within reach of the mouse skin up to 1 mm distance was counted every 24 h. The data are shown as the mean ± SE for eight skin discs from the same animal.

of fibroblasts on the collagen gel in a dose-dependent manner in the presence of 10% FBS. In other views, FBS enhanced the growth of fibroblasts on the collagen gel in a dose-dependent manner only in the presence of 200 nmol/mL Pro-Hyp (**Figure 7**). In the absence of Pro-Hyp, FBS showed no significant effect on the growth of fibroblasts on the collagen gel after 48 h.

DISCUSSION

Under physiological conditions, fibroblasts are buried in collagen fibrils and are quiescent (*18*, *19*). On the other hand, PDGF, which is released from wounded tissues, can stimulate the fibroblasts in the collagen gel, thereby enhancing the mobility of the fibroblasts (*20*). The activated fibroblasts migrate to the wounded spot and synthesize collagen, which has been considered to be a critical step in the early stage of wound healing (*12*). In the present skin culture system, fibroblasts migrate from the skin in the medium containing FBS, which is rich in PDGF and FGF. This phenomenon allows the collection of fibroblasts from the skin and other tissues. Even in the absence of the FBS, significant numbers of fibroblasts specifically migrate from the skin with epidermis and follicle, when endogenous PDGF and bFGF are secreted into the medium. This culture system that does not involve the addition of exogenous growth factors could be considered to mimic the wound healing process of the skin.

The addition of Pro-Hyp into the medium in the absence of FBS significantly increased the number of fibroblasts on the outside of the skin. However, this effect was abolished by mitomycin C, which arrests the cell cycle in the G2 phase and does not affect cell migration (*21*), suggesting that Pro-Hyp does not enhance the mobility of skin fibroblasts but enhances their growth. On the other hand, it has been demonstrated that Pro-Hyp and other collagen-derived peptides exert a chemotactic action on fibroblasts in Boyden chemotaxis chambers (*9*). However, the millimolar concentrations of the collagen peptides in that study were significantly higher than the micromolar concentrations of food-derived collagen peptides in human peripheral blood. Furthermore, the growth and mobility of fibroblasts depends on the presence of the extracellular matrix. Put together, we conclude that the plasma Pro-Hyp level has a minor effect on the mobility of fibroblasts in the mouse skin.

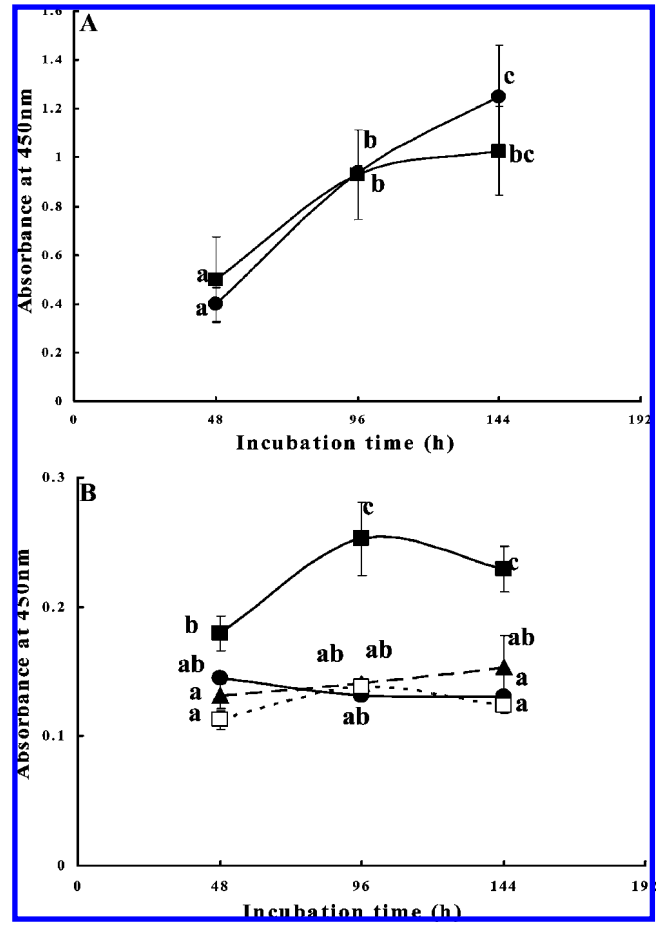

Figure 5. Effect of collagen gel on the growth of fibroblast in the absence and presence of Pro-Hyp and its constituting amino acids. Mouse skin primary cultures of fibroblasts (5×10^3 cells) were cultured on a 96-well plastic plate (A) and on a 96-well plate paved with collagen gel (B) for 144 h. Pro-Hyp (■), a mixture of Pro and Hyp (□), and Hyp (▲) were added into the control medium containing 10% FBS (●) to give 200 nmol/mL. Growth of fibroblasts was estimated by the absorbance of the medium treated with Cell Counting Kit-8 every 48 h. The data are shown as the mean ± SE; $n = 6$. The different letters on the values indicate significant difference ($p < 0.05$).

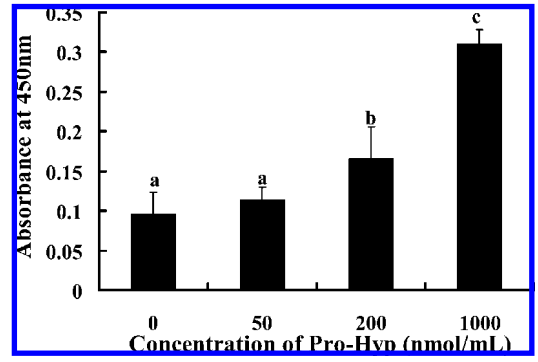

Figure 6. Dose response effect of Pro-Hyp on the growth of fibroblast on collagen gel in the medium containing 10% FBS. The mouse skin primary cultures of fibroblasts (5×10^3 cells) were cultured on a 96-well plate paved with collagen gel for 144 h. The data are shown as the mean ± SE; $n = 8$. The different letters on the values indicate significant difference ($p < 0.05$).

While the fibroblasts rapidly grew on the plastic plate, they stopped growing on the collagen gel to which Pro-Hyp was not added, even in the presence of FBS (**Figure 5**), which

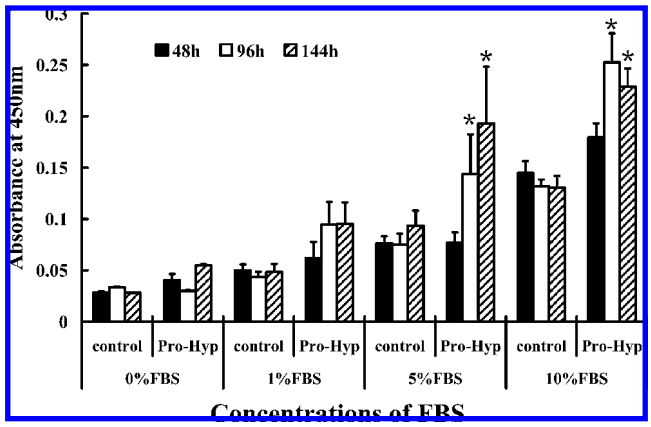

Figure 7. Effect of various concentrations of FBS on the growth of fibroblasts on collagen gel in the medium containing 200 nmol/mL of Pro-Hyp. Primary cultures of fibroblasts (5×10^3 cells) from mouse skin were cultured on a 96-well plate paved with collagen gel for 144 h. The data are shown as the mean ± SE; $n = 4$ for 0, 1, and 5% FBS groups; $n = 8$ for the 10% FBS group (Figure 5). The asterisks indicate significant difference as compared to the results obtained after incubation for 48 h ($p < 0.05$).

can be analogous to the quiescent fibroblasts in the dermis. In our preliminary experiments, the established cell line of human fibroblasts (MSU-2) was used for same experiment. However, the effect of the collagen gel on the growth of fibroblasts extensively depended on passage of the cell line. Then, inconsistent results were obtained. By using primary cultures of fibroblast as shown in the present study, the growth of fibroblasts was suppressed consistently. Therefore, the present culture system may better reflect the biological response of the fibroblasts in the wounded tissues than the conventional culture system on the plastic plate.

In the presence of FBS, Pro-Hyp enhanced the growth of fibroblasts on the collagen gel in a dose-dependent manner (**Figure 6**), while Pro-Hyp did not have any effect on fibroblast growth on the plastic plate. In the absence of FBS, Pro-Hyp did not enhance fibroblast growth on the collagen gel (**Figure 7**). These facts indicate that Pro-Hyp can abolish the suppression of fibroblast growth on the collagen gel and in the skin rather than acting as a growth factor, which cannot be detected by the conventional wound healing model on a plastic plate.

Addition of Pro-Hyp into the medium containing 10% FBS did not significantly increase the number of fibroblasts migrating from the mouse skin (**Figure 3**). FBS contains a higher level of PDGF and other growth factors. Since fibroblast rapidly grows on the plastic plate in the presence of FBS, it can be deduced that the effect of Pro-Hyp on the growth of fibroblasts in the skin did not significantly affect the number of fibroblasts on the outside of the skin.

The occurrence of food-derived collagen peptides circulating in the human blood at >4 h after the oral ingestion of collagen peptides has been demonstrated (*7*). Together with the present results, we propose a hypothesis that food-derived Pro-Hyp in human peripheral blood promotes wound healing by enhancing fibroblast proliferation in the wounded tissues in the presence of growth factors. This hypothesis could at least partially explain the improvement of damaged skin among women in winter by oral ingestion of collagen peptides (*2*).

No data is available on the mechanism behind Pro-Hyp-mediated enhancement of fibroblast growth on collagen. There are possibilities that Pro-Hyp might act by binding with the cell surface receptor or by passing through the transporter, if present. Alternatively, Pro-Hyp might affect the interaction between the extracellular matrix and fibroblasts.

The occurrence of some food-derived collagen peptides except Pro-Hyp, including Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp, and Phe-Hyp, has been demonstrated in human peripheral blood (*7*, *8*). Further studies on the effects of these peptides on fibroblasts and other cells in the skin are now in progress.

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