Identification of a Collagen Production-promoting Factor from an Extract of Royal Jelly and Its Possible Mechanism

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We have previously shown that royal jelly (RJ) promoted collagen production by skin fibroblasts in the presence of ascorbic acid-2-O-alpha-glucoside (AA-2G). In this study, we purified the honeybee RJ-derived collagen production-promoting factor (HBRJ-CPF) from an alkali-solubilized fraction of RJ by C18 reverse-phase column chromatography. The elution profile by the C18 column chromatography and the molecular mass of the purified HBRJ-CPF material coincided with those of 10-hydroxy-2-decenoic acid (10H2DA). We then examined the collagen production-promoting activities of several commercially available fatty acids contained in RJ. We found that 10H2DA and 10-hydroxydecanoic acid increased the collagen production in a dose-dependent manner. Furthermore, 10H2DA induced the fibroblast cell line, NHDF, to produce transforming growth factor-beta 1 (TGF-β1) which is an important factor for collagen production. As expected, the collagen production-promoting activity of 10H2DA was neutralized by the anti-TGF-β1 antibody. These results suggest that HBRJ-CPF identified as 10H2DA promoted the collagen production of AA-2G-treated fibroblasts by inducing TGF-β1 production.

Key words: royal jelly (RJ); collagen production; 10-hydroxy-2-decenoic acid (10H2DA); transforming growth factor-β (TGF-β1)

Royal jelly (RJ), which is secreted from the hypopharyngeal gland and mandibular gland of the worker honeybee, is the exclusive food for the queen honeybee and larvae. RJ has been reported to have such pharmacological characteristics as anti-tumor,1) anti-bacterial,2) anti-hypercholesterolemic,3) anti-allergic,4,5) anti-fatigue,6) insulin-like,7) and wound-healing properties.8) A chemical composition analysis has shown that RJ consisted mainly of proteins, sugars, lipids, vitamins and free amino acids.9) In particular, several substances contained in RJ, including 10-hydroxy-2-decenoic acid (10H2DA),10–13) royalisin,14) and apisin,15,16) have been found to exhibit these pharmacological activities.

Collagen is the predominant fibrous protein of the extra-cellular matrix and is a major protein constituting connective tissue in the human body. About 3–6% of total tissue protein in the body is collagen, and the functional properties of skin depend on the integrity of collagen in the dermis. The deposition of collagen is finely controlled, and is dependent on the physiological status of the body. Changes in the rate of collagen deposition occur during wound-healing and new bone development, and with aging.17) Therefore, the control of collagen metabolism may be useful for a variety of therapeutic and cosmetic applications.

We have demonstrated in our previous study that the medium-soluble fraction of RJ, which had been prepared by suspending RJ in Dulbecco’s modified Eagle’s medium (D-MEM) and then centrifuging at $3000 \times g$ for 10 min, increased collagen production by skin fibroblasts in the presence of ascorbic acid or ascorbic acid-2-O-alpha-glucoside (AA-2G).18) AA-2G is a vitamin C derivative that is synthesized from ascorbic acid and maltose or oligosaccharide by the use of trans-glucosylation enzymes.19–21) Since the hydroxyl group binding to the carbon at position 2 in ascorbic acid is protected by glucose, AA-2G is refractory to oxidation, and is therefore more stable than ascorbic acid under various conditions.22) We have observed that combined usage of the medium-soluble fraction of RJ with AA-2G increased the collagen production much more than the combination of the medium-soluble fraction of RJ with ascorbic acid. In this study, we report that the alkali-solubilized fraction of RJ strongly promoted collagen production by the human fibroblast cell line, NHDF. In addition, we purified a honeybee RJ-derived collagen production-promoting factor (HBRJ-CPF) from the alkali-solubilized fraction by C18 reverse-phase column chromatography.
**Materials and Methods**

*Preparation of samples.* 10H2DA and 3-hydroxidecanoic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 10-hydroxydecanoic acid was purchased from Sigma (St. Louis, MO, USA). Each of these fatty acids was dissolved in a 0.1 N NaOH solution. The alkali-solubilized fatty acid solutions were slowly neutralized with a 6 N HCl solution for use in the collagen production-promoting assay.

RJ was obtained from Anhui in China. The jelly was suspended in 9 volumes of distilled water for 1 h at room temperature, before the suspension was then centrifuged at 3000 \( \times \) g for 10 min to recover the precipitate. The precipitate was dissolved in a 0.1 N NaOH solution. The alkali-solubilized RJ solution was neutralized with a 6 N HCl solution for use in the collagen production-promoting assay and as a starting material for the identification of HBRJ-CPF.

*Cell culture.* NHDF cells, originating from human fetal foreskin fibroblasts, were purchased from Kurabo Company (Osaka, Japan). The cells were cultured in D-MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Thermo Fisher Scientific, Waltham, MA, USA) containing 0.1 mg/ml of streptomycin (Meiji Seika Company Ltd., Melbourne, Australia), 100 IU/ml of penicillin and 0.1 mg/ml of streptomycin (Meiji Seika Kaisha Ltd., Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air.

*Collagen production-promoting assay.* The NHDF cells were seeded in a 96-well microculture plate at 2.5 \( \times \) 10\(^4\) cells/well and incubated for 24 h. The culture medium was replaced by 200 \( \mu \)l of fresh D-MEM medium containing 10% FCS, a sample, and 50 \( \mu \)M of AA-2G purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). A cell viability assay was performed by the MTT method according to the procedure of Mosmann.\(^{23}\)

The amount of collagen in the culture supernatant was directly determined with a Sircol collagen assay kit (Biocolor Ltd., N. Ireland) according to the manufacturer’s instructions. The cell layer of an extract of the cell surface being increased in comparison with the control NHDF cells cultured with AA-2G alone. In respect of the statistical significance of differences in the neutralization experiment was assessed by Scheffe’s test, while the statistical significance of differences in the other experiments was assessed by Dunnett’s test.

**Results**

*Collagen production-promoting activity of the alkali-soluble fraction of RJ.*

Our experiment was designed to study the effects of RJ on collagen production. Figure 1 shows the time-course characteristics of collagen production when 1.3 and 4.3 mg/ml of the alkali-soluble fraction were added to NHDF cells on days 1–5.

NHDF cells cultured with the alkali-soluble fraction in the presence of AA-2G for 4–5 days resulted in the collagen production in both the culture supernatant and the cell surface being increased in comparison with control cells cultured with AA-2G alone. In respect of the total amount of collagen, the control NHDF cells had produced 3.9 ± 1.2 and 4.4 ± 0.6 \( \mu \)g/2.5 \( \times \) 10\(^4\) cells by
days 4 and 5, respectively. NHDF cells cultured with 4.3 mg/ml of the alkali-soluble fraction together with AA-2G resulted in 6.2 ± 0.5 and 7.6 ± 0.2 µg/2.5 × 10⁴ cells by days 4 and 5, respectively (p < 0.05 and p < 0.01, respectively). However, the alkali-soluble fraction did not affect the cell growth (Table 1). Furthermore, in the absence of AA-2G, the alkali-soluble fraction did not induce the NHDF cells to produce collagen either into the culture supernatant or on the cell surface (data not shown). These results suggest that the alkali-soluble fraction of RJ promoted the collagen production by NHDF cells in response to AA-2G.

**Identification of HBRJ-CPF**

Since the results of Fig. 1 suggested the presence of HBRJ-CPF in the alkali-soluble fraction of RJ, we purified the factor that promoted collagen production on the surface of NHDF cells after 5 days of culture. The low-molecular-weight fraction in the alkali-soluble fraction showed greater collagen production than the high-molecular-weight fraction did (data not shown). Therefore, the low-molecular-weight fraction was further separated into 12 fractions according to the appearance of peaks by C18 column chromatography (Fig. 2), and the collagen production-promoting activity of each fraction was determined (Fig. 3). Fractions 8 and 9 showed stronger activity than the other fractions (Fig. 3). Control NHDF cells produced 1.2 ± 0.1 µg/2.5 × 10⁴ cells, while the cells incubated with fractions 8 and 9 produced 1.7 ± 0.2 and 2.7 ± 0.1 µg/2.5 × 10⁴ cells, respectively (p < 0.05 and p < 0.01, respectively) of collagen on their cell surface.

We tried to identify HBRJ-CPF from fraction 9 that showed the strongest activity. The absorbance of fraction 9 was the most prominent of all the fractions in the C18 column (Fig. 2). We therefore considered that the peak in fraction 9 might have been derived from 10H2DA. To examine this possibility, fraction 9 was analyzed by the C18 column. Fraction 9 showed a retention time corresponding to that of 10H2DA by a mass spectrometric analysis (data not shown). These results indicate that HBRJ-CPF contained in fraction 9 was 10H2DA.

**Collagen production by the fatty acids contained in RJ**

As shown in Fig. 4, we examined the collagen production-promoting activities of 10H2DA [HO–(CH₂)₂–CH=CH–COOH], 10-hydroxydecanoic acid [HO–(CH₂)₉–COOH], and 3-hydroxydecanoic acid [H₃C–(CH₂)₉–CH(OH)–CH₂–COOH] which are known to be the comparatively abundant fatty acids contained in RJ. 24 10H2DA and 10-hydroxydecanoic acid

<table>
<thead>
<tr>
<th>Group</th>
<th>OD (570/650 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.482 ± 0.012</td>
</tr>
<tr>
<td>RJ (1.3 mg/ml)</td>
<td>0.475 ± 0.017</td>
</tr>
<tr>
<td>RJ (4.3 mg/ml)</td>
<td>0.468 ± 0.015</td>
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NHDF cells were incubated in D-MEM containing 10% (v/v) FCS and 50 µM of AA-2G for 5 days in the presence or absence of the alkali-soluble fraction of RJ. Each data value is presented as the mean±SD of triplicate cultures.
increased the total amounts of collagen in a dose-dependent manner at concentrations from 0.06 to 1.5 mM. On the contrary, 3-hydroxydecanoic acid showed no effect. These fatty acids did not affect the growth of NHDF cells at the concentration used in these experiments (data not shown).

Fig. 2. C18 Column Chromatography of the Low-molecular-weight Fraction in the Alkali-soluble Fraction of RJ.
The low-molecular-weight fraction in the alkali-soluble fraction (4.3 mg) was loaded into a C18 column (4.6 x 250 mm) that had been equilibrated with water containing 0.05% trifluoroacetic acid. The fraction was separated into 12 further fractions (fractions 1–12). The acetonitrile gradient profiles are indicated by the dotted lines. The collected fractions were each dried and dissolved in PBS for the collagen production-promoting assay.

Fig. 3. Effect of the 12 Fractions Separated in the C18 Column on Collagen Production.
NHDF cells were incubated with 50 μM of AA-2G for 5 days in the absence [□] or presence [■] of each fractionated sample. The loaded material (low-molecular-weight fraction in the alkali-soluble fraction of RJ) [■] was also added to the culture instead of a fractionated sample. The amounts of collagen produced on the cell surface were determined as described in the Materials and Methods section. Each data value is presented as the mean±SD of triplicate cultures. * p < 0.05; ** p < 0.01, significantly different when compared with the control culture (Dunnett’s test).

Fig. 4. Effect of Fatty Acids Contained in RJ on the Collagen Produced by NHDF Cells.
NHDF cells were incubated with 50 μM of AA-2G for 5 days in the absence (□) or presence of various concentrations of 10H2DA (●), 10-hydroxydecanoic acid (△), or 3-hydroxydecanoic acid (▲). The total amounts of collagen produced were determined as described in the Materials and Methods section. Each data value is presented as the mean±SD of triplicate cultures. * p < 0.05, significantly different when compared with the control culture (Dunnett’s test). $ p < 0.05, significantly different when compared with the control culture (non-parametric Dunnett’s test).
Fig. 5. Effect of 10H2DA on TGF-β1 Production by NHDF Cells.
NHDF cells were incubated for 24 h in the absence or presence of 0.5 mM or 1.5 mM of 10H2DA. The amount of TGF-β1 in each culture supernatant was determined by ELISA. Each data value is presented as the mean±SD of triplicate cultures. * p < 0.05; ** p < 0.01, significantly different when compared with the control culture (Dunnett’s test).

10H2DA promoted collagen production by inducing the TGF-β1 production by NHDF cells
Since TGF-β1 is well known to increase collagen production, the amount of TGF-β1 in the culture supernatant was determined by a Duoset® ELISA system. The amount of TGF-β1 in the culture supernatant after exposure to 10H2DA for 24 h was significantly higher than that of the vehicle control (Fig. 5). These results indicate that 10H2DA induced NHDF cells to produce TGF-β1.

We then investigated the effect of the mouse anti-human TGF-β1 monoclonal antibody on the 10H2DA-mediated promotion of collagen production. As shown in Fig. 6, the collagen production-promoting activity by 0.5 mM of 10H2DA was completely abrogated by adding 0.2 μg/ml of the anti-TGF-β1 antibody. This result suggests that 10H2DA promoted the collagen production by inducing TGF-β1 production by the NHDF cells.

Discussion
RJ has been reported to show anti-bacterial activity, anti-oxidation activity, growth-stimulating activity for fibroblasts, and stimulating activity for skin turnover.25,26) RJ also contains a variety of free amino acids, sugars, minerals, and vitamins. For these reasons, RJ has been widely used in commercial medical products, dietary supplements, and cosmetics in many countries.

We have demonstrated in our previous study that collagen production by AA-2G-treated fibroblasts was increased by the addition of the medium-soluble fraction of RJ to the culture.18) In this study, we found that the alkali-soluble fraction of RJ strongly increased collagen production by the human fibroblast cell line, NHDF, in response to AA-2G. We then tried to identify HBRJ-CPF from the alkali-soluble fraction. The alkali-soluble fraction of RJ was therefore further fractioned by C18 column chromatography. The elution profile from C18 column chromatography and the molecular mass of the purified HBRJ-CPF sample coincided with those of 10H2DA. 10H2DA is the hydroxyl fatty acid that is abundantly contained in RJ. It has been reported that 10H2DA has such pharmacological functions as anti-tumor activity,10–13) anti-bacterial activity, tyrosinase inhibitory activity, and sebaceous lipids synthesis-inhibitory activity.25–27)

Since RJ contains various fatty acids that are similar to 10H2DA in their molecular structure,24) we examined the effects of several commercially available fatty acids contained in RJ on the collagen production by NHDF cells in response to AA-2G. Interestingly, in addition to 10H2DA, 10-hydroxydecanoic acid significantly promoted collagen production, while 3-hydroxydecanoic acid had no effect (Fig. 4).

Although 10-hydroxydecanoic acid was not identified in the C18 column chromatographic analysis (Fig. 2), it has been reported that both 10H2DA and 10-hydroxydecanoic acid accounted for at least 60% to 80% of the fatty acids contained in RJ.24) We therefore consider that the collagen production-promoting effects of the alkali-soluble fraction of RJ may be attributed to the sum of the activities exerted by these hydroxyl fatty acids. Furthermore, since it is known that both 10H2DA and 10-hydroxydecanoic acid also exist in the water-soluble fraction of RJ,28) it is likely that the medium-soluble fraction of RJ, which was prepared by suspending RJ in the D-MEM medium and then centrifuging, promoted collagen production due to the presence of these hydroxyl fatty acids in the fraction.

Glycolic acid (HO–CH₂–COOH), which is a hydroxyl
acid similar to 10H2DA in its molecular structure, is known to increase collagen production in vitro.29,30 Additionally, glycolic acid can provide a protective anti-oxidative effect on photodamaged skin,31 increase the hyaluronic acid level, and increase the number and quality of elastic fiber tissues.32–34 Therefore, 10H2DA may exert its pharmacological activities through a mechanism similar to that of glycolic acid.

TGF-β has been shown to act on human dermal fibroblasts at the pretranslational level by stimulating the accumulation of fibronectin and type I procollagen mRNAs.35–38 To clarify the mechanism by which 10H2DA promoted collagen production, we paid particular attention to the involvement of TGF-β1. 10H2DA induced NHDF cells to produce a significant amount of TGF-β1 during 24 h of incubation (Fig. 5). Furthermore, the anti-TGF-β1 antibody completely inhibited the 10H2DA-mediated promotion of collagen production (Fig. 6). These results suggest the involvement of TGF-β1 in the possible mechanism for the promotion of collagen production by 10H2DA.

In addition, prolyl hydroxylase and lysyl hydroxylase are the key enzymes in collagen synthesis acting by the hydroxylation of proline and lysine residues in procollagen peptide.39 It remains unclear whether 10H2DA affected the activities and mRNA expression of these enzymes. Further studies are planned.

In conclusion, the results of our study demonstrate that HBRJ-CPF isolated from the alkali-soluble fraction of RJ was 10H2DA, and further suggest that 10H2DA and 1H2DA possibly by inducing TGF-β1 production. Thus, RJ and 10H2DA may stimulate increased collagen production and enhance the deposition of collagen in the dermis.

Acknowledgments

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References

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