STUDY OF COLLAGEN RECYCLING AND ITS MODULATION DURING AGING USING IN VITRO SENESCENT MODELS

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Summary

The dermis and its extracellular matrix (ECM) provide fundamental primary skin properties such as the maintenance of skin architecture. The main well-known structural protein of the dermis is collagen. The whole of its metabolism and its regulation is disturbed during aging, leading to an increase in collagen fiber degradation. Discovered this last decade, microRNAs play a fundamental role in post-transcriptional inhibition for gene expression. For example, miRNA-29a targets different key genes involved in collagen metabolism such as COL1A1, COL3A1 (alpha-1 type I or III collagen) and PEPD (prolidase). Thereby, we selected a peptide which has shown ability to affect collagen metabolism, due to the study of key markers: miRNA-29a, collagen I, collagen III, and prolidase. Our results showed a positive modulation of collagen I and III in in vitro fibroblasts or ex vivo skin treated with the peptide. Next, we investigated these key markers by using certain in vitro senescent models: miRNA-29a 3p expression was studied in in vitro fibroblasts from donors of different ages; collagen I expression was studied in senescent fibroblasts by transfection with FOXO3a-specific siRNA; and prolidase activity was examined using the model of replicative senescence. Regarding the in vivo experiment, after 42 days of treatment, the main visible wrinkle parameters (number, area, length and depth) were decreased in the treated sides compared to the placebo sides. In conclusion, the study of collagen regulation and its modulation are fundamental to understand the relationship between collagen metabolism and the visible signs of skin aging.

Introduction

The foremost purpose of dermal fibroblasts is the establishment of the ECM, which is mainly composed of collagen fibers that are responsible for maintenance of dermis architecture [1]. Continuously remodeled, the collagen metabolism is a process including different regulated steps: synthesis and secretion of procollagen by fibroblasts; formation of tropocollagen and collagen fibrils in ECM; collagen degradation by matrix metalloproteinases; and recycling of degraded collagen [2]. All the steps of collagen metabolism are subject to different regulators such as miRNA-29a that act on post-transcriptional expression of collagen and prolidase genes [3]. Prolidase, also known as peptidase D, is the enzyme responsible for the final step of collagen metabolism: recycling. This enzyme catalyzes hydrolysis of di- and tri-peptides containing C-terminal proline or hydroxyproline from the degradation of old collagen. Thereby, due to the prolidase action, a pool of proline is available for synthesis of new collagen, a molecule containing a quarter of proline and hydroxyproline [4]. The modulation of prolidase is even more important because the essential source of proline comes from this recycling and a decrease in its activity is observed in senescent fibroblasts [4-5].
Methods

In the present work, we first evaluated the effect of a previously selected peptide on collagen expression in *in vitro* fibroblasts and *ex vivo* skin by flow cytometry and immunostaining. We confirmed the positive effect of the peptide on collagen I expression in an *in vitro* artificial senescent model by using FOXO3a-specific siRNA. Next, we studied the impact of age on different key markers of the collagen regulation process: miRNA-29a 3p expression and prolidase activity. For that, we used fibroblasts from donors with different ages or fibroblasts aged by replicative senescence. Finally, an *in vivo* study was performed on 35 volunteers. After 42 days of application, the observed effect of the peptide on wrinkle appearances was evaluated by analysis of silicone replicas done on the crow’s feet.

Results

Study of collagen expression

We studied *in vitro* the expression of collagen I, by flow cytometry, on fibroblasts treated with the peptide or with TGF-β (positive control: inductor of collagen production).

![Collagen I expression in fibroblasts treated with 0.5% or 1% peptide or with TGF-β for 48 h by flow cytometry](image)

We observed an increase in collagen I expression in *in vitro* fibroblasts treated with the peptide or with TGF-β. To confirm this result, collagen I and III expressions were investigated in *ex vivo* skin biopsies treated with 1% peptide for 48 hours.
When *ex vivo* skin biopsies were treated with 1% peptide, the expression of collagen I and III was observed to have increased.

The next step of this study was to observe this peptide effect on collagen expression from senescent fibroblasts. For that step, fibroblasts were transfected with FOXO3a-specific siRNA, inducing the accelerated senescence of these cells. At the same time, these cells were treated with the peptide or with TGF-β for 48 hours.
After the transfection with FOXO3a-specific siRNA, we observed a decrease in collagen I expression in the \textit{in vitro} fibroblasts. When the cells were transfected and treated with the peptide, a weaker decrease was observed. With TGF-\(\beta\) treatment, the effect of the transfection was totally reversed: we observed an increase in collagen I expression.

**Study of key markers on collagen regulation despite the aging**

To pursue this study, we focused on the regulation of collagen metabolism during aging. miRNA-29a 3p expression in fibroblasts extracted from donors of different ages was investigated by qPCR.

We observed \textit{in vitro} an increase in miRNA-29a 3p expression. The magnitude of this increase appeared to vary based on the age of the donor. Indeed, in the literature, the level of miRNA-29a expression is linked with the senescence. Interestingly, when fibroblasts were treated with the peptide at 1% overnight, we observed a decrease in miRNA-29a 3p expression, irrespective of the age of the donors.
Following this result, we began observing a target of miRNA-29a 3p that regulates the recycling of collagen: prolidase. We used the well-documented method of replicative senescence; fibroblasts were cultured during three months. To confirm the senescence induction, we performed senescence-associated (SA) β-galactosidase activity staining on fibroblasts, at different passages, that were either treated with 1% peptide for 48 hours or untreated.

![Graph showing SA β-galactosidase staining in senescent fibroblasts treated with 1% peptide for 48 h](image)

We observed, as expected, an increase in SA β-galactosidase activity on *in vitro* cells continuously along passages. Interestingly, with the treatment, the comparison of the level of SA β-galactosidase activity staining on the same passage clearly showed a decrease in SA β-galactosidase activity level in treated cells. We observed a significant limitation of the accumulation of SA β-galactosidase in senescent cells treated with the peptide *in vitro*.

Then, the prolidase activity was observed on these cells. In this stage, two passages were studied: P5 (cells were considered as young) and P21 (senescent cells).
We observed a statistically significant decrease in prolidase activity in \textit{in vitro} senescent cells (P21). Senescent cells treated with 1% peptide showed a beneficial effect: the decrease of prolidase activity was delayed.

\textbf{In vivo study}

In order to assess the peptide effect on wrinkle appearance, a clinical study was performed on thirty-five Asian volunteers. This double blind study was carried out in the winter season. During a 6-week period, the volunteers applied twice daily the peptide formulated at 1% in a cream and the placebo cream on the face according to a randomization list. Silicone replicas of the crow’s feet area, which are negative replicas of skin micro relief, were performed before the first cream application and at the end of the study. The analysis of the imprint with Violine® software (Courage + Khazaka) showed a significant reduction of the observed wrinkle parameters on the treated sides with the peptide compared to the placebo sides.

\begin{center}
\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Treated sides} & \textbf{Mean} & \textbf{SEM} & \textbf{p} & \% \textbf{of} & \% \textbf{of improved} \\
\hline
\textbf{Number of wrinkles} & Floosor & 7.60 & 7.47 & 0.0088 & 1\% peptide & 6.29 & 6.94 & 0.0005** & 57\% \\
& 1\% peptide & 6.31 & 17.26 & 0.028* & 57\% & 27.9\% & 63\% \\
\hline
\textbf{Total area (mm²)} & Floosor & 5.01 & 1.33 & 0.0004** & 1\% peptide & 5.17 & 11.84 & 0.0081 & 66\% \\
& 1\% peptide & 6.15 & 0.89 & 10.67 & 0.0001 & 66\% & 66\% & 57\% \\
\hline
\textbf{Total length (mm)} & Floosor & 5.04 & 7.15 & 0.0009** & 1\% peptide & 5.46 & 10.68 & 0.0041 & 66\% \\
& 1\% peptide & 6.20 & 0.89 & 10.67 & 0.0001 & 66\% & 66\% & 57\% \\
\hline
\textbf{Total depth (μm)} & Floosor & 5.00 & 7.15 & 0.0009** & 1\% peptide & 5.46 & 10.68 & 0.0041 & 66\% \\
& 1\% peptide & 6.20 & 0.89 & 10.67 & 0.0001 & 66\% & 66\% & 57\% \\
\hline
\textbf{Maximal depth (μm)} & Floosor & 8.68 & 10.81 & 0.0092** & 1\% peptide & 8.68 & 10.81 & 0.0092** & 69\% \\
& 1\% peptide & 7.35 & 12.09 & 0.0001 & 66\% & 66\% & 57\% \\
\hline
\end{tabular}
\caption{Violine® analysis results}
\end{table}
\end{center}

\textit{***} significant; \textit{**} very significant, Student’s \textit{t}-test (unpaired) or Wilcoxon signed-rank test (unpaired) are used depending on whether the data followed a normal distribution, n=50’s sem = 12 ± sem
In parallel, on the pictures of the area of interest, we observed a significant reduction of the wrinkle appearance.

**Pictures of the crow’s feet area**

<table>
<thead>
<tr>
<th>Placebo</th>
<th>1% Peptide</th>
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<tbody>
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<td>D0</td>
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**Conclusion**

Prolidase and miRNA-29a play an essential role in collagen metabolism. During aging, the reduction of collagen synthesis is closely related to the reduction of prolidase activity and to the increase in miRNA-29a expression. Our present results suggest that targeting different key steps of the collagen metabolism process by the modulation of prolidase and miRNA-29a could help reduce visible signs of aging *in vitro*. The *in vivo* study suggests a reduction of wrinkle appearance.

**Bibliography**


