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사람 피부 섬유아세포에서의 파이브로넥틴 조각(70, 45 kDa)에 의한 MMP-1 발현 증가와 MMP-2 활성 증가 연구

김 혜 경 · 손 의 동 · 이 진 영 · 황 재 성† · 강 학 희 · 장 이 섭

(주)아모레퍼시픽 기술연구원 피부과학연구소

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The Fragments of Fibronectin (Fn-fr's 70, 45 kDa) Increase MMP-1 Expression and MMP-2 Activity in Normal Human Fibroblasts

Hyae Kyoung Kim, Eui Dong Son, Jin Young Lee, Jae Sung Hwang†, Hak Hee Kang, and Ihseop Chang

Skin Research Institute, Amore-Pacific Corp/R&D Center, 314-1, Bora-dong, Gilheung-gu, Yongin-si, Gyeonggi-do 442-729, Korea

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요 약: 노화 과정 중에 일어나는 extracellular matrix (ECM)의 변성은 피부의 주름과 탄력 감소를 유발한다. 현재까지 항노화의 주요 타겟은 metalloproteases 혹은 콜라겐이나 엘라스틴같은 구조 단백질에 집중되어 있지만, 최근 세포와 ECM 단백질(콜라겐, 피브릴린, 파이브로넥틴) 간의 상호작용이 세포의 생존과 증식, 조직의 재건에 중요한 역할을 한다고 알려졌다. 파이브로넥틴은 다른 ECM 단백질이나 인테그린 같은 세포 표면 수용체와 결합할 수 있는 부위를 가진 부착 단백질이다. 최근 보고에 따르면 세린 프로티아제들에 의해 분해된 파이프로넥틴 조각이 골아세포에서 MMPs 발현을 증가시킨다. 그러나 파이브로넥틴 조각의 사람 피부에서의 역할은 보고된 바 없다. 본 연구에서는 노인의 피부에서 파이브로넥틴 조각이 현저히 증가되어 있으며, 섬유아세포에 파이브로넥틴 조각을 처리하였을 시, MMP-1의 발현과 MMP-2의 활성이 증가한다는 것을 입증하였다. 이 결과는 파이브로넥틴 조각이 피부 노화를 유발하는 새로운 인자일 가능성을 제시하고 있다.

Abstract: The alternation of extracellular matrix (ECM) protein in aging process is associated with symptoms such as wrinkling and loss of elasticity in skin. Now, the major target proteins for anti-aging have been metalloproteases and the structural proteins such as collagen and elastin. Recently, the interaction of cell and ECM proteins (collagen, fibrillin, and fibronectin) is reported to have an important role in survival, proliferation and tissue reconstruction. Fibronectin is a matrix adhesion protein which binds to collagen and integrin and degraded by serine proteases. It has been reported that fragments of fibronectin (Fn-fr's) were involved in matrix metalloproteases (MMPs) expression in osteoblast. But, the role of Fn-fr's in human skin and in skin cells has not been reported yet. Therefore, we investigated the differences of fibronectin fragmentation pattern between young and aged human skin, and demonstrated that the fragmentation of fibronectins is significantly increased in aged human skin. Also, treatment of Fn-fr's (70, 45 kDa) increased MMP-1 expression and MMP-2 activity in human dermal fibroblasts. Our results suggest that Fn-fr's as a potential new factor to accelerate skin aging.

Keywords: *fibronectin, Fn-fr's, MMPs, skin aging, human dermal fibroblast*

1. Introduction

The symptoms of skin aging such as wrinkling and loss of elasticity are associated with alternation of extracellular matrix (ECM) proteins by ultraviolet radiation (UV). In photoaged skin, the dermis results in reduction in the amount and organization of connective tissue. At present, the major target proteins to prevent alternation of ECM for anti-wrinkle have been focused on the structural proteins such as collagen and elastin, and the mechanisms of ECM alternation have been

† 주 저자 (e-mail: hkkim@amorepacific.com)

proposed to damage of elastic fiber and increase of matrix metallopeotease-1 (MMP-1) by UV irradiation. But, we have investigated that some of the micromolecular constituents of ECM such as fibronectin fragments will play as signaling molecules that have important correlation to mechanism of skin aging.

Recently, the interaction of cell-extracellular matrix proteins (collagen, elastin, fibrillin, and fibronectin) was reported to have an important role in survival, proliferation of cell and tissue reconstruction. Among these matrix proteins, fibronectin (Fn) has been known as an adhesion protein containing binding site to ECM proteins (collagen, fibulin) and cell surface receptor (integrin).

Fn is an extracellular matrix glycoprotein found in plasma and ECM. The expression of Fn is elevated in tissues undergoing repair or remodeling. Also, it is a major adhesion molecule processing important biological activities in the cell-ECM interaction. Fn has been proteolytically fragmented into 30 to 200 kDa size in cartilage[1,2]. Fragmentation of fibronectin was reported in UVB irradiated hairless mouse skin extract[3]. Fn-fr's have been reported as stimulating the expression of MMPs in chondrocytes[4]. Fn-fr (45 kDa) induces MMP-13 synthesis in chondrocytes. Fn-fr (29 kDa) stimulated the release of several MMPs from cartilage explants in culture, including MMP-3, 2, 9[5].

At present, the functional studies of Fn-fr's were unclear in skin cell and skin tissue. The purpose of this study was to investigate the change of fibronectin fragmentation in human skin and the effects of MMPs boosting by Fn-fr's in normal human fibroblast.

Our results demonstrate that Fn-fr's (70, 45 kDa) induce MMP-1 expression and MMP-2 activity in normal human fibroblast. In immunofluorescence staining and Western blot, the level of fibronectin expression increased in young skin compared to aged skin. The fragmentation of fibronectin was increased in aged skin compared to young skin.

2. Materials and Methods

2.1. Primary Human Dermal Fibroblasts Culture

Human dermal fibroblasts from the infant foreskin were grown under proper culture conditions (37 ℃, 5 % CO_2) in Dulbecco's modified Eagle's medium (DMEM) containing 0.48 mg/mL glutamine, 100 IU/mL penicillin, 50 mg/mL streptomycin, and 10 % fetal bovine serum (FBS). Between the fourth and seventh passage, cells were used for the experiment.

2.2. Human Skin Samples

Six elderly -(four men; mean age, 68 yr; age range, 60 ~ 75 yr; two women; mean age 66 yr) and four young (four men; age range 31 ~ 35 yr, mean age 33 yr) Koreans, without current or prior skin diseases, provided skin samples. The skin samples were obtained by punch biopsy. The Western blot specimens were snap frozen in liquid nitrogen while the immunohistochemistry specimens were placed immediately in cryomatrix (Shandon, Pittsburgh Pennsylvania, USA) and stored at -70 ℃. This study was conducted according to the Declaration of Helsinki Principles. All procedures involving human subjects received prior approval from Seoul National University Institutional Review Board, and all subjects were provided with written informed consent.

2.3. MTT Assay

Cytotoxicity was determined by MTT, which is based on the reduction of the soluble yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to its blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase. For treatment, primary human dermal fibroblasts (1×10^4 cells/well) were maintained on culture media without FBS for 24 h. After Fn-fr's treatment, the cells were cultured for 24 h. The cells were washed with PBS, 200 μL of MTT (0.05 mg/mL) was added to each well, and the cells were incubated for 4 h at 37 ℃. The supernatant was then removed, and 200 μL of DMSO was added to each well to dissolve the formazan product. Wells without cells were used as blanks. Absorbance was determined at 570 nm, spectrophotometrically, using an ELISA reader.

2.4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA from human dermal fibroblasts was isolated using Trizol reagent (Gibco, USA) according to the manufacturer's instructions. The cDNA coding for

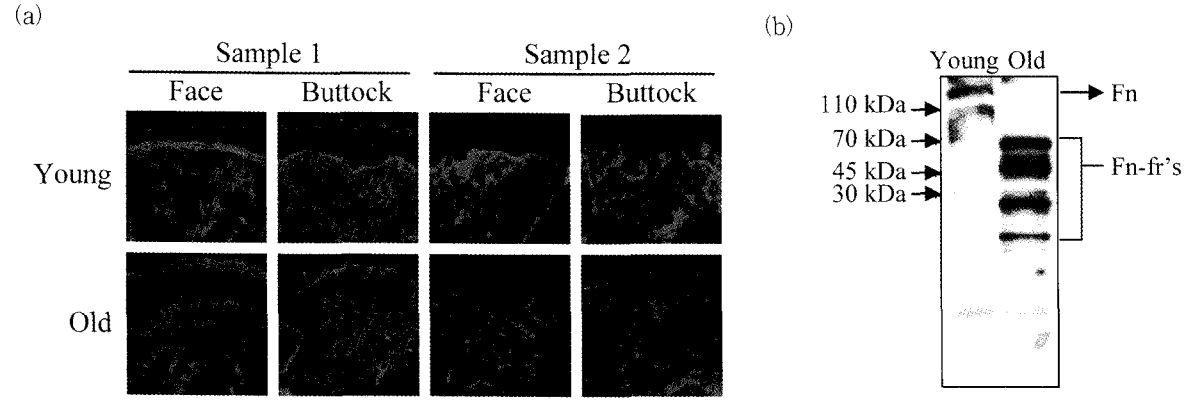

Figure 1. Immunofluoresence staining of fibronectin in young and old human skin (a) and the expression of fibronectin in young and old human skin (b). The fibronectin antibody was stained in dermal area, especially stained strong immunoactivity beneath the dermo-epidermal junction in young skin. The expression of fibronectin in young skin increased to compare with aged skin (a). And the fragments of fibronectin were shown in aged human skin (b, n = 4).

target gene was synthesized from the total RNA using first strand cDNA synthesis kit (MBI fermentas, USA) and then was amplified using Ex Taq™ (Takara, Japan). PCR primers were produced by custom oligonucleotide synthesis service (Bioneer, Korea). GAPDH was amplified in parallel and the results were used for normalization.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The measurement for the expression level of MMP-1 after Fn-fr's treatment in cultured foreskin fibroblast was performed by ELISA kit (Amersham, UK). The test was used according to the manufacturer's instructions. All reactions were performed in duplicate. The repeatability of the optical density (OD) values on a single microtiter plate corresponded to a coefficient of variation of < 10 %.

2.6. Western Blot Analysis

The levels of MMP-1 were determined by immunoblot analysis. The target proteins were electroblotted onto nitrocellulose membrane and detected by chemiluminescence method. Supernatant extracts were centrifuged at 12,000 g for 10 min at 4 ℃ and used for Western blot analysis. The monoclonal antibody for MMP-1 was purchased from oncogene. Anti-mouse IgG-HRP conjugate was used as secondary antibody. The antibody-antigen complexes were detected using the ECL system (Amersham Pharmacia Biotech; Little Chalfont, UK). To determine the amounts of MMP-1 (42/46 kDa) secreted into culture media, equal aliquots of conditioned culture media from an equal number of cells were fractionated by 10 % SDS-PAGE, transferred to Hybond ECL membrane. From membrane stain, we validate equal loading. Membranes were subsequently blocked with 5 % skimmed milk in TBS/T [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.05 % Tween-20] and incubated with the indicated antibodies. Blotting proteins were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, England). Signal strengths were quantified using a densitometric program (TINA; Raytest Isotopenmeβgerate, Germany).

2.7. Immunohistochemistry

For Immunohistochemistry, we purchased Histostain Plus kit (Zymed Laboratories Inc., USA) for the use of LAB method staining. The samples were sectioned 4 μm and fixed in acetone (5 min at -20 ℃). Then the sections were rehydrated in DW and endogenous peroxidase activity was quenched using 3 % hydrogen peroxide (dilution in PBS) for 10 min. Sections were blocked with blocking solution (Zymed Laboratories Inc., USA) for 30 min and the blocking solution was removed from around the section. Then the sections were incubated with anti fibronectin (Sigma, USA), in humidified chamber at 4 ℃ for 18 h. After washing in PBS, sections were incubated with FITC-goat anti rabbit secondary antibody (Zymed Laboratories Inc., USA) for 1 h.

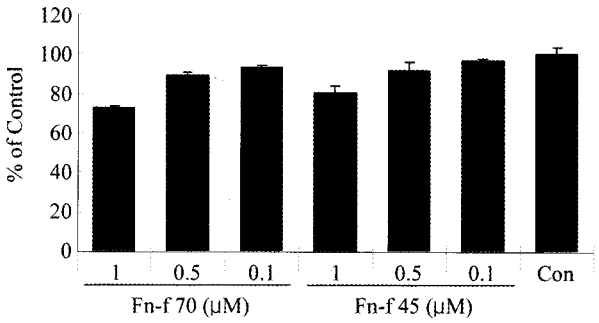

Figure 2. The cytotoxicity of Fn-fr's. Cytotoxicity of Fn-fr's was tested by an MTT-based cytotoxicity assay. The results are from 3 independent experiments. The bars indicate the standard error.

Then sections were washed in running tap water for 5 min, and the samples were mounted using fara-mounting medium (Dako, USA).

2.8. Statistics

Statistical analysis was performed with Student's *t*-test. All p values of were selected as the limit of statistical significance when p was ≤ 0.05.

3. Results and Discussion

3.1. The Expression of Fn in Young and Aged Human Skin *in vivo*

To investigate the expression of Fn in the face and buttock skins of young and elderly volunteers, we performed immunofluorescence staining with antibody for Fn. The fluorescence of Fn antibody stained in extracellular matrix and dermo-epidermal junction. Especially, the expression of Fn increased young skin compared to aged human skin and strongly stained in buttock skin compare with face skin (Figure 1(a)). Also, we performed Western blot analysis for Fn protein using the dermis protein extracted samples of young skin and elderly skin. The pattern of Fn protein in young skin showed 200 kDa band but in aged skin, Fn was degraded into four fragments (Figure 1(b)).

3.2. Cytotoxicity

Cytotoxicity of the Fn-fr's was also quantified by an MTT-based cell viability assay, and it was found that both 45 kDa and 70 kDa fragments were cytotoxic by dose-dependent manner. But, under 0.5 μM, Fn-fr's were not cytotoxic then that concentration was employed (Figure 2).

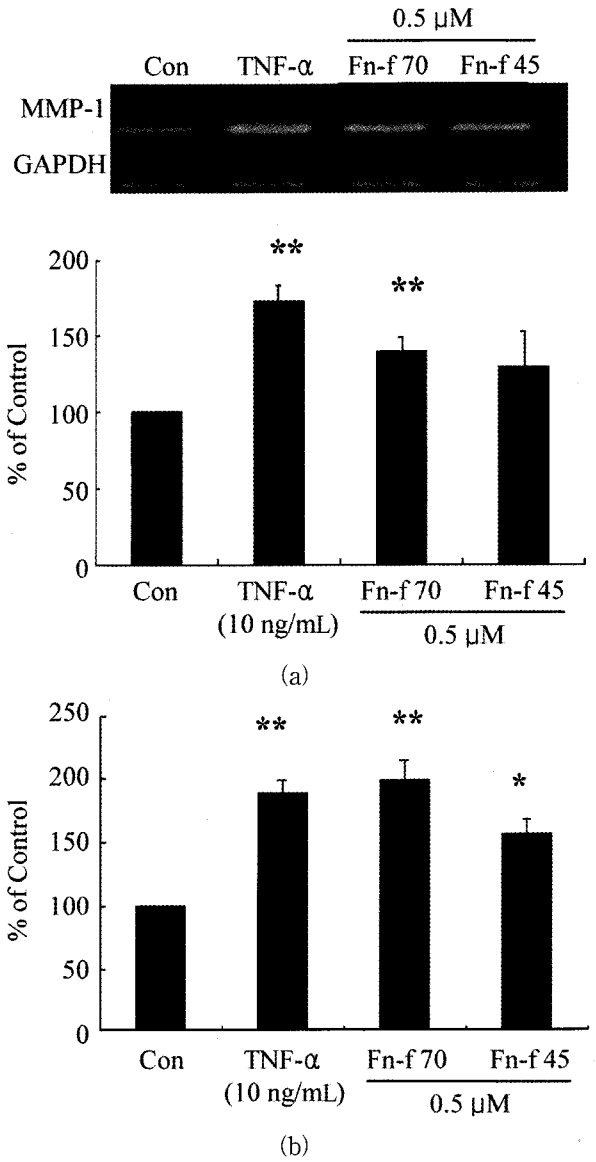

Figure 3. Effects of Fn-fr's (70, 45 kDa) on MMP-1 expression. The mRNA (a) and the protein expression (b) of MMP-1 after treatment with Fn-fr's were analyzed by RT-PCR and Western blot.

3.3. Fn-fr's Increased MMP-1 Expression in Normal Human Fibroblasts

It is also known that Fn-fr's induced MMP-1 expression by MAP kinase signaling in cartilage[1]. So we tested the induction abilility of Fn-fr's on MMP-1 expression in cultured normal human fibroblasts. Fn-

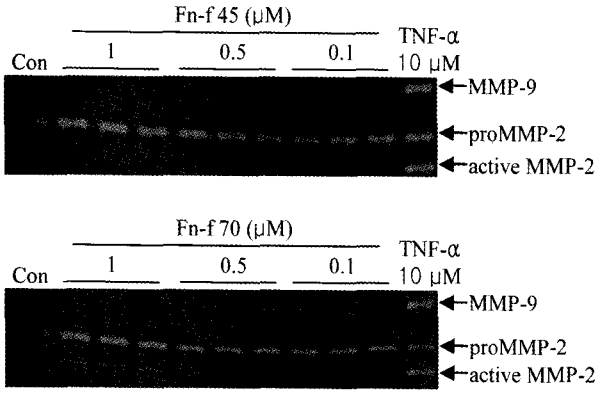

Figure 4. The fragments of fibronectin increased MMP-2 activity in normal human fibroblasts. The expression of MMP-2, 9 was measured in dose dependently treated Fn-fr's (70, 45 kDa) in normal human fibroblasts. The activity of MMP-2 was increased by Fn-fr's 70 and 45 kDa, but the activity of MMP-9 was not increased.

fr's (0.5 μM) increased mRNA transcription and protein expression of MMP-1 in fibroblasts and especially the expression was markedly induced compared to control cells (Figure. 3). Accordingly, we suppose that Fn-fr's act as a stimulating factor of MMP-1 expression.

3.4. Fn-fr's Increased MMP-2 Activity in Normal Human Fibroblast

It is also known that Fn-fr's can stimulate the release of several MMPs from cartilage explants in culture, including MMP-3, 2, 9[5]. So we tested the ability of Fn-fr's on MMP-2, 9 activity in cultured fibroblasts. Fn-fr's (70, 45 kDa) increased activity of MMP-2 in fibroblasts but the activity of MMP-9 was not increased (Figure. 4). Accordingly, we suppose that Fn-fr's act as stimulating factor of MMP-2 activity in human fibroblasts.

4. Conclusion

We demonstrated that Fn-fr's (70, 45 kDa) stimulate the synthesis of MMP-1 in human fibroblasts and increased fibronectin fragmentation in aged human skin. This is the first report that fragments of fibronectin increased the expression of MMP-1 in normal human fibroblasts and the fragmentation of fibronectin increased in aged skin.

Now, the major target proteins for anti-aging have been focused to the structural proteins such as collagen and elastin. Many cosmetic companies or anti-aging research groups have been developing anti-aging materials to prevent induction of MMPs in UV-damaged cell or to increase collagen.

In conclusion, we demonstrated that Fn-fr's were able to increase MMP-1 production and MMP-2 activity by directed or indirect mechanisms in chronic aging. We showed the fragmentation of fibronectin increased in aged human skin compared with young skin.

We believe that these findings may contribute to a better understanding ECM damage in chronic aging without UV-radiation and may be helpful in regulation of ECM alternation.

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