Augmentation of Gene Expression and Production of Promatrix Metalloproteinase 2 by Propionibacterium acnes-Derived Factors in Hamster Sebocytes and Dermal Fibroblasts: A Possible Mechanism for Acne Scarring

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Acne vulgaris, a common inflammatory skin disease,1—3) is characteristic to i) excess sebum production and hyperplasia of sebaceous glands; ii) the formation of microcomedones, which is closely associated with the hyperkeratinization of the follicular wall and infundibulum; and iii) the induction of inflammatory reactions such as the acceleration of cytokine production and the biosynthesis of arachidonic acid metabolites in keratinocytes, sebocytes, and invaded inflammatory cells.4) Furthermore, the aggravation and duration of the inflammation have closely been associated with the formation of acne scars, a severely disfiguring and permanent sequel, which may result in a psychological and social impact that affects the patient’s quality of life.5) Although recent works have reported that abnormal extracellular matrix (ECM) remodeling may progress in sebaceous glands and pilosebaceous units, which is associated with acne scar formation.

Aberrant extracellular matrix (ECM) remodeling in sebaceous glands and pilosebaceous units in the skin is associated with scar formation under acne conditions. To investigate the involvement of Propionibacterium acnes (P. acnes), a Gram-positive anaerobic microbial species, in ECM remodeling in sebaceous glands and pilosebaceous units, we examined the effects of P. acnes culture media, formalin-fixed P. acnes, and peptidoglycan (PGN) from Gram-positive bacteria walls on the production of promatrix metalloproteinase 2 (proMMP-2)/progelatinase A in hamster sebocytes and dermal fibroblasts. When hamster sebocytes (1.8×10^5 cells) and dermal fibroblasts (1×10^5 cells) were treated with P. acnes culture media and formalin-fixed P. acnes (corresponding to 1×10^4 and 1×10^5 bacterial cells), the production of proMMP-2 was augmented. In addition, PGN (5—50 μg/ml) dose-dependently augmented the production of proMMP-2 in both cells. Furthermore, the PGN (50 μg/ml)-augmented proMMP-2 production was resulted from an increase of its transcript. In contrast, there were no changes in cell proliferative activity in either the P. acnes or PGN-treated sebocytes and dermal fibroblasts, indicating that the augmented proMMP-2 production was not due to an increase in cell numbers. Therefore, these results provide novel evidence that PGN transcriptionally up-regulates the production of proMMP-2 in hamster sebocytes and dermal fibroblasts. Given an increase in the quantity of Gram-positive bacteria, including P. acnes in acne lesions, the aberrant ECM degradation may progress in sebaceous glands and pilosebaceous units, which is associated with acne scar formation.

Key words sebocyte; Propionibacterium acnes; peptidoglycan; matrix metalloproteinase; scar formation; extracellular matrix remodeling

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ster sebocytes and dermal fibroblasts. Furthermore, PGN transcriptionally augmented proMMP-2 production in both cells. These results provide novel evidence that P. acnes, as well as PGN, directly up-regulates both sebaceous and dermal proMMP-2 production along with an increase of its transcript.

MATERIALS AND METHODS

Preparation of Acnes-CM and F-Acnes Acne-CM and F-acnes were prepared from P. acnes (UCM6473) (RIKEN BioResource Center, Ibaraki, Japan) according to the method of our previous paper.10 Briefly, P. acnes was incubated in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Tokyo, Japan) under anaerobic conditions at 37°C for 72 h. The culture was separated into the supernatant and bacterial cells by centrifugation. The supernatant filtered using a membrane (φ 0.45 nm) was used as acnes-CM. The bacterial cells were washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS(−)), and then fixed with 4% formaldehyde/PBS(−) for 30 min. After centrifugation, the cell pellet was re-suspended in distilled water of an equal volume to the culture medium. The cell suspension was used as F-acnes. The number of P. acnes was determined by plating serial dilutions of the culture onto a GAM agar plate.

Cell Culture and Treatments Hamster sebocytes and dermal fibroblasts were prepared from sebaceous glands of auricles and back skin, respectively, of 5-week-old male golden hamsters as previously described.16,19 Sebocytes in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (1 : 1) (DMEM/F12) (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 6% heat-denatured fetal bovine serum (Invitrogen), 2% human serum (MP Biomedicals, Solon, OH, U.S.A.), 0.68 mm L-glutamine (Invitrogen) and recombinant human epidermal growth factor (10 nm) (Progen Biotechnik GmbH, Heidelberg, Germany) and dermal fibroblasts in DMEM (Invitrogen) with 10% fetal bovine serum were incorporated into the cells was measured with excitation at 540 nm and emission at 590 nm according to the manufacturer’s instructions. Relative amounts of proMMP-2 protein were quantified by densitometric scanning using an Image Analyzer LAS-1000 Plus (Fuji Film, Tokyo, Japan), and the relative expression level was expressed as the mean value of the control as 100%.

Real-Time Polymerase Chain Reaction (PCR) for ProMMP-2 For the quantification of proMMP-2 mRNA, total RNA was isolated from cells using ISOGEN (Nippon Gene, Toyama, Japan) and then subjected to reverse transcriptase reaction for the synthesis of cDNA using a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. Aliquots (an equivalent of 2.5 ng of total RNA) of the transcript were subjected to real-time PCR using SYBR Premix Ex Taq II (Takara Bio) and primers for hamster proMMP-2 (Accession No. AF260254): sense and antisense primers, 5’-TATCCAAACCAGCTAGACCA-3’ (126—143 bp) and 5’-GTATCCAGAACACTTCTCC-3’ (225—245 bp), respectively. As an internal control gene, hamster glyceraldehyde-3-phosphate dehydrogenase (Accession No. X52123) was similarly amplified using the following primers: sense and antisense primers, 5’-CACGGAACTCATCCCTGCA-3’ (677—694 bp) and 5’-TAGGAAACCGGAAAGGCCAT-3’ (758—776 bp), respectively. The amplification cycle was performed at 94°C for 5 s and 60°C for 30 s using a Thermal Cycler Dice Real Time System TP-800 (Takara Bio). The obtained threshold cycle (Ct) value for proMMP-2 was normalized by that for glyceraldehydes-3-phosphate dehydrogenase, and the relative expression level was expressed as the mean value of the control as 100%.

Assay for Cell Proliferation Hamster sebocytes and dermal fibroblasts (1 × 10⁴ cells/well) in 96-well multiplates (Becton Dickinson) were treated with acne-CM, F-acnes (1 × 10⁶ and 1 × 10⁷ cells), and PGN (5—50 μg/ml) in DMEM/F12 supplemented with 2% serum for 48 h, and then cell proliferation was monitored by an Alamar blue assay (Biosource International, Camarillo, CA, U.S.A.) as previously described.20 The fluorescence of the Alamar blue incorporated into the cells was measured with excitation at 540 nm and emission at 590 nm according to the manufacturer’s instructions.

Statistical Analysis Data are presented as means±standard deviation (S.D.), and were analyzed by a one-way analysis of variance and by the Fisher test for multiple comparisons. A value of p<0.05 was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

Gelatinolytic activity and gelatinases such as MMP-2 and MMP-9 have been reported to be detectable in sebum from acne patients and immortalized human SZ95 sebocytes, respectively.10 Recently, we have reported that hamster sebocytes and dermal fibroblasts constitutively produce proMMP-2, which is immunologically similar to human proMMP-2.20 In the present study, we demonstrated that hamster dermal fibroblasts as well as sebocytes constitutively and transcriptionally produced proMMP-2 (Figs. 1A, B, see untreated cells). We have also confirmed that gelatinolytic activity at approximately 92 kDa, which might correspond to proMMP-9, was negligible in sebocytes and dermal fibroblasts (data not shown). Since keratinocytes, monocytes, and dermal fibroblasts in hu-
mans play important roles for MMP-mediated cutaneous ECM degradation in acne,\textsuperscript{10,11,17,18} these results suggest that hamster sebocytes as well as dermal fibroblasts are useful for the investigation of sebaceous and pilosebaceous ECM remodeling in acne lesion.

Acne vulgaris has been classified as both a non-inflammatory and inflammatory skin disease and, in the latter, the inflammatory reactions are likely to be induced and enhanced by \textit{P. acnes}.\textsuperscript{1,2} Exacerbation of the inflammation in acne has been reported to cause the destruction of integrity in sebaceous glands and pilosebaceous units, resulting in the formation of intractable acne scars.\textsuperscript{22} Scar formation, as in the case of keloids, has been reported to be associated with aberrant ECM production and degradation.\textsuperscript{5} Although cutaneous ECM remodeling under physiological and pathological conditions has been reported to be regulated by MMPs,\textsuperscript{9} it is not fully understood whether \textit{P. acnes} may directly regulate MMP expression in sebaceous glands and/or pilosebaceous units. So far, Choi \textit{et al.}\textsuperscript{18} have also reported the augmentation of proMMP-2 production by \textit{P. acnes} in human skin fibroblasts. In the present study, acne-CM and F-acnes, which exert the enhancement of sebaceous lipogenesis in hamsters \textit{in vivo} and \textit{in vitro},\textsuperscript{20} augmented the production of proMMP-2 in hamster sebocytes and dermal fibroblasts (Fig. 1). Since \textit{P. acnes} has been reported to exist within follicular canals,\textsuperscript{2} sebaceous glands as well as follicular walls and infundibulum are likely to be spatially susceptible to \textit{P. acnes}-itself and/or \textit{P. acnes}-derived factor(s) rather than dermis in the skin. Therefore, our finding that \textit{P. acnes} augments proMMP-2 production in sebocytes suggests a possible mechanism whereby sebocyte-derived MMP-2 participates in the onset of aberrant sebaceous ECM remodeling in acne.

PGN is a component of the Gram-positive bacteria cell walls\textsuperscript{12,13} and exhibits inflammatory actions through Toll-like receptor 2 (TLR2).\textsuperscript{23–25} In the present study, PGN transcriptionally facilitated the production of proMMP-2 in both hamster sebocytes and dermal fibroblasts (Fig. 2). Romics \textit{et al.}\textsuperscript{26} reported that \textit{P. acnes} as well as PGN derived from \textit{Staphylococcus aureus} induced the production of pro-inflammatory cytokines via TLR2 in human TLR2-overexpressing
Chinese hamster ovary cells and human embryonic kidney cells, but not in TLR2-deficient mouse peritoneal macrophages. In addition, Kim et al.\(^{27}\) reported that TLR2 was requisite for the \(P.\) acnes-mediated activation of monocytes. Therefore, these findings allow us to hypothesize that \(P.\) acnes-derived PGN plays an aberrant role due to the enhancement of proMMP-2 production in acne lesions via a TLR2-dependent pathway. Furthermore, since MMP-2 has higher substrate specificity to type-IV collagen, a structural element of the basement membrane,\(^{28}\) the acceleration of MMP-2 expression by \(P.\) acnes in sebaceous glands in acne lesions is likely to result in the destruction of the basement membrane between the epithelium and dermis, subsequently leading to dermal ECM degradation for acne scarring.

Nagy et al.\(^{29}\) reported that the growth of human keratinocytes is facilitated by a clinically isolated \(P.\) acnes strain, but not another reference \(P.\) acnes strain. In the present study, neither \(P.\) acnes nor PGN influenced the cell proliferation of hamster sebocytes and dermal fibroblasts (Fig. 3). Although differences in the regulation of cell proliferation by \(P.\) acnes may be due to distinct cell-type species and/or genetic and functional variations of \(P.\) acnes strain(s) that is/are derived from acne lesions in different individuals,\(^{25,29}\) our findings suggest that the augmentation of proMMP-2 production by \(P.\) acnes is not due to increases in cell proliferation in sebocytes and dermal fibroblasts.

In conclusion, we demonstrated for the first time that in hamster sebocytes and dermal fibroblasts, both \(P.\) acnes and PGN enhance the production of proMMP-2 by facilitating its gene expression. Together with our previous report that \(P.\) acnes facilitates the sebum production in the sebaceous glands,\(^{20}\) it is suggested that \(P.\) acnes works cooperatively not only to facilitate sebum production but also to form acne scars due to the enhanced ECM degradation. These findings may provide novel insights into acne therapy in that compounds that inhibit MMP-2 production can be effective as anti-acne agents for the prevention and remission of scar formations.

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