The Effect of TNF-α and INF-γ on the Telomerase Activity of Cultured Human Keratinocyte

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Psoriasis is a chronic inflammatory skin disease characterized by hyperproliferation and abnormal differentiation of the lesional epidermis. In pathogenesis, inflammatory cytokines such as TNF-α and IFN-γ from infiltrated T-cells seem to act a central role. Although many chronic inflammatory conditions can lead to cancer development, there is no evidence of increased incidence of cancer in psoriatic skin lesion.

Telomerase is an enzyme reverse transcriptase that protects chromosomes from degradation by stabilizing telomere length. Recent studies suggest that telomerase activity may be responsible for some part of nonmalignant proliferatory skin disease. In addition, there is evidence that telomerase activity is related with proliferation and differentiation of keratinocyte.

In this experiment, we tried to evaluate the effect of TNF-α and IFN-γ to the telomerase activity and its differential effect thought the passage. The results showed increased telomerase activity according to stimulation and this extent was different from the various passage. These results suggest that the key cytokines of psoriasis, namely, TNF-α and IFN-γ increase telomerase activity at proliferative cells, which could contribute to hyperproliferation and abnormal differentiation of lesional keratinocyte. Moreover, this increased telomerase activity could partially explain the cancer incidence of psoriasis that is not increased compared to the normal population.

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INTRODUCTION

Psoriasis is a common chronic inflammatory skin disease. It frequently develops in early adulthood although all ages can be affected. Once psoriasis appears, it is usually a life-long disease without spontaneous improvement. The skin lesion of the disease is characterized by erythematous scaly patches or plaques, caused by hyperproliferation and abnormal or incomplete differentiation of epidermal keratinocyte, together with chronic inflammatory cell infiltrate in both the dermis and epidermis. In its pathogenesis, psoriasis is considered as T-cell mediated inflammatory disease and various inflammatory cytokines such as tumor necrotic factor (TNF)-α and interferon (IFN)-γ are likely to play major functions. Although inflammatory cells seem to play a central role, it is probably best to conceptualize the pathogenesis as an interactive response between genetics, inflammatory cells, resident skin cells and an array of immunologic cascades between these. A strong association has been well established between chronic inflammatory conditions and carcinogenesis, and although psoriasis also has many characteristics prone to malignant transformation, conversion of a psoriatic...
lesion to skin cancer is extremely rare.\textsuperscript{6,7}

Telomere is the nucleoprotein structure that caps the end of eukaryotic chromosomes, which maintains chromosomal stability.\textsuperscript{9} It contributes not only to senescence, but also to the provision of an effective tumor suppressor mechanism.\textsuperscript{10,11} Among somatic cells, telomerase activity is demonstrated in the keratinocyte of the proliferatory basal layer of the epidermis.\textsuperscript{12} There is also evidence of increased telomerase activity in non-malignant skin conditions such as psoriasis vulgaris.\textsuperscript{12,13}

\textbf{MATERIALS AND METHODS}

\textbf{Preparation of primary human keratinocyte.}

Primary human keratinocytes were obtained from fresh tissue of excised neonatal foreskin after informed consent. After removal of the subcutaneous tissue and much of the reticular dermis, the tissue samples were cut into $2 \times 2$ mm strip and incubated in 0.05\% Tripasin, 0.53 mM EDTA solution overnight at 4°C. The following day, the epidermis was peeled off the residual dermis and aspirated using a Pasteur pipette to aid cell dissociation. The primary suspension of primary epidermal cells was washed with PBS (Phosphate Buffered Saline, pH7.4) twice then prepared in Eplife basal culture medium (Cascade Biolibics Inc., Portland, Or, USA) with Human keratinocyte growth supplement (Cascade Biolibics Inc., Portland, Or, USA).

\textbf{Subculture and treatment with TNF-α and IFN-γ}

Prepared primary keratinocytes were seeded into culture plates and maintained at 37°C in a humidified incubator with 5\% CO$_2$ until forming the monolayer then trypsinized and cells were plated in secondary culture ($5 \times 10^4$ cells/cm$^2$) and serially subcultured at every 60-70\% confluence. Cells from each passage were seeded at 6-well culture plates ($3 \times 10^5$ cells/well) and cultured in presence or absence of 10 ng/mL of TNF-α (R&D Systems Inc, Minneapokis, USA) and/or IFN-γ (R&D Systems Inc, Minneapokis, USA) for another 24 hours.

\textbf{HaCaT cell culture and treatment with TNF-α and IFN-γ}

The human keratinocyte cell line HaCaT was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10\% fetal bovine serum and 100 μ/mL penicillin/streptomycin at 37°C and in 5\% CO$_2$. Forming monolayer cells were trypsinized, then seeded at 6-well plates ($1 \times 10^5$ cells/well). At approximately 70\% of confluence, 10 ng/mL of TNF-α and/or IFN-γ were treated and cells were cultured for another 24 hours.

\textbf{Growth pattern analysis}

Proliferation patterns of cultured keratinocyte was calculated using an inverted phase-contrast microscope and cells were collected at planned time for counting the number with hemocytometer. The number of population doublings was calculated according to the previous report.\textsuperscript{17}

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\text{Number of population doublings (P.D)} = \frac{\log N_t - \log N}{\log 2}
\]

Where $N_t$ was the number of viable cells at the end of the growth period and $N$ was the number of cells attached to the flasks after plating.

\textbf{Detection of Telomerase activity}

Tissue extraction and PCR-ELISA were performed according to the instructions for the Telomerase PCR-ELISA kit (Roche Diagnostics, Mannheim, Germany). Briefly, 12 frozen sections (10 μm) of each sample was homogenized in 200 μL of ice cold lysis buffer and incubated for 30 minutes on ice. After centrifugation at 16,000g for 20 minutes at 4°C, the supernatant was collected, quickly frozen in liquid nitrogen, and stored at -80°C. Protein concentration was measured using the DC protein assay kit (Bio-Rad Laboratories). Tissue extracts
Fig. 1. When cultured human keratinocytes were treated with TNF-α and/or IFN-γ, it showed inhibitory effect on the proliferation rate. Through every passage of the TNF-α and/or IFN-γ showed synergic effect on proliferation inhibition. TNF-α and/or IFN-γ did not show inhibitory effect on HaCaT cells (*: p<0.05, n=3).

RESULTS

Effect of TNF-α and IFN-γ on the number of population doublings according to the passage.

Each of TNF-α and IFN-γ showed inhibitory effect on cultured human keratinocyte (Fig. 1). When added together, they showed more inhibitory effects than any of the single cytokines. These inhibitory effects were more potent in the early passage and became weaker through the following passages. There was no specific effect on HaCaT cell (*: p<0.05, n=3). Because inhibitory effects of TNF-α and IFN-γ on the keratinocyte are well known, and considering that cells become senescent according to passage, we can postulate that the effect of these cytokines is more pronounced in the early passage of proliferative young cells.

The effect of TNF-α and IFN-γ on the telomerase activity.

We evaluated the effect of both cytokines on the telomerase activity through each passage (Fig. 2). Telomerase activity was presented as relative telomerase activity (RTA). To do this, the same number of cells from each passage was collected, and treated with each or both cytokines. The result showed increased telomerase activity on the cells of the early passage (P0, P1). After passage 2, these effects became obscured. This was thought to relate to cellular senescence or differentiation. There was no difference of telomerase activity in the HaCaT cells. TS 8.0 cells were used for negative control according to manufacturer’s direction (*: p<0.05, n=3).

Statistical analysis

The statistical significance of differences in P.D and RTA were tested using the Student’s t-test. p-values<0.05 were considered statistically significant.
Comparison of effect of TNF-α and/or IFN-γ on telomerase activity levels according to passage.
To evaluate the different effect of each cytokine according to the passage, we compared the change of RTA of each passage separately. Fig. 3A shows changes of RTA according to passage of normal control. It showed decreased RTA according to the passage. Fig. 3B-D showed RTA changes by the TNF-α and/or IFN-γ. In the presence of TNF-α, RTA increased until P1 and decreased from P2. In

Fig. 2. There was a significant increase of RTA especially in the early passage (P0, P1). Elevated RTA level was more prominent when cells were treated with TNF-α and TNF-γ together. These effects became faint from P2 and RTA level showed no significant differences compared to the control of the same passage. In HaCaT cell, the cells treated with TNF-α and/or TNF-γ showed no changes in RTA level (*: p<0.05).

Fig. 3. Comparing the RTA levels of cultured human keratinocyte through the passage, RTA increased until P2. However, after P2, RTA decreased more than the level of the P0. (A) Control. There was a mild increase in telomerase activity on P1. However, it decreased after P2 compared with P0. (B) RTA levels of each passage treated with TNF-α were compared. RTA levels were elevated until P1 than decreased according to passage. (C) RTA levels of each passage treated with IFN-γ were compared. There were no significant changes until P3. (D) After combined treatment with TNF-α and INF-α, degree of the changes of RTA were enhanced when compared to the result of the single treatment of TNF-α or INF-α.
the presence of INF-γ, it also showed increased RTA in the early passage. These effects seemed to be enhanced when it was stimulated by both TNF-α and IFN-γ together (*: p<0.05, n=3).

DISCUSSION

Psoriasis is a chronic inflammatory skin condition that varies in severity, which is an important implication in terms of medical costs and treatment strategies1. Various factors such as genetic components, environmental factors, microorganism and immunologic reactions seem to play a role in the disease development. The skin lesion of psoriasis is characterized by focal formation of inflamed, raised plaques that constantly shed scales. Previously, it was assumed that keratinocyte hyperproliferation associated with abnormal epidermal differentiation was the primary cause of psoriasis4,14. However, it is now conceptualized that T-cell mediated immunologic reaction play a central role during its pathogenesis, as the trigger for keratinocyte hyperproliferation and incomplete abnormal differentiation14,15. During this process, TH1-type cytokines, such as IFN-γ and TNF-α are known to play an important role and downregulation of epidermal IFN-γ and TNF-α level has been shown to correlated with clinical improvement of psoriasis3,16.

In this study, we treated cultured human keratinocyte with IFN-γ and/or TNF-α and measured the number of population doubling to evaluate indirectly which stage or compartment of cells are mostly affected17. In the previous report about the effect of TNF-α in epidermal keratinocyte, it showed the inhibitory effect on cell proliferation18. IFN-γ also showed strong inhibitory effects on cell proliferation and these effects showed synergy in present action of TNF-α19,21. Our results showed significant inhibitory effects on cell proliferation in the early passage (0, P1) of serial cultures. Considering early passage contains more proliferatory cells such as stem cells and transit amplifying cells, we could postulate that those effects of TNF-α and TNF-γ affect mostly the proliferatory cell components. However, some specific methods using cell marker or certain proteins seemed to be necessary to find which cell components were mostly affected.

Telomere serves essential roles in preventing checkpoint activation and it maintains chromosomal stability1. Telomerase is an enzyme-reverse transcriptase that stabilizes chromosomal length. It serves multiple functions in preserving chromosome stability; including protecting the end of chromosomes from degradation and preventing chromosomal end fusion10. Through these actions, telomerase was known to play a central role in series of cellular biology such as proliferation, differentiation and apoptosis9,11. In the previous reports, telomerase was also known to play a central role in carcinogenesis. During the early stages of carcinogenesis, telomere shortening leads to loss of telomerase capping function, chromosomal fusion and chromosomal instability. In this stage, if additional pressures exist to lose p53 function to allow further cell proliferation in the presence of chromosomal instability, it could finally result in cellular transformation and initiation of cancer15,22. Increased telomerase activity was reported in not only malignant cancers, but also nonmalignant skin conditions such as psoriatic skin lesions12,13.

Chronic inflammatory diseases are also known to contribute to carcinogenesis6,7. However, the conversion of a psoriatic plaque to cancer is very rare8.

Considering all the above reports, we hypothesized that TNF-α and IFN-γ, key cytokines on the psoriasis, might result in the increased telomerase activity, and this elevated telomerase activity could partially explain the reason why conversion of psoriatic plaque to cancer is very rare. To evaluate the above hypothesis, we investigated the effect of TNF-α and IFN-γ on telomerase in the keratinocyte. To examine which compartment of cells are more sensitive, we treated human keratinocytes form various passages with TNF-α and/or IFN-γ. The results showed significant increased telomerase activity in the early passage of cultures and these effects became faint during the passage. Elevated relative telomerase activity was more significant when cells were treated with TNF-α and IFN-γ together. Those results were similar to previous reports about the synergic effect of TNF-α with IFN-γ in the induction of keratinocyte apoptosis20. Interestingly, our results about the pattern of increased telomerase activity seemed to parallel with the inhibitory effect of TNF-α and IFN-γ on cultured human keratinocyte. In concordance with our results, we could postulate that TNF-α and IFN-γ elevate the telomerase activity in the psoriatic skin lesions which is partially responsible for the reason of its rare cancer incidence. Moreover, if we consider...
that reports about epidermal differentiation, apoptosis and senescence share a series of common pathways, our results could also help explain the abnormal differentiation of the psoriatic skin lesions.

REFERENCES