The inhibitory effect of A20 on the inflammatory reaction of epidermal keratinocytes

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Abstract. A20 is a negative regulator of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling, and has been implicated in the pathogenesis of psoriasis through genome-wide association study (GWAS). In the present study, we investigated the putative role of A20 in epidermal keratinocytes. Immunohistochemical analysis showed that A20 was expressed in all layers of the epidermis, with an increasing pattern in the upper layers. In our model of calcium-induced keratinocyte differentiation, A20 expression was increased in a time-dependent manner. To investigate whether A20 affected keratinocyte differentiation, we overexpressed A20 in cultured keratinocytes. As a result, we noted that A20 overexpression did not affect keratinocyte differentiation, suggesting that A20 is not a direct modulator of keratinocyte differentiation. Interestingly, we found that A20 levels were decreased in psoriatic lesional skin compared to non-lesional areas. To investigate whether A20 played a role in the innate immune response of keratinocytes, we overexpressed A20 and then examined poly(I:C)-induced cytokine expression. We noted that A20 significantly inhibited poly(I:C)-induced cytokine production, and this effect was related to the inhibition of NF-κB signaling. These results suggest that the downregulation of A20 increased the susceptibility of keratinocytes to external stimuli, thus contributing to the development of psoriasis.

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Introduction

The primary function of skin is to protect the organism from environmental insults such as chemicals, ultraviolet (UV) radiation and microbial infection (1). Epidermal keratinocytes contribute to the protective function by forming the skin barrier structure through a sophisticated program of differentiation (2). Since keratinocyte differentiation is the pivotal process that results in a proper skin barrier against harmful environmental insults, dysregulation of keratinocyte differentiation is directly linked to skin diseases (3). In addition to their essential role in the formation of the physical barrier, keratinocytes exert an important effect as primary defense cells. Keratinocytes express different pattern recognition receptors (PRRs), such as various Toll-like receptors (TLRs), which are important to human innate immunity. Keratinocytes recognize the bacterial pathogen-associated molecular patterns (PAMPs) through TLRs, and inflammation-related intracellular signaling such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) is activated. Thus, keratinocytes produce a range of inflammatory cytokines linked to several skin diseases (4-6).

Psoriasis is a common chronic inflammatory skin disease. The characteristic features of psoriasis include keratinocyte hyperproliferation, altered keratinocyte differentiation and inflammation (7). For the past 30 years, psoriasis has been regarded as an adaptive immune-mediated disease, in which T helper (Th)1-type immune cells and their cytokines play critical roles in terms of the development of disease (8). It has previously been demonstrated that a unique interleukin (IL), IL-17, is produced by Th17 cells, and these IL-17 cytokines also play important roles in the pathogenesis of psoriasis (9,10). Several growth factors, such as fibroblast growth factor-10 (FGF-10) and FGF-7, have also been implicated in the pathogenesis of psoriasis (11).

Additionally, previous research has emphasized the important role of keratinocytes in the pathophysiology of psoriasis. Stimulation of keratinocytes with various PAMPs or damage-associated molecular patterns (DAMPs) resulted in the activation of innate immunity, leading to the production of inflammatory cytokines related to psoriasis (12-14).

Another important factor for psoriasis is the genetic background. Several chromosomal regions are thought to harbor genes relating to psoriasis (8). Interestingly, a previous investigation termed the Genome-Wide Association Study (GWAS) identified new psoriasis susceptibility loci including A20 [also known as tumor necrosis factor α-induced protein 3 (TNFAIP3)] and TNFAIP3-interacting protein 1 (TNIP1) (15). A20 is a cytoplasmic zinc finger protein that acts as a negative regulator in the NF-κB signaling pathway (16). Although it has been suggested that A20 is linked to psoriasis by GWAS, there is still limited evidence as to whether A20 is directly involved in the pathogenesis of psoriasis.

In the present study, we investigated the putative role of A20 in keratinocytes and found that A20 decreased PAMP-induced inflammation of keratinocytes. These results provide insight into the molecular mechanism of inflammation, emphasizing the importance of A20-regulated innate immunity in the pathogenesis of psoriasis.

Materials and methods

Immunohistochemical analysis. We biopsied psoriatic skin samples from lesions and non-lesional areas of patients, in order to perform immunohistochemical analysis. These paraffin-embedded sections of skin specimens were de-waxed, re-hydrated and washed three times with PBS. Sections were then incubated with proteinase K (Dako, Carpinteria, CA, USA) for 5 min at 37˚C, and treated with H_2O_2 for 10 min at room temperature, blocked in 0.1% Tween-20, 1% bovine serum albumin (BSA) in PBS for 30 min, and this was followed by reaction with anti-A20 antibody (cat. no. ab92324; Abcam, Cambridge, MA, USA) for 1 h. Sections were incubated sequentially with peroxidase-conjugated secondary antibody (cat. no. P0448) and visualized with a ChemMate EnVision detection kit (both from Dako).

Cell culture. For the cell culture, human skin tissues (five circumcised foreskins) were obtained after written informed consent was obtained from donors, in accordance with the Ethical Committee approval process of the Institutional Review Board of Chungnam National University Hospital (Jung-gu, Korea). Keratinocytes and fibroblasts were primary cultured using these skin specimens. We did not isolate and culture the keratinocytes from patients with psoriasis. Skin specimens were briefly sterilized in 70% ethanol, minced, and then treated with dispase overnight at 4˚C. The epidermis was separated and placed in a solution containing 0.05% trypsin and 0.025% ethylenediaminetetraacetic acid (EDTA) at 37˚C for 15 min. After vigorous pipetting, cells were precipitated by centrifugation (200 x g for 5 min) and resuspended in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (BPE) and recombinant human epidermal growth factor (rhEGF; Life Technologies Corporation, Grand Island, NY, USA).

For the 3-dimensional artificial skin, type I collagen solubilized in 0.1% acetic acid (Bioland, Cheonan, Korea) was mixed with primary cultured dermal fibroblasts, neutralized with NaOH, poured into the Transwell plate (Corning, Tewksbury MA, USA), and polymerized. After 2 days of incubation, keratinocytes were loaded onto the dermal matrix and incubated with FAD medium. When the cells reached confluence, the cultures were lifted to the air-liquid interface and incubated for 2 weeks.

Model of calcium-induced keratinocyte differentiation. We constructed a model of calcium-induced keratinocyte differentiation, as previously reported by Seo *et al* (17). Cultured keratinocytes were treated with 1.2 mM calcium for 0, 1, 3, 7 and 14 days. We chose four time points that revealed the specific situation of differentiating keratinocytes: the situation of day 1 after calcium treatment is similar to that of the cells leaving the basal layer in the skin, day 3 is for the early spinous layer, day 7 is for the middle spinous layer, and day 14 is for the late spinous or granular layer (17).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In the present study, total RNA was isolated using an easy-BLUE RNA extraction kit (Intron, Daejeon, Korea). Two micrograms of total RNA was reverse transcribed with Moloney-murine leukaemia virus (M-MLV) reverse transcriptase (RTase; Elpis Biotech, Daejeon, Korea). Aliquots of the RT mixture were subjected to PCR cycles with the appropriate primer sets. The sequences for primers were as follows: A20 forward, 5'-AAGGGTGTCTGAGCAGGAGA-3 and reverse 5'-TACGTCCATTTTCCCTGAGC-3; involucrin forward, 5'-GAACAGCAGGAAAAGCACCT-3 and reverse 5'-CACCCTCACCCCATTAAAGA-3; and β-actin forward, 5'-CTCTTCCAGCCTTCCTTCCT-3 and reverse 5'-CACCTT CACCGTTCCAGTTT-3.

For qPCR, aliquots of RT mixture were amplified using SYBR Green Master Mix in a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The following primer sequences were used: tumor necrosis factor (TNF)-α forward, 5'-CTCCTTCAGACACCCTCAACCT-3 and reverse 5'-CGACCCTAAGCCCCCAATT-3; IL-1β forward, 5'-TTA AAGCCCGCCTGACAGA-3 and reverse 5'-GCGAATGAC AGAGGGTTTCTTAG-3; IL-6 forward, 5'-CTGCGCAGC TTTAAGGAGTTC-3 and reverse 5'-CCATGCTACATTTGC CGAAGA-3; IL-8 forward, 5'-CCTTTCCACCCCAAATTT ATCA-3 and reverse 5'-TTTCTGTGTTGGCGCAGTGT-3; chemokine (C-C motif) ligand 20 (CCL20) forward, 5'-CCA CCTCTGCGGCGAAT-3 and reverse 5'-TGTGTATCC AAGACAGCAGTCAAA-3; and GAPDH forward, 5'-TGC ACCACCAACTGCTTAGC-3 and reverse 5'-GGCATGGAC TGTGGTCATGAG-3.

Western blot analysis. Cells were lysed in Pro-Prep solution (Intron). Total protein was measured using BCA protein assay reagent (Pierce Biotechnology, Rockford, IL, USA). Samples were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred onto nitrocellulose membranes and incubated with appropriate antibodies. Blots were then incubated with peroxidase-conjugated secondary antibodies (cat. no. P0160, cat. no. P0161, cat. no. P0448; all from Dako), visualized by enhanced chemiluminescence (Intron). The following primary antibodies were used in western blot analysis: A20 (Abcam), involucrin and loricrin (sc-21748 and sc-51130; both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-p65 (Cell Signaling Technology, Beverly, MA, USA), and actin (Sigma, St. Louis, MO, USA).

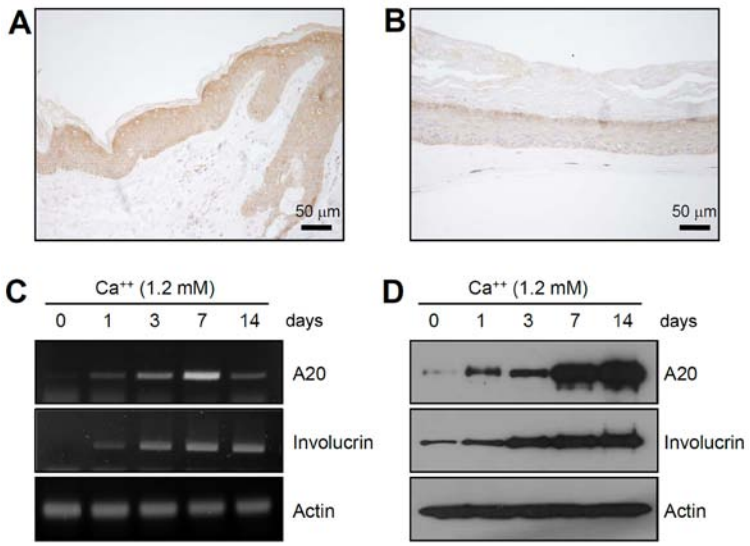

Figure 1. Expression of A20 in epidermal keratinocytes. (A) A20 was detected in all layers of normal skin epidermis by immunohistochemical analysis. In the upper layer of the epidermis, A20 expression was increased. (B) A20 was detected in the 3-dimensional artificial skin model in a similar way to normal skin. (C) Expression of A20 in model of calcium-induced keratinocyte differentiation. Cultured keratinocytes were treated with 1.2 mM calcium at the indicated time points. mRNA level was determined by RT-qPCR. The expression of A20 was increased in a time-dependent manner. (D) The protein level was determined by western blot analysis. Involucrin served as a positive control. Actin was used as the internal control.

Keratinocyte differentiation and transfection. To measure the effect of A20 overexpression on keratinocyte differentiation, keratinocytes were transfected with 10 MOIs of adenovirus expressing A20 or LacZ (control) for 6 h. Cells were replenished with fresh medium and treated with 1.2 mM calcium for 2 days. Keratinocyte differentiation, as shown by involucrin and loricrin expression, was determined by western blot analysis.

Poly(I:C) exposure. To investigate whether A20 exerted an effect on the PAMP-induced innate immune response of keratinocytes, we transfected normal human epidermal keratinocytes (NHEKs) with adenovirus-expressing A20 (Ad/A20) for 6 h and exposed them to poly(I:C), a dsRNA mimic (InvivoGen, San Diego, CA, USA). Cells were replenished with fresh medium and incubated overnight. Cells were exposed to 1 μg/ml poly(I;C) for 6 h. The mRNA level for inflammatory cytokines was determined by RT-qPCR. To measure the effects of A20 overexpression on poly(I:C)-induced nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activation in keratinocytes, after adenovirus transfection, cells were incubated with fresh medium overnight. Cells were then exposed to 1 μg/ml poly(I;C) for 45 min. Phosphorylation of the NF-κB p65 subunit was determined by western blot analysis. Cells were transfected with NF-κB-luciferase reporter adenovirus together with adenovirus expressing A20. After being replenished with fresh medium, cells were exposed to 1 μg/ml poly(I;C) for 24 h. Cells were then lysed and assayed for luciferase activity.

Adenovirus creation. A full-coding fragment of A20 cDNA was amplified by PCR with the following primer sets: 5'-AGATCTATGGCTGAACAAGTCCTTCC-3 and 5'-CTC GAGTTAGCCATACATCTGCTTG-3. A20 cDNA was subcloned into the pENT/CMV vector, and subsequently the replication-incompetent adenoviruses were created, using a ViraPower adenovirus expression system (Life Technologies Corporation) according to the manufacturer's instructions.

Luciferase reporter assay. Keratinocytes were grown to 50% confluence in a 6-well culture plate, and then co-transfected with reporter adenovirus and A20-expressing adenovirus. After adenoviral transfection for 6 h, cells were replenished with fresh medium. Cells were further incubated for 24-48 h, and luciferase activity was then determined using a Luciferase assay system (Promega, Madison, WI, USA). For the creation of involucrin-luc reporter adenovirus (Ad/Inv-luc) and loricrin-luc reporter adenovirus (Ad/Lor-luc), genomic DNA isolated from keratinocytes was used as a template for PCR, as previously described (17). To measure the effect of A20 on involucrin and loricrin luciferase promoter activities, keratinocytes were transfected with 1 MOI of involucrin-luc or loricrin-luc reporter adenovirus, in which about 3.7 kb of involucrin promoter fragment and 2.0 kb of loricrin promoter fragment were fused to luciferase gene, respectively, as previously described (18), together with A20 expressing adenovirus (10 MOIs). Cells were lysed and luciferase activity was studied.

Statistical analysis. Data were evaluated statistically using one-way analysis of variance (ANOVA) with the SPSS software (v 22.0; IBM, Seoul, Korea). A $P < 0.01$ was considered to indicate a statistically significant different.

Results

Expression of A20 in epidermal keratinocytes. To investigate the expression of A20 in the epidermis, we performed immunohistochemical analysis. We noted that A20 expression was increased in the upper layers of normal epidermis samples and also in 3-dimensional artificial skin (Fig. 1A and B). To further verify the expression of A20, NHEKs were cultured and differentiated using calcium, a best-known keratinocyte differentiation inducer, as previously described (2). After calcium treatment, we noted that the expression of involucrin, a marker of keratinocyte differentiation, was increased in a

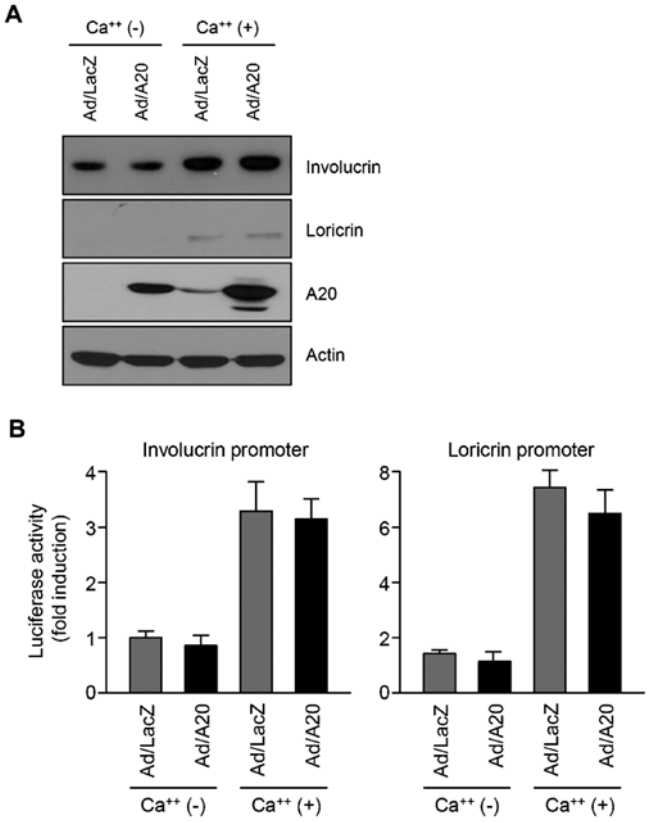

Figure 2. Effect of A20 overexpression on keratinocyte differentiation. (A) Overexpression of A20 did not markedly affect keratinocyte differentiation. (B) Effect of A20 on involucrin and loricrin promoter activities. Data are represented as fold induction and SD, measured in three independent experiments. Overexpression of A20 did not markedly affect the involucrin and loricrin promoter activities.

time-dependent manner, indicating that differentiation was well-induced, although A20 mRNA expression rose until day 7, and it then decreased on day 14. A20 mRNA and protein expression was also increased by calcium in a time-dependent manner (Fig. 1C and D). On the basis of these results, we suggest that A20 plays a role in keratinocyte differentiation.

Effect of A20 on keratinocyte differentiation. Since the expression of A20 was increased during the keratinocyte differentiation process, we examined the putative role of A20 in keratinocyte differentiation. To this end, we overexpressed A20 in NHEKs using a recombinant adenovirus. Overexpression of A20, however, did not markedly affect the protein levels of involucrin and loricrin, in both the absence and presence of calcium (Fig. 2A). Consistent with the data obtained from western blot analysis, overexpression of A20 did not markedly affect the luciferase activity of involucrin or loricrin (Fig. 2B). These data suggest that A20 is not a direct modulator of keratinocyte differentiation.

Effect of A20 on the inflammatory reaction of keratinocytes. Since A20 has been shown to be strongly associated with psoriasis using a GWAS (15), we wondered whether A20 expression was altered in psoriatic samples. Interestingly, immunohistochemical analysis showed that A20 expression was decreased in the psoriatic lesional area, compared to in the non-lesional skin sample (Fig. 3). These data support the theory that A20 is linked to the pathogenesis of psoriasis.

The pivotal role of keratinocytes in the development of psoriasis has been emphasized in the context of the PAMP-induced innate immune response: for example, double-stranded RNA (dsRNA) activates TLR3 and leads to excessive inflammation, which is relevant to psoriasis (13). To investigate whether A20 exerted an effect on the PAMP-induced innate immune response of keratinocytes, we transfected NHEKs with adenovirus-expressing A20 (Ad/A20) and then exposed them to poly(I:C), a dsRNA mimic. Exposure of keratinocytes to poly(I:C) resulted in a marked increase in the levels of inflammatory cytokines and chemokines, namely TNF-α, CCL20, IL-1β, IL-6 and IL-8. As expected, overexpression of A20 significantly inhibited poly(I:C)-induced cytokine production (Fig. 4A). Similar to these results, overexpression of A20 markedly inhibited poly(I:C)-induced phosphorylation of p65 (NF-κB subunit) and consequent NF-κB activation (Fig. 4B and C). These results suggest that downregulation of A20 increased the susceptibility of keratinocytes to PAMPs.

Discussion

A20 was originally identified as a TNF-inducible gene, and previous investigations have demonstrated that A20 is also induced in various cell types and by a wide range of stimuli (19,20). A20 expression is low under basal conditions; however, stimuli activating intracellular NF-κB lead to quick induction of A20 transcription. Once induced, it has been noted that A20 functions as a dual inhibitor of NF-κB activation and cell death. A20 ubiquitinates receptor interacting protein 1 (RIP1), a critical signaling intermediate protein in TNF-mediated NF-κB activation, resulting in proteasomal degradation of RIP1 and termination of NF-κB activation (21). The importance of A20 has been further demonstrated in a mouse model: A20-knockout mice developed severe inflammation and cachexia; they were hypersensitive to TNF and died prematurely. A20-deficient cells fail to terminate TNF-induced NF-κB responses and are also more susceptible to TNF-mediated programmed cell death (22). It has been also demonstrated that A20 is required for terminating NF-κB signaling in response to microbial products such as muramyl dipeptide (23). Several reports have suggested that A20 restricts innate immune signaling in response to viral infection (24-26).

In this study, we demonstrated that A20 was expressed in the epidermis, and an upregulated pattern was noted in the upper layers. Although the expression of A20 was increased in the upper layers, it is unlikely that A20 was directly involved in keratinocyte differentiation. We found that expression of A20 was decreased in the psoriatic lesional area compared to the non-lesional skin samples. This suggests that A20 is linked to the innate immune response of keratinocytes. Previous research supports the primary involvement of keratinocytes in psoriasis, through the recognition of PAMPs by TLRs (27,28). Many PAMPs elicit an inflammatory reaction through the NF-κB signaling cascade (12). Thus, if there is a loss of feedback relating to the NF-κB pathway, the PAMP-induced inflammatory reaction is exacerbated even under low-threshold conditions. In this regard, A20 is an important candidate that is critically involved in the development of psoriasis: we noted

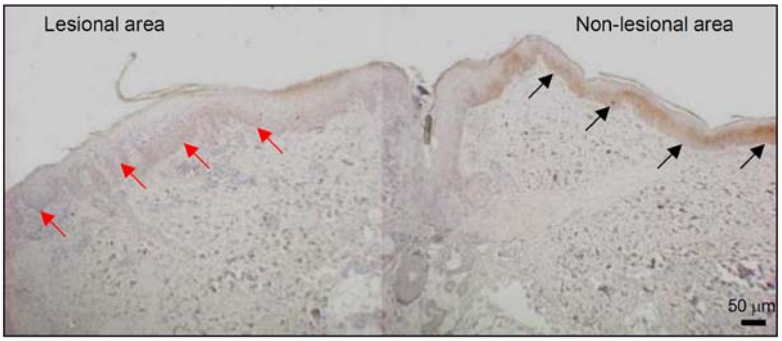

Figure 3. Expression of A20 in psoriatic lesion samples. Immunohistochemical analysis showed that A20 expression was detected in the non-lesional area of the patient with psoriasis (black arrows), but not in the lesional area (red arrows).

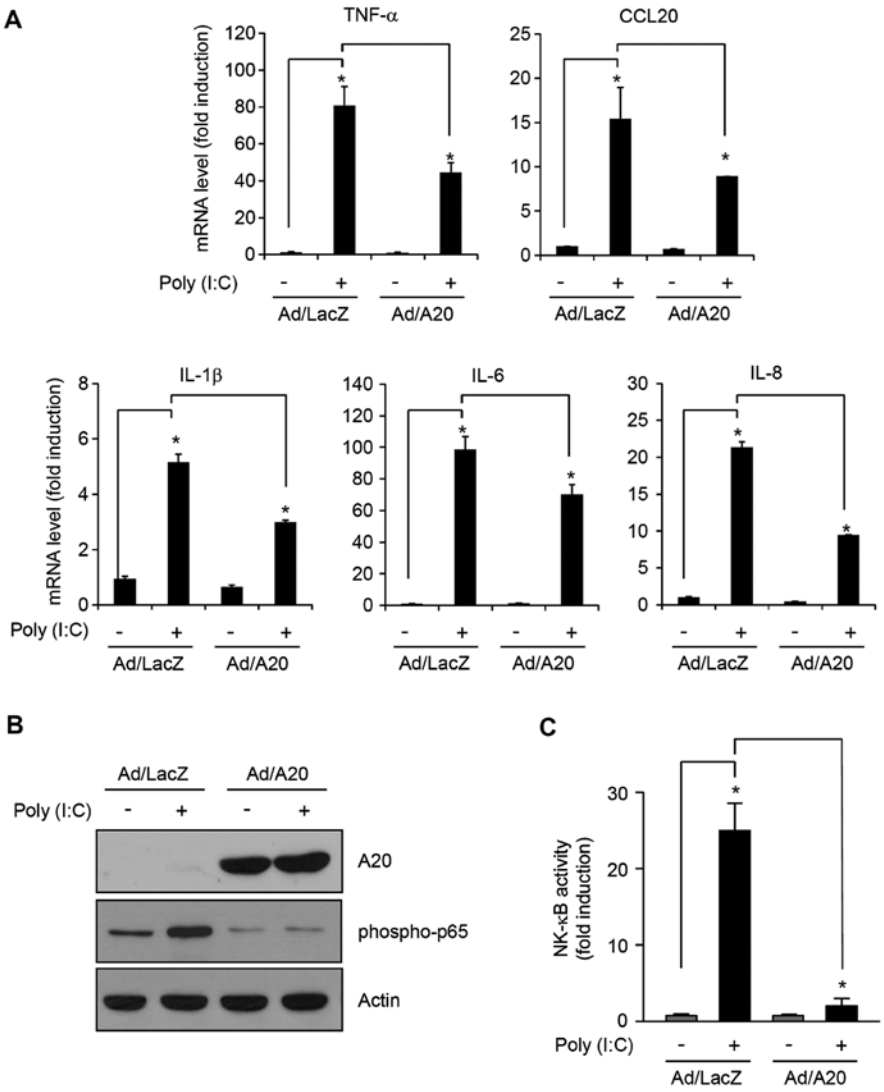

Figure 4. Effect of A20 overexpression on poly(I:C)-induced inflammatory reaction in keratinocytes. (A) Overexpression of A20 significantly inhibits poly(I:C)-induced cytokine induction in keratinocytes. Data are represented as fold induction and SD, measured in three independent experiments. *P<0.01. (B) Effect of A20 overexpression on poly(I:C)-induced nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activation in keratinocytes. (C) Overexpression of A20 significantly inhibits poly(I:C)-induced NF-κB activity. *P<0.01. TNF-α, tumor necrosis factor; CCL20, chemokine (C-C motif) ligand 20; IL, interleukin.

that in normal skin, A20 expression was increased in upper epidermal layers, and we suggest that there is a possibility that A20 decreases commensal-induced innate immunity and inflammatory reaction. This functional role contributes to maintain the non-pathological status of skin. If there is a condition which downregulates A20, it likely causes weakening of feedback regulation of the inflammatory loop in keratinocytes, thus increasing susceptibility to exogenous pathogens. Elucidation

of the regulatory mechanism underlying A20 downregulation in psoriasis will be an interesting future study.

In summary, we demonstrated that A20 was decreased in psoriatic samples, and that A20 decreased the poly(I:C)-induced inflammatory reaction of keratinocytes. Our results contribute to a better understanding of the causes of psoriasis, and may help to develop new targets for psoriasis treatment.

Acknowledgements

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