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ORIGINAL ARTICLE

INHIBITORY EFFECT OF TGF-β ON EXPRESSION OF 230KD BULLOUS PEMPHIGOID ANTIGEN

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Abstract 230-kD bullous pemphigoid antigen (BPAG1) is known as an autoantigen in bullous pemphigoid and is expressed exclusively in proliferating basal keratinocytes. TGF-β is a growth factor that has pleiotropic effects on a wide range of target cells and induces differentiation of basal keratinocytes. Therefore, TGF-β is postulated to inhibit BPAG1 expression. However, previous report conversely demonstrated an increase of BPAG1 expression by TGF-β. In this study, to understand regulatory role of TGF-β on BPAG1 functions, we examined the effect of TGF-β on BPAG1 gene expression using cultured keratinocytes. This study showed that BPAG1 mRNA expression was inhibited by TGF-β1 in concentration higher than 1.0 ng/ml. Furthermore, incubation of the cells with TGF-β1 in the presence of cycloheximide demonstrated that newly synthesized protein was required for BPAG1 regulation. To understand the detailed mechanisms of BPAG1 modulation by TGF-β, we preformed transient transfection assay with a BPAG1 promoter-CAT construct to know the detailed mechanisms of BPAG1 modulation by TGF-β. The results revealed that calcium and IFN-γ inhibited BPAG1 expression at transcriptional level, but TGF-β1 is not responsible for that transcriptional inhibition, suggesting that TGF-β may have differential molecular mechanism for down-regulation of BPAG1 gene expression from the events induced by IFN-γ.
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Key words: Keratinocyte; BPAG1; TGF-β; IFN-γ.

原 著

230-kD 類天疱瘡抗原発現に対する TGF-β の抑制効果

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抄録 230-kD 類天疱瘡抗原（BPAG1）は類天疱瘡の自己抗原として知られ，増殖する基底細胞にのみに発現する．TGF-βはいろいろな標的細胞に多彩な効果を示す増殖因子であり，基底細胞にはその分化を誘導する．そのため，TGF-βはBPAG1の発現を抑制すると予想されるが，過去の報告は逆にその発現を促進することを示している．本研究では，BPAG1の発現に対するTGF-βの効果を明らかにするため，その効果を培養表皮細胞にて検討した．その結果，BPAG1の遺伝子発現は，TGF-β1 1.0 ng/ml以上の濃度で抑制された．さらに，cycloheximideの存在下で細胞をTGF-β1処理すると抑制がみられなくなることから，TGF-βの効果には蛋白の新たな合成が必要であることが示された．さらに，TGF-βによるBPAG1調節の詳細なメカニズムを明らかにするため，BPAG1 promoter-CAT遺伝子を一過性に細胞に導入し，プロモーター活性に与える影響を検討した結果，カルシウムやIFN-γのBPAG1発現抑制は転写レベルで起こっているが，TGF-βのそれは転写の抑制を介していないことが示された．これらの結果から，TGF-βはIFN-γとは異なる分子メカニズムにより，BPAG1の遺伝子発現を抑制することが示唆された．
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キーワード：表皮細胞；BPAG1；TGF-β；IFN-γ.

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Introduction

Epidermal keratinocytes move upward through the epidermis after losing their attachment to the basal lamina in terminal differentiation. This differentiation of epidermal keratinocytes from basal layer to horny layer is regulated by activation and inactivation of a variety of genes at different levels of the epidermis. The first step of the differentiation consists of three parts including growth arrest of basal cells, inhibition of specific gene expression in basal cells, and induction of differentiation specific gene expression.

230-kD bullous pemphigoid antigen (BPAG1) belongs to the plakin family of proteins that crosslink the cytoskeleton to each other and connects it to cell junctions. BPAG1 is known as an autoantigen in a blistering skin disease, bullous pemphigoid and well characterized as a component of a hemidesmosome, which anchor proliferating basal keratinocytes on the basement membrane of the skin[1-3]. BPAG1 is expressed exclusively in proliferating basal keratinocytes while BPAG1 expression is terminated when basal keratinocytes start to differentiate into suprabasal keratinocytes.

TGF-β is a protein of 25,000 daltons, and composed of two 12,500-dalton subunits held together by disulfide bonds[4]. TGF-β is a growth factor that has pleiotropic effects on a wide range of target cells. The TGF-β family is composed of TGF-β1, β2, and β3 and the biological activities of these molecules are known to be very similar. For the skin, it is well known that TGF-β induces the synthesis of ECM proteins including collagens and fibronectin, and inhibits the synthesis of ECM-degrading enzymes[5]. Many in vitro and vivo studies have demonstrated that TGF-β enhances differentiation of basal keratinocytes, resulting in enhancement of keratinization and suppression of growth[6-9].

To understand regulatory role of TGF-β on BPAG1 functions, we examined the effect of TGF-β on BPAG1 gene expression. Furthermore, we performed transient transfection with a BPAG1 promoter-CAT construct to know the detailed mechanisms of BPAG1 modulation by TGF-β.

Methods

Keratinocytes Cultures

Human epidermal keratinocyte cultures were established from neonatal foreskin (Cascade Biologics, Inc.). The cultures were maintained in serum-free, low calcium (0.09mM) keratinocyte growth medium supplemented with epidermal growth factor, L-leucine, L-proline, phenol red, $CaCl_2 \cdot H_2O$, L-methionine, sodium phosphate dibasic, hydrocortisone, insulin, T-3, and bovine pituitary extract (KERATINOCYTE-SFM, GIBCO). Cultures were passaged by trypsinization and studied in passage 2. Some cultures were incubated with TGF-β1 from human platelets, which were purchased from COSMO BIO, Inc. Japan. The lyophilized TGF-β1 was dissolved in 5 mM hydrochloric acid in use.

Reverse transcriptase-polymerase chain reaction (RT-PCR) method

The cells were incubated with TGF-β1 at concentrations varying from 0 to 10 ng/ml for 24 hours. Total RNA was then isolated by the Phenol/chloroform method and cDNA was synthesized by reverse transcriptase with random heximer primer, using 1st-Strand^{TM} cDNA Synthesis Kit (CLONTECH) .

The synthesized cDNA was used as a template in PCR. PCR conditions were following; denaturation at 94℃ for 1min, annealing at 55℃ for 1 min and extension at 72℃ for 1 min. The primer sequences were 5'-TAAGCCCTTC TTAAGGAAATC-3' (exon 21, sense primer) and 5'-CAGATAACTTTTGA TGAGACA-3' (exon 22, antisense primer) according to the sequence of BPAG1 gene[10]. As

control, the primers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used, 5'-TGAAGGTCGGAGTCA ACGGATTTGGT-3' (sense primer) and 5' -TGAAGGTCGGAGTCACGGAT TTGGT-3' (antisense primer). PCR products were analyzed by 2% agalose gel electrophoresis.

Southern analyses

After the electrophoresis of the PCR products, the gels were alkalized twice in 0.2 M NaOH / 0.6M NaOH for 40 minutes, and neutralyzed in 0.2 M Tris-HCl pH 7.5 / 0.6 M NaCl for 30 minutes. DNA in the gels was transferred to nitrocellulose filters (Zeta-Probe Blotting Membrane, BIORAD). The filters were prehybridized in 50% formamide / 0.25 M sodium dihydrogenphosphate / 0.25 M NaCl / 7% sodium dodecyl sulfate / 0.001 M ethylenediami netetraacetic acid at 42℃ for 12 hours, and then hybridized with a 1.7-kb human BPAG1 cDNA radioactively labeled with [α-^{32}P] ATP at 37℃ for 24 hours. Then the filters were washed twice in 2 x SSC / 0.1% sodium dodecyl sulfate for 15 minutes and exposed to X-ray films (X-Omat, Eastman Kodak Co.). The signals were scanned by densitometer (DENSITRON MODEL-PAN, JOKO, Tokyo, Japan) for quantitative analysis.

Chloramphenicol acetyltransferase (CAT) assay

The human BPAG1 promoter-CAT reporter gene plasmid, pBP2.6CAT, containing the human BPAG1 promoter region extending from positions -2536 to -1 (in relation to the transcription initiation site[10] was used for transient transfection of cultured keratinocytes. The transfections were performed with a commercial kit (DOTAP), and TGF-β1, IFN-γ, and $CaCl_2$ were added to some of the cultures at the time of the transfection.

Results

Determination of PCR cycles for semi-quantitative RT-PCR

PCR cycles were determined for quantitative RT-PCR. Ten μl of PCR products from the reaction mixture of various PCR cycles (0, 10, 20, 30, 40, 50 cycles) were electrophoresed. After each product was Southern-hybridized, they were analyzed quantitatively by densitometer and relative amount of each product were determined. 30 cycles for BPAG1 and 20 cycles for GAPDH were suitable for semi-quantitative analysis for following experiments (data not shown).

Dose-dependent down regulation of BPAG1 mRNA expression by TGF-β1 treatment

To examine the effects of TGF-β1 on BPAG1 mRNA expression, cultured human epidermal keratinocytes were incubated with TGF-β1 of varying concentrations for 24 hours, and the corresponding mRNA levels were determined by RT-PCR and Southern hybridizations (Fig.1A). BPAG1 mRNA expression was dose-dependently down regulated by TGF-β1, (Fig.1B). GAPDH mRNA expression was essentially the same levels in the samples of various TGF-β1 concentration.

Time-dependent down regulation of BPAG1 mRNA by TGF-β1 treatment

To examine the time course of the inhibition of BPAG1 mRNA expression by TGF-β1, cultured human epidermal keratinocytes were incubated with 10 ng/ml of TGF-β1 for various incubation times. The corresponding mRNA levels were determined by the same protocol as described above (Fig.2). Time-dependent inhibition of BPAG1 mRNA expression was observed (Fig.2).

Requirement of on-going protein synthesis for the TGF-β1 effects

To examine whether the TGF-β1-elicited down-regulation of the BPAG1 mRNA required newly synthesized proteins, similar inhibition

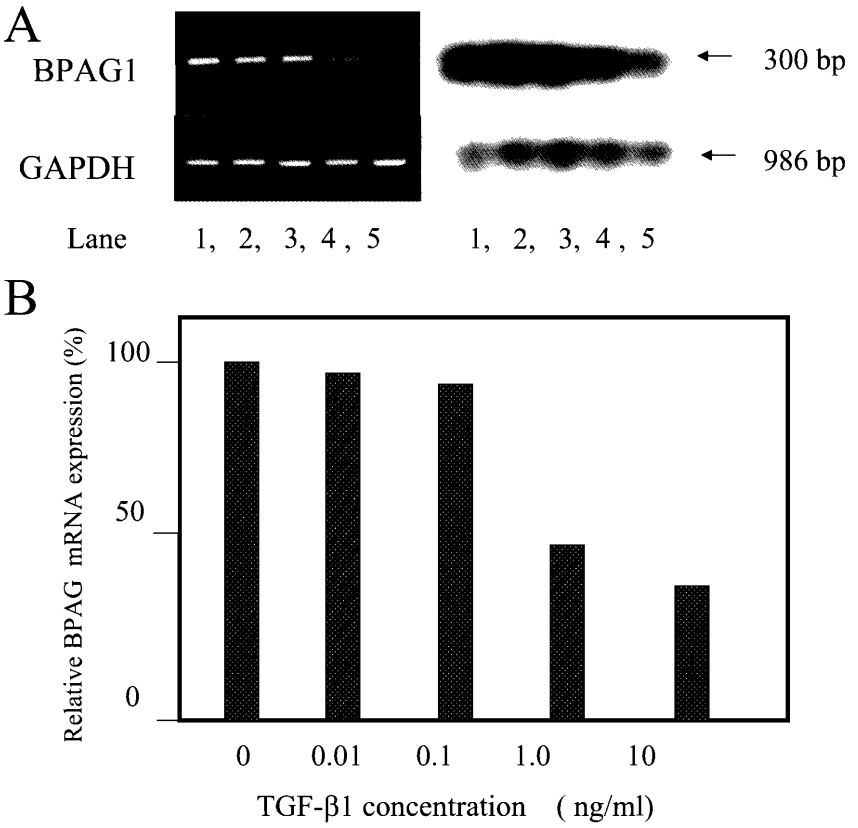

Fig. 1 Dose-dependent down regulation of BPAG1 mRNA expression by TGF-β1.
To examine the effects of TGF-β1 on BPAG1 mRNA expression, cultured human epidermal keratinocytes were incubated with TGF-β1 of varying concentrations for 24 hours, and the corresponding mRNA levels were determined by RT-PCR and Southern hybridizations (A). TGF-β concentrations: lane 1; 0 ng/ml, 2; 0.01 ng/ml, 3; 0.1 ng/ml, 4; 1.0 ng/ml, 5; 10 ng/ml. The densitometer analysis showed that the inhibition was found in the presence of 1.0 and 10 ng/ml of TGF-β1 (B).

studies were performed in the presence and absence of cycloheximide (10μg/ml). Incubation of cultured keratinocytes with TGF-β1 (1.0 ng/ml) for 24 hours resulted in essentially more than 50% inhibition of BPAG1 mRNA steady state-level (Fig.3). Incubation of keratinocytes with cycloheximide alone resulted in about 30% inhibition of BPAG1 mRNA steady state-level. On the contrary, cycloheximide reduced inhibitory effect of TGF-β1 on the BPAG1 mRNA expression (Fig.3). GAPDH expression was essentially not altered by cycloheximide as well as TGF-β1 (data not shown).

Evidence of transcriptional regulation of BPAG1 gene expression by TGF-β1

To examine the potential mechanisms that might be responsible for TGF-β1-elicited down-regulation of BPAG1 gene expression as detected at the mRNA level, transient transfections with a BPAG1 promoter-CAT construct were performed in cultured keratinocytes. This construct consists of a 2.6 kb of 5'- flanking DNA of the BPAG1 gene, a segment that has been previously shown to contain cis-elements conferring tissue-specific expression of the gene in keratinocytes[10]. Transfection of control keratinocytes with this plasmid construct revealed strong activity of the promoter, as detected by CAT assay (Fig.4). Thus, using this transfection system, we examined

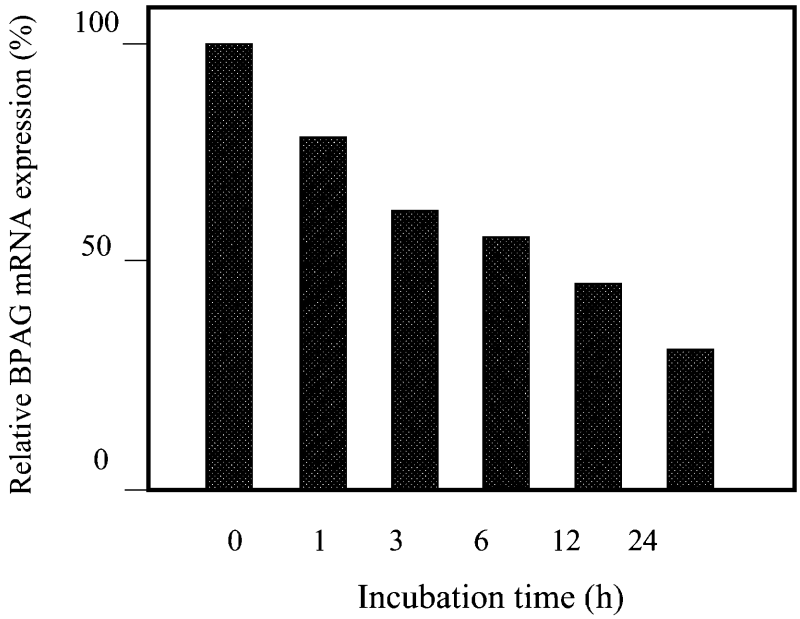

Fig. 2 Time-dependent down regulation of BPAG1 mRNA by TGF-β1.
To examine the time course of the inhibition of BPAG1 mRNA expression by TGF-β1, cultured human epidermal keratinocytes were incubated with 10 ng/ml of TGF-β1 for various incubation times. The densitometer analysis showed the inhibition of BPAG1 mRNA expression was time-dependent.

effect of TGF-β1 on promoter activity of BPAG1 gene.

TGF-β1 treatment failed to exhibit transcriptional down regulation of the BPAG1 promoter, while Ca^{2+} (2.0mM) or IFN-γ (100 U/ml) treatment resulted in inhibition of promoter activity about 40% and 10% of the control, respectively.

Discussion

Epidermis is composed of several cell types of different features morphologically and functionally. Among those, keratinocyte is a major constituent. Epidermis is formed by proliferation of mitotic basal cells and by consecutive differentiation. Homeostasis of the epidermis is maintained by control of the proliferative ability of keratinocytes, but precise molecular mechanism of proliferative regulation in vivo still remains to be clarified.

TGF-β1 has been shown to regulate epidermal keratinocyte growth and differentiation. Until now, very few study of TGF-β1 for BPAG1 gene expression has been reported[11]. They examined expression of BPAG1, BPAG2 and β4-integrin, which are important structural proteins for basal keratinocytes. Interestingly, the results indicated detectable expression of all three genes in normal keratinocytes, whereas extremely low or undetectable levels of expression were noted in two transformed cell lines. Furthermore, addition of TGF-β up-regulated mRNA levels for all three proteins.

It was shown that TGF-β1 caused normal human and murine epidermal keratinocytes to arrest growth predominantly in the G1 phase of the cell cycle[12]. Transgenic mice where K14 promoter drove TGF-β1 gene showed hyperkeratosis, hypogranulosis, and epidermal thinning[8]. Recently, we introduced the TGF-β gene into keratinocytes using the HMG-1-DNA injection method, and we found hyperkeratosis

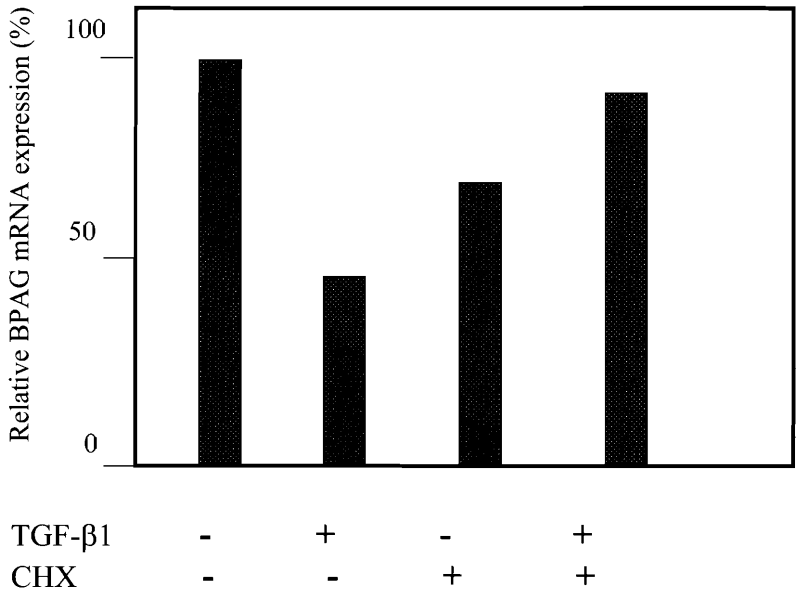

Fig. 3 Requirement of on-going protein synthesis for the TGF-β1 effect.
To examine whether the TGF-β1-elicited down-regulation of the BPAG1 mRNA was dependent on on-going protein synthesis, similar inhibition studies were performed in the presence and absence of cycloheximide. Incubation of parallel cultures with cycloheximide (CHX) alone resulted in inhibition of BPAG1 mRNA. However, the adition of TGF-β1 failed to down-regulate the BPAG1 mRNA levels in the presence of CHX.

and hypogranulosis[9]. Those results clearly indicate that TGF-β may induce keratinocyte differentiation from basal cells. In this regard, TGF-β is postulated to down-regulate expression of BPAG1, which is expressed exclusively in basal keratinocytes.

In this study, we examined the effects of TGF-β1 on BPAG1 gene expression in cultured normal keratinocytes. Since the biological activities of TGF-β1, β2, and β3 are known to be very similar, this study examined only main molecule TGF-β1. Here, we showed that BPAG1 mRNA expression was inhibited by TGF-β1 in concentration higher than 1.0 ng/ml. Also, previous reports demonstrated that growth of cultured human keratinocytes was inhibited significantly in the medium containing TGF-β1 at a dose higher than 2.0 ng/ml[7]. These suggest that BPAG1 expression is relating to the regulation of keratinocytes proliferation.

We have previously shown that calcium and IFN-γ have similar effects to TGF-β1 as regards to inhibition of BPAG1 mRNA expression[3,10]. It was shown that IFN-γ inhibited the BPAG1 expression at both mRNA level and promoter level, and the effects of IFN-γ was shown to require newly synthesis of protein. In this study, incubation of the cells with TGF-β1 in the presence of cycloheximide also demonstrated that newly synthesis of protein was required for BPAG1 regulation. As indicated in Fig.5, calcium and IFN-γ inhibited promoter activity of the BPAG1 gene, but TGF-β1 is not responsible for the inhibition, suggesting that TGF-β1 may give down-regulation of BPAG1 gene expression mediated by different pathway of IFN-γ.

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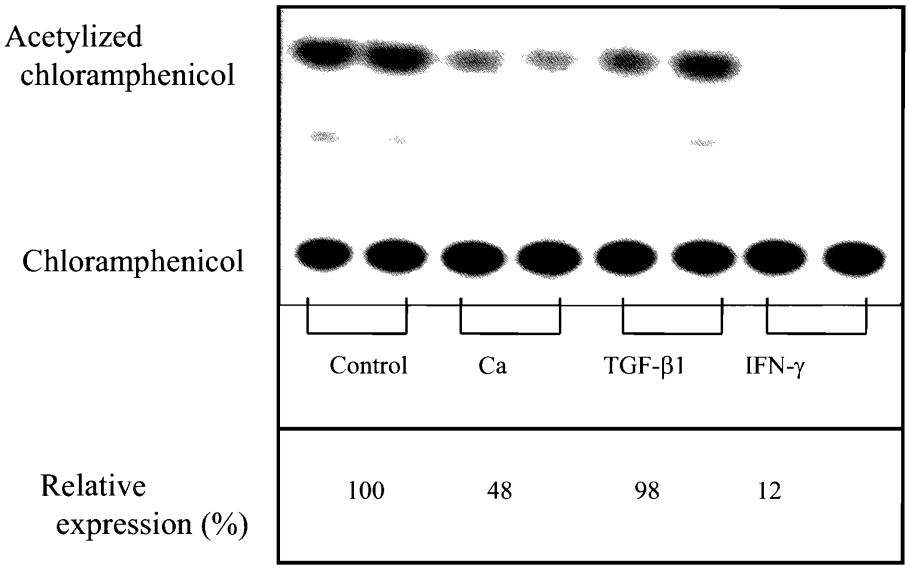

Fig. 4 Evidence of transcriptional regulation of BPAG1 gene expression by TGF-β1. Transient transfections with a BPAG1 promoter-CAT construct were performed in cultured keratinocytes. Promoter activity was measured by acetylation of chloramphenicol. Calcium and IFN-γ inhibited BPAG1 expression in transcriptional level, but TGF-β1 did not evidently inhibited it.

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