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Regulation of CCAAT/enhancer Binding Protein-alpha in Ultraviolet B Responses Involves the Cooperation of p53 and Glycogen Synthase Kinase-3

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자외선 B 조사시 p53와 glycogen synthase kinase-3에 의한 CCAAT/enhancer binding protein alpha의 발현조절

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요 약

태양광선, 특히 자외선 B에 대한 환경적 노출은 편평세포암과 기저세포암을 포함하는 흑색선종 이외의 피부암과 크게 관련된다고 알려져 있다. 염기 류신 지퍼계 전사조절인자인 CCAAT/enhancer binding protein-alpha는 표피 각질형성세포에서 다량으로 발현되었고, 각질형성세포의 증식을 억제하며 피부암 발생을 억제하는 유전자로서의 역할이 암시된 바 있다. 최근 자외선 B가 각질형성세포에서 p53에 의한 CCAAT/enhancer binding protein-alpha의 발현을 강력하게 유도한다는 것이 보고되었다. 이러한 CCAAT/enhancer binding protein-alpha 단백질 발현의 유도는 세포 성장 억제, 세포고사와 함께 일어났다. 이 연구는 glycogen synthase kinase-3 길항제가 자외선 B에 의한 CCAAT/enhancer binding protein-alpha 유도를 억제하며 변이 kinase-불활성 GSK의 강제 발현은 자외선 B가 CCAAT/enhancer binding protein-alpha 전사조절부위 활성의 증가를 억제한다는 것을 보여주었다. 즉 자외선 B에 의한 CCAAT/enhancer binding protein-alpha의 유도가 p53과 활성 glycogen synthase kinase-3에 의한 것이라는 것을 증명하였다.

Key words : ultraviolet B, CCAAT/enhancer binding proteins, keratinocytes, p53, glycogen synthase kinase

INTRODUCTION

CCAAT/Enhancer Binding Proteins (C/EBPs) are basic leucine zipper class of transcription factors and the genes for six C/EBP members (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ) have been identified (Ramji and Foka, 2002). C/EBPα is abundantly expressed in terminally differentiated cells and is involved in mitotic growth arrest and/or differentiation in numerous cell types, including keratinocytes (Umek *et al.*, 1991; Diehl *et al.*, 1996; Radomska *et al.*, 1998; Zhu *et al.*, 1999). The antip-

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roliferative function of C/EBPα is multifaceted and involves the regulation of cell cycle regulatory proteins mainly in G_1 such as p21, E2F, retinoblastoma proteins, cyclin-dependent kinase 4 (cdk4) and cdk2 by its transcription activity and/or direct interaction (Timchenko *et al.*, 1999; Timchenko *et al.*, 1999; Porse *et al.*, 2001; Wang *et al.*, 2001). Moreover, the antiproliferative activity of C/EBPα was shown to require a SWI/SNF chromatin remodeling complex, supporting a transcriptional basis for its antiproliferative activity (Muller *et al.*, 2004). More recently, it has been shown that C/EBPα has a role in the DNA damage-induced G_1 checkpoint (Yoon and Smart, 2004). The importance of C/EBPα in the regulation of growth arrest and differentiation was highlighted in the studies of human acute myeloid leukemia in which C/EBPα was implicated as a human tumor suppressor gene (Wang *et al.*, 1999). C/EBPα expression is also reduced in hepatocellular carcinomas (Tomizawa *et al.*, 2002), lung cancer, and lung cancer cell lines (Halmos *et al.*, 2002), supporting a possible tumor suppressor function in these organs. It has been observed that C/EBPα protein levels were greatly diminished or undetectable in mouse skin squamous cell carcinomas (SCCs) and mouse skin SCC cell lines and reintroduction of C/EBPα in SCC cells inhibited cell proliferation (Shim *et al.*, 2005). Taken together, it appears that the loss of C/EBPα expression may contribute to the altered growth characteristics of skin SCCs.

Skin cancer is the most prevalent form of human cancer and is caused by exposure to solar radiation, primarily ultraviolet B (UVB). UVB radiation (280~315 nm wavelengths) is responsible for approximately one million nonmelanoma skin cancer cases in the United States each year and it accounts for nearly 50% of the total annual cancer incidence (Miller and Weinstock, 1994). UVB radiation has DNA damaging effects and can also produce reactive oxygen species (Brash, 1997). UV-induced DNA damages activate cell cycle checkpoint pathway and DNA repair system, and induce apoptosis depending on the dose (Neades *et al.*, 1998; Ouhtit *et al.*, 2000). UVB is known to produce an increase in p53 protein levels. p53 is considered to play a critical role in the cellular response to UVB and the abrogation of p53 function is important in UVB-induced skin cancer (Brash *et al.*, 1991; Zhan *et al.*, 1993). However, UVB can also induce apoptosis in p53-null cells although at a reduced level compared to wild type cells and UVB effectively induces mitotic growth arrest in p21-null embryonic fibroblasts (Ziegler *et al.*, 1994; Neades *et al.*, 1998). Thus, the exact role of specific p53 inducible proteins as well as p53 independent events in UVB-responses is of interest. Recently, it has been discovered that C/EBPα protein and mRNA in keratinocytes was potently induced by UVB in a p53-dependent manner (Yoon and Smart, 2004). However, it is still questionable that p53 directly activate C/EBPα transcription upon UVB irradiation or indirectly induces C/EBPα through interaction with other signaling pathways or modulating intermediate molecules.

Studies have identified a novel link between p53 activation and the inhibition of WNT signaling pathway. p53 inducible proteins, Siah-1 and Dkk-1 inhibit WNT/β-catenin signaling by enhancing the degradation of β-catenin or by functioning as WNT antagonist by interfering co-receptor Lrp-6. Importantly, genotoxic stresses such as UVB or adriamycin treatment inhibit WNT/β-catenin signaling through activation of p53 and subsequent induction of Siah-1 and Dkk-1 (Matsuzawa and Reed, 2001; Sadot *et al.*, 2001; Grotewold and Ruther, 2002). GSK3 has a major role in blocking WNT pathway by regulating the proto-oncogene, β-catenin. Phosphorylation of β-catenin by GSK3 targets β-catenin for ptoteosomal degradation. Activation of WNT signaling results in the inhibition of GSK3 activity and stabilization and accumulation of cytosolic β-catenin levels. β-catenin then translocates to the nucleus where it cooperates with the TCF/LEF transcription factor to alter gene expression (Cohen and Frame, 2001; Frame and Cohen, 2001). And thus, GSK inhibitors have been reported to function as WNT signaling mimics in the cell. Studies in 3T3-L1 preadipocytes have demon-

strated that expression of WNT or a stabilized mutant β-catenin or treatment with GSK3 inhibitors block C/EBPα mRNA and protein expression and adipocyte differentiation (Ross *et al.*, 2000). The present study is to determine if p53 induces C/EBPα expression through inhibition of WNT signaling pathway.

MATERIALS AND METHODS

1. Cell culture and UVB irradiation

BALB/MK2 keratinocytes were cultured in Ca^{2+}-free Eagle's minimal essential medium (BioWhittaker) supplemented with 8% Chelex-treated fetal bovine serum (Invitrogen), 4 ng of human epidermal growth factor/mL (Invitrogen), and 0.05 mM calcium. The UVB lamp (Spectronics) emits wavelengths between 280 to 320 nm with a spectrum peak at 312 nm. The light intensity of the lamp was measured by the IL-1700 Research Radiometer (International Light) equipped with a SED 240 sensor. The UVB lamp was positioned 15 cm above the cells. Cells were washed with PBS and irradiated in the presence of PBS for the amount of time corresponding to the indicated UVB dose. After irradiation, PBS was removed and replaced with the specified medium.

2. Western blot analysis

Cells were harvested and lysed in hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail [Roche], 1 mM sodium orthovanadate with 0.6% NP-40) by sonication, and then a 1/10 volume of 5 M NaCl was added. The cell lysates were incubated for 15 min on ice, centrifuged at 14,000×g for 10 min and stored at −80°C until use. Equal amounts of protein (20~30 μg) were precipitated by the addition of an equal volume of 20% trichloroacetic acid and washed with cold acetone. Protein samples were dissolved in sodium dodecyl sulfate (SDS) sample buffer, subjected to SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer, the membranes were probed with rabbit polyclonal immunoglobulin G raised against C/EBPα (sc-61). The membranes were washed and then probed with a horseradish peroxidase-linked secondary antibody and detection was made with an enhanced chemiluminescence reagent (Amersham). Membranes were stained with Coomassie blue to confirm equal protein loading.

3. Construction of C/EBPα promoter-reporter and luciferase assay

The 2.9 Kb region of the C/EBPα promoter was amplified by PCR and inserted into the pGL3-basic vector (Promega) using KpnI and XhoI sites. Colonies were screened by restriction enzyme analysis. BALB/MK2 keratinocytes were plated and transfected 24 h later with Tfx-10 (Promega) with the pGL3-promoter-reporter containing the C/EBPα promoter (C/EBPα-pGL3), the pCMV-p53 or pCMV control vector. Cells were harvested 24 h after transfection, and luciferase activity was determined.

4. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were blocked with normal goat serum and incubated with the anti-C/EBPα antibody at 4°C overnight and Texas Red-conjugated goat anti-rabbit IgG at room temperature for 1 hour. Detection was made with the fluorescence microscope.

5. Thymidine incorporation assay

Cells were pulse-labeled with 3 μCi of [^{3}H-methyl]thymidine per ml (20 Ci/mmol) for 1 h before collection. Cells were collected by trypsinization, resuspended in 1 mM EDTA buffer, and sonicated; aliquots were collected onto glass fiber filters; and the filters were placed in a liquid scintillation cock-

tail and subjected to scintillation counting. For DNA quantitation, an aliquot of each sample and 5 μL of 0.1 mg of Hoechst 33258 solution/ml were mixed in 1 mL of 0.01 M Tris (pH 7.0)-0.1 M NaCl-0.01 M EDTA buffer. The fluorescence units were determined with a fluorometer (excitation at 365 nm and emission at 450 nm).

6. Caspase 3/7 assay

Cells were plated in fluorescence-readable 96-well plates and grown in keratinocyte medium. The Apo-ONE Homogeneous Caspase-3/7 assay (Promega) was used to measure apoptosis, following the manufacturer's protocol. Caspase 3/7 activity was normalized to the total amount of protein.

RESULTS AND DISCUSSION

To begin to determine whether C/EBPα is involved in the keratinocytes' response to UVB, BALB/MK2 keratinocytes, a nontransformed immortalized mouse cell line, were irradiated with a single dose of UVB (10 mJ/cm^2). As shown in Fig. 1A, immunofluoscence staining for C/EBPα revealed a very large increase of C/EBPα in the nucleus at 18 h post-UVB treatment. UVB treatment of keratinocytes is known to be a potent inducer of mitotic growth arrest and depending upon the dose, apoptosis. UVB treatment (10 mJ/cm^2) was a potent inhibitor of BALB/MK2 keratinocyte proliferation as ^{3}H-thymidine incorporation into DNA (1 hr pulse) was decreased by 82% at 14 hours after UVB treatment (Fig. 1B). In addition, there was a 3~7 fold increase in caspase 3/7 activity at 10 and 20 mJ/cm^2 UVB treatment after 24 hr (Fig. 1C). These results are consistent with the observation that cell density was less in UVB-irradiated group compared to control (Fig. 1A) and suggest that the increase in C/EBPα protein levels are temporally related to growth arrest and apoptosis.

UVB can activate a variety of MAP kinase pathways including p38, JNK, ERK1/2. In addition, UVB stimulate IGF and EGFR signaling resulting in the

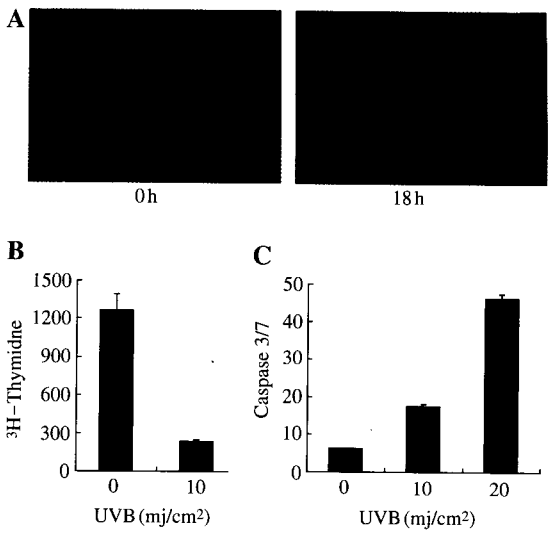

Fig. 1. C/EBPα induction by UVB is temporally related to growth arrest and apoptosis. A. BALB/MK2 cells were irradiation with UVB 10 mJ/cm^2. At indicated time after UVB, cells were stained for C/EBPα. Photographs were taken at a magnification of ×400. B. 14 hr after UVB irradiation, cells were pulse-labeled with [^{3}H-methyl]thymidine for 1 h and the amount of [^{3}H-methyl]thymidine incorporated into DNA was determined. C. Keratinocytes were irradiated with UVB (10 mJ/cm^2) and twenty four hours later, caspase 3/7 activity was measured. Data are expressed as the means ± standard deviation of a representative experiment done in triplicate.

activation of PI3K and Akt (Kulik *et al.*, 1997; Ramaswamy *et al.*, 1998). UVB activation of one or more of these pathways may be responsible for the upregulation of C/EBPα. It was found that UVB induction of C/EBPα was not affected by treatment with pharmacological inhibitors of EGFR (PD153035), JNK (JNK inhibitor II) and MEK1/2 (PD98059) (Fig. 2). However, induction of C/EBPα by UVB was effectively blocked by GSK3 inhibitors, lithium chloride and SB216763 (Fig. 3A). Lithium chloride also produced a 5-fold increase in TCF/ LEF reporter activity demonstrating that GSK inhibitors stimulate TCF/LEF transcription activity in keratinocytes at the same dose and time point as it inhibit UVB-induction of C/EBPα while wild type β-catenin, a positive control, activated transcription activity by 3

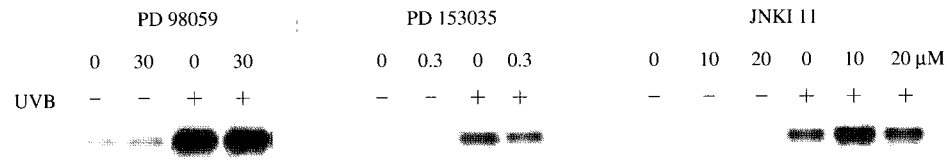

Fig. 2. UVB induction of C/EBPα was not affected by treatment with pharmacological inhibitors of EGFR (PD153035), JNK (JNK inhibitor II) and MEK1/2 (PD98059). Balb/MK2 cells were pretreated with indicated doses of inhibitors for 0.5~1 hr and irradiated with 10 mJ/cm^2 UVB. Then cells were further incubated for 12 hr in the presence of inhibitors specified. Whole cell lysates were prepared for Western blot analysis.

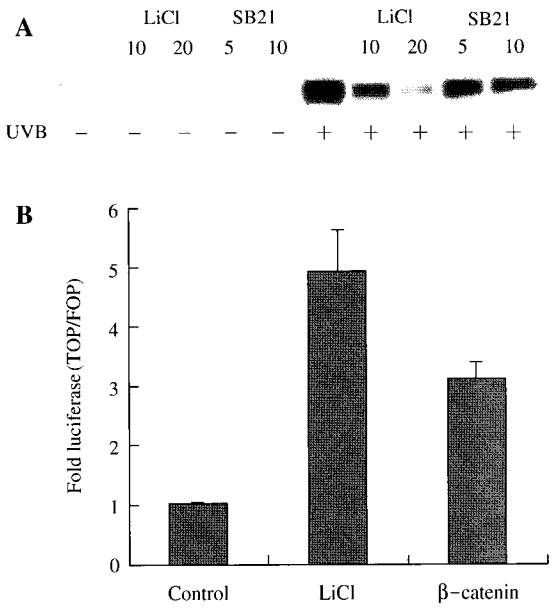

Fig. 3. UVB-induction of C/EBPα was effectively blocked by GSK3 inhibitors. A. Cells were pretreated with 10 or 20 mM LiCl or 5 or 10 μM SB216763 for one hour, and irradiated with UVB at the dose of 10 mJ/cm^2. B. BALB/MK2 cells were transfected with TCF reporter (TOPflash) or mutant TCF site containing reporter (FOPflash) with or without wild-type β-catenin expression vector. 20 mM LiCl were treated 24 hours after transfection in groups without β-catenin transfection. Cells were further incubated for 12 hr and luciferase assay was conducted. Data are expressed as the means±standard deviation of a representative experiment done in triplicate.

fold (Fig. 3B). In addition, it has been recently discovered that p53 is required for UVB-induction of C/EBPα as exposure of p53-null or mutant p53 containing keratinocytes to UVB fails to induce C/EBPα (Yoon and Smart, 2004). Collectively, our results indicate that UVB-induction of C/EBPα is dependent on the presence of catalytically active GSK as well as p53.

Two pathways have been identified that link p53 and active GSK in the cellular response to DNA damage. One pathway involves the formation of a p53/nuclear GSK complex which functions as a multiprotein complex to regulate cellular responses to DNA damage (Watcharasit *et al.*, 2002). The other pathway involves p53-dependent inhibition of WNT signaling pathway which ultimately regulates LEF/TCF transcription activity. Activation of WNT signaling results in the inhibition of GSK3 activity and stabilization and accumulation of cytosolic β-catenin levels. β-catenin then translocates to the nucleus where it cooperates with the TCF/LEF transcription factor to alter gene expression (Matsuzawa and Reed, 2001; Sadot *et al.*, 2001; Grotewold and Ruther, 2002), possibly repressing C/EBPα expression. If the WNT signaling pathway suppresses C/EBPα gene expression in keratinocytes, UVB-p53-induced repression of WNT signaling pathway could result in de-repression of C/EBPα expression. Consistent with the ideas, UVB inhibited basal TCF/LEF reporter activity (Fig. 4A). In addition, p53 completely abrogated β-catenin-induced TCF/LEF promoter activity in keratinocytes as shown in Fig. 4B, suggesting that the UVB-p53 induces C/EBPα through inhibition of WNT/β-catenin signaling pathway which normally repressed the expression of C/EBPα.

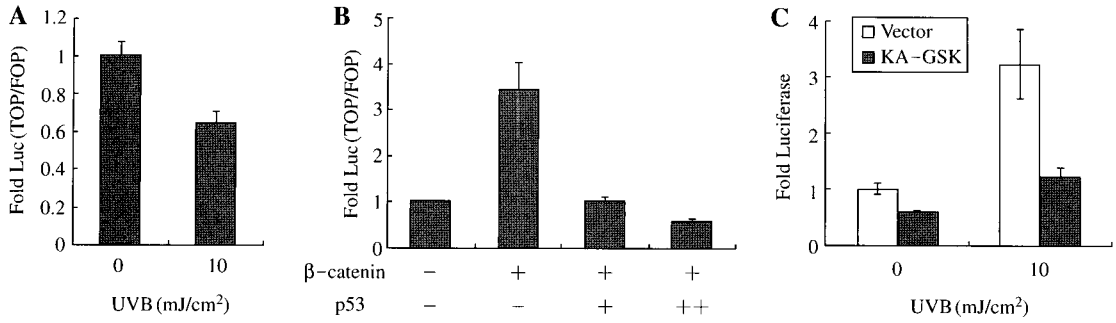

Fig. 4. UVB-induction of C/EBPα is dependent on the presence of catalytically active GSK as well as p53. A. BALB/MK2 cells were transfected with TOPflash vector or FOPflash and 24 hr after transfection, cells were irradiated with 10 mJ/cm^2 UVB. 12 hr after irradiation, luciferase assay was conducted. B. BALB/MK2 cells were transfected with TOPflash vector or FOPflash and 24 hr after transfection, cells were irradiated with 10 mJ/cm^2 UVB. 12 hr after irradiation, luciferase assay was conducted.BALB/MK2 cells were transfected with TOPflash vector or FOPflash along with b-catenin and/or p53 expression vectors (0.3 μg or 0.6 μg/6 well plate) and 36 hr after transfection, cells were harvested. C. BALB/MK2 cells were transfected with C/EPBα-pGL3 basic, which contains the 2.9 Kb proximal region of C/EBPα promoter including p53 sites and LEF/TCF binding sites, or empty pGL3 basic with or without kinase kinase inactive GSK-3β expression vector (KA-GSK). 24 hr after transfection, cells were irradiated with UVB and luciferase assay was done after 12 hr incubation. Data are expressed as the means ± standard deviation of a representative experiment done in triplicate.

Analysis of proximal 2.9 Kb of the C/EBPα promoter revealed that this region contains several LEF/TCF consensus (A/T) (A/T)CAA (A/T)GG sites including a perfect match and 2 putative, overlapping p53 consensus binding sites at ~0.5 Kb upstream transcription start site. In order to begin to determine if the C/EBPα promoter is responsive to p53, we cloned the proximal 2.9 Kb of the C/EBPα promoter and inserted it into the promoterless pGL3-basic luciferase vector. As shown in Fig. 4C, UVB treatment of cells transfected with C/EBPα promoter/reporter construct demonstrated that UVB produced a 3-fold increase in C/EBPα promoter activity. Cotransfection with pCMV-p53 increased C/EBPα promoter activity by ~7 fold (data not shown). Furthermore, enforced expression of kinase inactive GSK-3β (KA-GSK) inhibited UVB-induced C/EBPα promoter activation, indicating that UVB-induction of C/EBPα is dependent on the presence of p53 and catalytically active GSK maybe through repression of WNT signaling pathway. Further studies are required to determine if this is a direct effect of p53 binding to C/EBPα promoter or an indirect effect and how GSK is involved in this process.

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