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Role of Pin1 on UVA-induced cyclooxygenase-2 induction in mouse epidermal cells

조선대학교 대학원

약학과

부이 뚜 퀴엔

마우스 표피세포에서 자외선 A 조사에 의한 cyclooxygenase-2 유도에서 Pin1 활성화의 역할

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UVA: ultraviolet A

COX-2: cyclooxygenase-2

Pin1: a peptidyl prolyl isomerise

NF-κB: Nuclear factor kappaB

CREB: cAMP response element binding protein

C/EBP: CCAAT-enhancer-binding protein

AP-1: activator protein-1

PGE2: prostaglandin E_2

JB6: JB6 Cl41 mouse epidermal cell

국문 초록

마우스 표피세포에서 자외선 A 조사에 cyclooxygenase-2 유도에서 Pin1 활성화의 역할

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피부노출 태양광의 대부분이 자외선A(UVA, 320–400 nm) 로 알려져 있으며, 피부광노화와 피부암의 가장 주요한 원인이다. 피부세포에서 cyclooxygenase-2 (COX-2)의 과다발현에 따르는 prostanoid 생성은 피부세포의 증식과 사멸 사이의 평형에 영향을 미치며, 이는 발암과정의 촉진인자로 작동한다. Pin1 (peptidyl prolyl isomerase)는 대부분의 종양조직에서 과다발현되며, 이는 발암의 발생 및 진행에서 중요한 역할을 하는 것으로 알려져 있다. 본 연구실에서는 저에너지 준위의 UVA 조사가 마우스 피부조직 및 JB6 Cl41 표피세포에서 Pin1의 발현을 증가시킨다는 사실을 규명한 바 있다. 본 학위논문에서는

JB6 Cl41 세포에 UVA 조사시 COX-2의 발현이 영향 받는 지를 평가하였고, 이 과정에서 Pin1 활성화가 관여하는 지를 평가하였다. UVA 조사시 COX-2 단백질 발현양과 촤종 산물인 prostaglandin E2 생성이 조사양 의존적으로 증가하였다. COX-2 발현 증가는 UVA 노출시 3시간 만에 증가하였으며, 이는 Pin1 억제시 유의성있게 억제되었다. UVA 노출에 의한 COX-2 유전자 전사는 다양한 전사인자 활성화에 의하여 매개된다. 즉, nuclear factor-κB (NF-κB), cAMP response element binding protein (CREB), CCAAT-enhancer-binding protein α, β (C/EBPα 와 C/EBPβ) 및 c-Jun/activator protein-1 (AP-1) 전사인자 활성화가 UVA 조사시 관찰되었다. 더 나아가, Pin1 과다발현 JB6 C141 세포의 경우, COX-2의 기초발현양이 현저하게 증가하였으며, NF-κB, CREB, C/EBP 및 AP-1의 활성화가 관찰되었다. 또한, UVA를 미리 JB6 Cl41 세포에 노출시켰을 경우, 암세포 형질변화의 지표인 epidermal growth factor (EGF)-유도성 군집 형성 (colony formation) 이 증가하였으며, 이는 COX-2 차단제인 meloxicam 처치에 의하여 유의성있게 억제되었다. 이상의 결과는 UVA 노출시 Pin1 활성화가 COX-2 발현 증가를 일으키며, 이는 표피세포의 암세포 형질변화를와 관련이 있음을 시사한다

ABSTRACT

Role of Pin1 on UVA-induced cyclooxygenase-2 induction in mouse epidermal cells

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Ultraviolet (UV) A (320–400 nm), which constitutes more than 90% of UV radiation in the sunlight that reaches the earth's surface, is considered a major cause of human skin photo-aging and skin cancer. Cyclooxygenase-2 overexpression and subsequent prostanoids formation in skin tissue disturbs the equilibrium between proliferation and apoptosis required for normal maintenance of organ homeostasis, thereby promoting tumorigenesis. Pin1, a peptidyl prolyl isomerase, is overexpressed in most types of cancer tissues and plays an important role in oncogenesis. In previous study, we demonstrated that Pin1 expression was enhanced by low energy UVA irradiation in both skin tissues of hairless mice and JB6 Cl41 epidermal cells. Here, we studied whether UVA exposure to JB6 Cl41 cells affects the expression of COX-2 and possible involvement of Pin1 activation. UVA increased COX-2 protein expression and

prostaglandin E2 production in an energy-dependent manner. The elevation of COX-2 levels was observed as early as 3 hours post-irradiation. Pin1 inhibition significantly blocked UVA-stimulated COX-2 expression. The increased COX-2 gene transcription in response to UVA was preceded by activation of several transcription factors, nuclear factor-κB (NF-κB), cAMP response element binding protein (CREB), CCAAT-enhancer-binding protein α and β (C/EBPα and C/EBPβ) and c-Jun/activator protein-1 (AP-1). Moreover, JB6 C141 cells overexpressing Pin1 increased basal expression of COX-2 and the activities of NF-κB, CREB, C/EBP and AP-1. Finally, we found that pre-exposure of JB6 Cl41 cells to UVA potentiated epidermal growth factor-induced anchorage-independent growth, and this effect was significantly suppressed by COX-2 and Pin1 inhibition. These results suggest that Pin1-mediated COX-2 induction by UVA exposure is associated with malignant transformation of epidermal cells.

Keywords: COX-2, Pin1, UVA, PGE2, skin cancer, JB6 epidermal cells.

1. Introduction

Skin cancer is one of the most commonly diagnosed types of cancer in Western people. Melanoma and non-melanoma skin cancers combined are more common than lung, breast, colorectal or prostate cancer (1). Limitation for the treatment of skin cancers and its high incidence necessitate finding of novel therapeutic targets. The major risk factor for skin cancer is excessive exposure to the ultraviolet (UV). The component of sunlight which can be divided into three ranges: UVA (320 - 400 nm), UVB (280 - 320 nm) and UVC (200 - 280 nm) (2, 3). Since UVC and the majority of UVB radiation are absorbed by the ozone layer, most solar radiation (90 ± 99%) reaching the Earth's surface is UVA radiation (3). UV radiation leads to the development of skin cancer and pathogenesis of other skin damage, and the cell proliferation signaling pathways initiated by UVA radiation are believed to contribute to skin tumor promotion and progression (4). Nevertheless, our understanding of the molecular mechanisms underlying the ability of UVA to induce skin cancer is still unclear.

Cyclooxygenase (COX)-2 and COX-1 are isoforms of an enzyme which

catalyses the first stage in the oxidation of arachidonic acid to the prostanoids, but they differ in their regulation of expression and tissue distribution. The important biological difference between the isoforms is that COX-1 is normally present in most types of cells and is a constitutive, housekeeping enzyme. By contrast, COX-2 protein is normally absent from most cells - with some notable exceptions - but appears rapidly (2 - 4 h) in large amounts in a range of pathological, often inflammatory, situations and in many cell types (5). COX-2 is frequently overexpressed in various types of human tumor tissues (6). Selective COX-2 inhibitor significantly suppressed growth of gastric and breast cancer xenografts (7, 8). Epidemiologic and mice studies also suggest that COX-2 inhibitors hold promise in chemoprevention of skin cancer development (9, 10).

Pin1 (a peptidyl prolyl isomerise), originally discovered in a screening for mitosis-associated molecules, specifically recognizes phosphorylated serine (Ser) or threonine (Thr) immediately preceding a proline (Pro) residue (pSer/Thr-Pro) and isomerizes the peptide bond (11). Because protein kinases and phosphatases recognize their substrates in a conformation-dependent manner, Pin1-dependent

isomerization of Ser/Thr-Pro motifs is critical to diverse enzyme activities (12). Pin1 overexpression is frequently observed in several types of cancer tissues and it has proven to be a prevalent and specific event in human cancers (13). We have previously reported that Pin1 expression was enhanced by low energy UVA irradiation in both skin tissues of hairless mice and JB6 Cl41 epidermal cells (14).

In the present study, we tested whether UVA exposure to JB6 C141 epidermal cells affects the expression of COX-2 and possible involvement of Pin1 activation. In an attempt to elucidate the signaling pathways leading to the expression of COX-2 in UVA-irradiated JB6 cells, we further assessed Pin1-dependent activation of nuclear factor-κB (NF-κB), cAMP response element binding protein (CREB), CCAAT-enhancer-binding protein (C/EBP) or activator protein-1 (AP-1) in GFP- and Pin1-overexpressing JB6 C141 cells. Finally, we studied the effects of inhibitors targeting COX-2 or Pin1 on UVA/epidermal growth factor (EGF)-inducible transformation.

2. Materials and Methods

2-1. Materials

The antibodies against Pin1, COX-2, CREB, C/EBPα, C/EBPβ, c-Jun and c-Fos were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-linked anti-rabbit and anti-mouse IgGs were obtained from Cell Signaling Technology (Beverly, MA). The siRNA targeting mouse Pin1 was acquired from Ambion (Austin, TX). 5-Bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium and phRL-SV40 plasmid were obtained from Promega (Madison, WI). The anti-actin antibody, as well as other reagents for molecular studies, were purchased from Sigma Chemical (St. Louis, MO).

2-2. Cell culture and UVA irradiation

JB6 Cl41 mouse epidermal cells were maintained in Minimum Essential medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. For UVA irradiation to JB6 Cl41 cells, the culture medium was removed, and cells

were washed with sterile PBS. UVA exposure was performed with fluorescent lamps (UVP, Upland, CA) with the dish lids off. In selected experiments, cells were pre-treated with inhibitors for 30 min prior to irradiation.

2-3. Preparation of the nuclear fraction

Cells in dishes were washed with ice-cold PBS, scraped, and transferred to microtubes. The cells were allowed to swell after addition of 100 μl of lysis buffer containing 10 mM HEPES (pH 7.9), 0.5% Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride. The cell membranes were disrupted by vortexing, and the lysates were incubated on ice for 10 min and centrifuged at 7,200 *g* for 5 min. Pellets containing the crude nuclei were resuspended in 60 μl of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride, and then incubated for 30 min on ice. The samples were centrifuged at 15,800*g* for 10 min to obtain the supernatants containing the nuclear extracts. The nuclear extracts were stored at -80°C until needed.

2-4. Immunoblot analysis

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to previously published procedures(13). The cells were lysed in EBC lysis buffer containing 20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 μg/ml leupeptin. The cell lysates were centrifuged at 10,000 *g* for 10 min to remove cell debris. Proteins were fractionated using 10% separating gels. The fractionated proteins were then electrophoretically transferred to nitrocellulose membranes, and the proteins were immunoblotted with each specific antibody. The secondary antibodies were either HRP- or AP-conjugated anti-IgG antibodies. The nitrocellulose papers were developed using either 5-bromo-4-chloro-3-indolylphosphate/4-nitroblue tetrazolium chloride or an enhanced chemiluminescence system. For chemiluminescence detection, LAS3000-mini (Fujifilm, Japan) was used.

2-5. Reporter gene assay

Promoter activity was determined using a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, cells were transiently transfected with 1 μg of either AP-1, COX2, CRE, C/EBP, NF-κB reporter plasmid, respectively and 10 ng of phRL-SV plasmid (hRenilla luciferase expression was used for normalization) (Promega, Madison, WI) using Hilymax® reagent (Dojindo Molecular Tech., MD). The cells were then exposed to UVA and incubated in serum-free medium for 2 h. The firefly and hRenilla luciferase activities in the cell lysates were then measured using a luminometer (LB941, Berthold Tech., Bad Wildbad, Germany).

To determine the transcriptional activity of the *COX-2* gene, we used the pGL-COX-2-574 luciferase reporter gene. A C/EBP mutant with an NF-IL-6 site (-132/-124) mutation, an NF-κB mutant (-223/-214), or a CRE/AP-1 mutant (-59/-53) are either co-transfected with pGL-COX-2-574 luciferase reporter gene. A total of 1 μg of the plasmid was transfected into the cells using Hilymax reagent (Dojindo Molecular Technologies), according to the manufacturer's instructions. After 6 h, the transfection medium was replaced with the basal culture medium without serum

and incubated for 30 minute. The cells were exposed to UVA and then further incubated for 2 h. The luciferase activities in the cell lysates were then measured using a luminometer (LB941, Berthold Tech., Bad Wildbad, Germany).

2-6. Prostaglandin E_2 (PGE2) determination

To determine PGE2 concentrations in culture medium, we used a commercial enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, U.S.A.) according to the manufacturer's protocol. Briefly, cells were plated in 12-well culture plates in serum-free medium for 24 h. Cells were exposed to UVA irradiation. Juglone and Meloxicam were added to the medium and cells then were incubated for 24 h. The medium was then collected to determine the PGE2 concentration by measuring absorbance at 420 nm.

2-7. Anchorage-independent cell transformation assay (soft agar assay)

Briefly, JB6 C141 epidermal cells (8×10^3) were irradiated to UVA. Then, cells were exposed to meloxicam in different concentrations or with or without

epidermal growth factor (EGF) in 1 ml of 0.3% basal Eagle's agar containing 10% FBS. The cultures were maintained at 37 ℃ for 14 days, and the cell colonies were scored using an Axiovert 200M florescence microscope and Axio Vision software (Carl Zeiss Inc., Thornwood, NY).

2-8. Data analysis

Scanning densitometry was carried out using an Image Scan and Analysis System (LAS-3000mini, Fujifilm, Japan). One-way analysis of variance (ANOVA) was used to assess the significance of differences between the treatment groups. The Newman-Keuls test was used to compare multiple group means for each significant treatment effect. Statistical significance was accepted at either $p<0.05$ or $p<0.01$.

3. Results

3-1. UVA irradiation-stimulated COX-2 expression in epidermal cells

Several reports have indicated that COX-2 is highly expressed in neoplastic epithelial cells in a wide variety of human tumor types (15, 16, 17). Bachelor et al. reported for the first time that UVA induced COX-2 expression in the human keratinocyte cell line, HaCaT, and showed the possibility that COX-2 plays a key role in the promotion step during skin tumorigenesis (4). To investigate whether UVA irradiation also affects COX-2 expression in JB6 Cl41 epidermal cells, we determined protein levels of COX-2, the rate-limiting enzyme in the production of prostaglandin. Western blot analyses showed the COX-2 expression increased in an energy- and time-dependent manner (Fig. 1A and 1C). The media production of PGE2, one of the end products catalyzed by COX-2 was also enhanced by UVA exposure (Fig. 1D). It has been shown that up-regulation of COX-2 expression in tumors is mainly caused by enhanced transcription (19). Reporter gene analyses using pGL-COX-2-574 luciferase reporter gene (a wild-type COX-2 promoter-luciferase chimeric construct that contained the 574-bp 5'-flanking region of COX-

2 gene) revealed that COX-2 gene transcription was stimulated by UVA (Fig. 1B). Interestingly, UVA exposure to epidermal cells also resulted in Pin1 induction and this showed very similar patterns to COX-2 expression (Fig. 1C).

3-2. Activation of AP-1, NF-κB, CREB and C/EBP transcription factors by UVA

We then examined the possible transcription factors required for UVA-mediated COX-2 gene transactivation. The COX-2 promoter contains binding sites for several transcription factors, such as NF-κB, C/EBP and cyclic AMP response element (CRE)/AP-1 (19), which are activated upon UVB stimulation (20-22). Therefore, JB6 Cl41 epidermal cells were transfected with either a wild type pGL-COX-2-574, a C/EBP mutant with an NF-IL-6 site (-132/-124) mutation, an NF-κB mutant (-223/-214), or a CRE/AP-1 mutant (-59/-53). Wild-type COX-2 promoter activity was ~1.6-fold increased by UVA irradiation (Fig. 2A). Even the cells were exposed to UVA, all mutants in each binding site (C/EBP, NF-κB, or CRE/AP-1) showed very low COX-2 reporter activities (89%, 97% and 90% inhibition, respectively) and the basal reporter activity was also significantly reduced by each

mutation (Fig. 2A). These results demonstrated C/EBP, NF-κB, and CRE/AP-1 elements are all involved in both the basal and UVA-stimulated transactivation of COX-2 gene.

To further confirm whether these transcription elements are activated by UVA in JB6 cells, we determined the activity of each transcription factor using Western blot analyses and reporter gene assays. The COX-2 promoter contains two NF-κB consensus sequences, and NF-κB heterodimer binding to these sites are known as key event for the gene transcription (23, 24). In fact, the NF-κB minimal reporter activity and nuclear p65 levels were significantly up-regulated in UVA- stimulated JB6 cells (Fig. 2B).

UVA exposure also sharply enhanced CRE-Luc and pC/EBP-Luc activities, especially C/EBP minimal reporter activity. Western blot analyses also illustrated higher expression of nuclear levels of CREB and two isoforms of C/EBP (C/EBP α and C/ EBP β) after UVA irradiation (Fig. 2C and 2D). It has been known that c-Jun activation by platelet-derived growth factor or serum increases COX-2 protein levels via CRE/AP-1 binding (25). We also found that UVA exposure increased AP-

1 minimal reporter activity in an energy-dependent manner (Fig. 2D). Furthermore, nuclear c-Jun expression was elevated by UVA irradiation, while the nuclear level of c-Fos, Jnu B and Jun D remained constant (Fig. 2E). These data demonstrates that UVA-stimulated COX-2 upregulation is preceded by activation of NF-κB, CREB, C/EBP and AP-1.

3-3. Suppression of malignant transformation by COX-2 inhibition

Prostanoids are critical mediators in many biologic processes, including inflammation, angiogenesis, and ultimately transformation (26) and PGE2 has been known as a major prostaglandin produced in human skin (27, 29). Meloxicam at low therapeutic doses selectively inhibits COX-2 over COX-1 (30). Because UVA-exposed JB6 C141 epithelial cells simultaneously expressed high levels of COX-2 and PGE2, we evaluated the effect of meloxicam on UVA-mediated PGE2 production in culture medium. 24 hours after 300 mJ/cm^2 UVA exposure, PGE2 levels showed approximately 2 fold increase compared with control. The enhanced PGE2 production was significantly diminished in JB6 C141 cells pretreated with

meloxicam in a concentration-dependent manner (Fig. 3A). COX-2 has been characterized as one of the induced proteins during the transformation of cells by viral oncogenes (31-33). In order to determine whether COX-2-stimulated prostanoids production is related with transformation of epidermal cells, we performed the colony formation assays in UVA-exposed and/or EGF-treated JB6 C141 cells. Sham-operated or UVA-irradiated cells were exposed to EGF on soft agar. UVA enhanced the number and the size of colonies promoted by EGF stimulation (Fig. 3B), whereas pretreatment with meloxicam concentration-dependently abrogated the stimulatory effect of UVA on colony formation (Fig. 3B and 3C). These data suggest that UVA-stimulated COX-2 expression and subsequent production of prostanoids were closely related with malignant transformation of epidermal cells.

3-4. Involvement of Pin1 in UVA-stimulated COX-2 expression

We recently demonstrated the enhanced Pin1 expression by UVA in both skin tissues of hairless mice and epidermal cell line (14). In this study, we confirmed

that the protein expression of Pin1 was also increased by UVA exposure in an energy-dependent manner (Fig. 4A). To evaluate whether the increased Pin1 in respond to UVA exposure is associated with the activation of COX-2 gene transcription in JB6 C141 cells, Pin1 siRNA and juglone were used as Pin1 blocking tools. As shown in Fig. 4B and Fig. 4C, UVA-mediated COX-2 upregulation was significantly reversed by either 3 μM Juglone treatment or Pin1 siRNA transfection (Fig. 4B and 4C). Moreover, PGE2 production in response to UVA exposure was completely blocked by juglone treatment (Fig. 4D). We have previously shown that Pin1 inhibition suppresses colony formation of JB6 C141 cells on soft agar assay (14). Hence, the increased COX-2 expression via Pin1 induction is a possible mechanism for malignant transformation of epidermal cells in response to UVA.

3-5. The increased COX-2 expression and activation of NF-κB, CREB and C/EBP in Pin1-overexpressing epidermal cells

To further confirm the role of Pin1 in UVA-induced COX-2 gene transcription,

we established stably Pin1-overexpressing JB6 Cl41 cells (Pin1-JB6) using retroviral infections. Pin1-JB6 cells had higher Pin1 levels than GFP-JB6 (GFP-overexpressing) cells (Fig. 5A). As expected, Western blot and pGL-COX-2-574 reporter gene analyses showed that gene transcription and protein expression of COX-2 were up-regulated in Pin1-JB6 cells compared to control GFP-JB6 cells (Fig. 5A and 5B). Moreover, Pin1-JB6 cells showed higher nuclear levels of NF-κB, CREB, C/EBPα and C/EBPβ, essential transcription factors for UVA-mediated COX-2 gene transcription (Fig. 5B, 5C, 5D, 5E). These results clarify that Pin1-mediated transcriptional activation of COX-2 was coordinately regulated by NF-κB, CREB and C/EBP. Thus, Pin1 elevation may act as initial signal to subsequently activate COX-2 gene transcription and ultimately causes the enhanced proliferation and malignant transformation of epidermal cells under UVA exposing condition.

4. Discussion

UV light in sunlight is composed of ultraviolet B (UVB 280–320 nm) and ultraviolet (UVA 320–400 nm). UVA has been considered far less carcinogenic based on limited direct damage to DNA (34). However, UVA is approximately 20-fold more abundant than UVB in the sunlight and much more UVA penetrates the epidermis and reaches the basal germinative layers (35). Recently, there has been compelling evidence that UVA alone, at environmentally relevant doses, has the potential to be a human skin carcinogen. Most skin cancers are caused by repeated exposure to sunlight (36, 37). Continued UV exposure may serve to induce mutations that result in sustained expression of COX-2 leading to sustained keratinocyte proliferation (4). As in rodent chemical carcinogenesis models, a causal relationship between induced COX-2 expression and the UV-induced development of skin cancer has been demonstrated in hairless SKH-1 mice (18, 28). Another study demonstrated transient induction of COX-2 expression in proliferating basal keratinocytes by environmentally relevant levels of UV irradiation, equivalent to 60 min of summer noonday sun in Rochester, New York

(38).

As a rate-limiting enzyme in the synthesis of PGs, cyclooxygenase (COX) exists as two isoforms. COX-1 is considered as a housekeeping gene which modulates physiologic responses such as regulation of renal and vascular homeostasis, and gastro protection of the stomach. By comparison, COX-2 appears rapidly (2 - 4 h) in large amounts in a range of pathological, often inflammatory, situations and in many cell types (5). In the present study, we investigated that COX-2 expression was increased by UVA exposure in JB6 C141 mouse epidermal cells (Fig. 1A, 1B). COX-2 protein induction occurs as early as ~3 h post-irradiation (Fig. 1C) and the activation of the related transcription factors for COX-2 induction have been appeared earlier. The overexpression of COX-2 might be caused by cooperative upregulation of AP-1, CREB, C/EBP and NF-κB (Fig. 2).

In addition, this study showed the role of COX-2 as an important enzyme mediating PGE2 synthesis in response to UVA irradiation in JB6 cells. UV irradiation also induces prostaglandin (PGs) production in the skin (39, 40). PGs are critical mediators in many biologic processes, including inflammation,

angiogenesis, and platelet aggregation (26). PGE2 is the major PGs produced in human skin (27, 29). The importance of the 'post COX' enzymes has recently been underlined by the identification of an inducible isoform of PGE2 synthase. Recent study has clarified the role of COX-2 as an important enzyme mediating PGE2 synthesis in response to acute UVB irradiation in human epidermal keratinocytes (41). Elevation of PGE2 levels are thought to play a role in carcinogenesis through inhibition of apoptosis, promotion of angiogenesis and increased cell proliferation (16, 42-45). The increased PGE2 expression have been observed in squamous and basal cell carcinomas of the skin and may correlate with an increased propensity for metastatic and invasive behavior (45, 46). Thus the final biological effect of COX-2 activity may be predominantly expressed by PGE_2. As shown in the present study, PGE2 production was also increased in respond to UVA exposure (Fig. 1D). Meloxicam, a COX inhibitor, were also illustrated the potential of reducing UVA-induced PGE2 production in epidermal cells after 24 h treatment (Fig. 3A). However, reducing prostaglandin levels within tumors by use of nonselective NSAIDs in conjunction with a chemotherapeutic or radiation therapeutic regimen

would probably be limited because of toxicity that could occur to proliferating tissues such as the gastric and intestine tract. Discovery of distinct isoforms of cyclooxygenase led to the notion that selective inhibition of COX-2 would avoid the gastric toxicity associated with inhibition of both isoforms. The observation that COX-2 is highly expressed in a majority of human tumors suggests that COX-2 inhibition may be a useful therapeutic mechanism for inhibiting tumor progression and growth of human cancer (6, 47–51).

Several reports have shown that both non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2-selective inhibitors have the potential efficacy of anticancer in laboratory models. They work by inhibiting the activity of cyclooxygenases, enzymes that catalyze the conversion of arachidonic acid to prostaglandins. A selective inhibitor of COX-2, Celecoxib, reduced the tumor yield by 89% in a dose-dependent manner in UVB-irradiated hairless mice (18, 44). In the same study, Indomethacin, a nonspecific COX-2 inhibitor, also showed a significant reduction (78%) in tumor number in UVB-irradiated mice (18).

Considering the vital role of COX-2 in cell proliferation and carcinogenesis,

UVA-stimulated COX-2 induction may cause the overgrowth and transformation of epidermal cells. In fact, with the presence of EGF, UVA-exposed JB6 cells could be increased colony formation than JB6 cells and this effect was significantly diminished by COX inhibition (Fig.3B and 3C).

Recent study has demonstrated that expression of Pin1, a key regulator of cancer development, is induced by low energy UVA in epidermal cells and mouse skin and the increased Pin1 expression is associated with UVA-induced AP-1 activation (14), the gene which related to COX-2 transactivation. In our work, we also observed the elevation of Pin1 in respond to UVA exposure (Fig. 4A). The proper relation between Pin1 and COX-2 in UVA-irradiated JB6 cells was clearly illustrated by the efficacy of Pin1 inhibitors on inhibiting COX-2 and PGE2 production (Fig. 4B, 4C and 4D). It was further confirmed in these experiments on stably Pin 1- overexpressing JB6 cells (Fig 5). Hence, Pin1 expression in response to UVA irradiation may act as an initial signal that subsequently activates COX-2 and exaggerates proliferation and malignant transformation of epidermal cells.

Taken together, Pin1-mediated COX-2 induction by UVA exposure is

associated with malignant transformation of epidermal cells. These data reported here suggest that COX-2 induced PG potentially play an early significant role in the pathogenesis of UV-induced epidermal neoplasia and provide further support for the importance of COX-2 as a potential pharmacological target mediating human skin tumor development. Likewise, these results suggest a role for the prophylactic use of selective COX-2 inhibitors to prevent the development of epidermal skin cancers due to their capacity to increase apoptosis in proliferating.

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6. Figure Legends

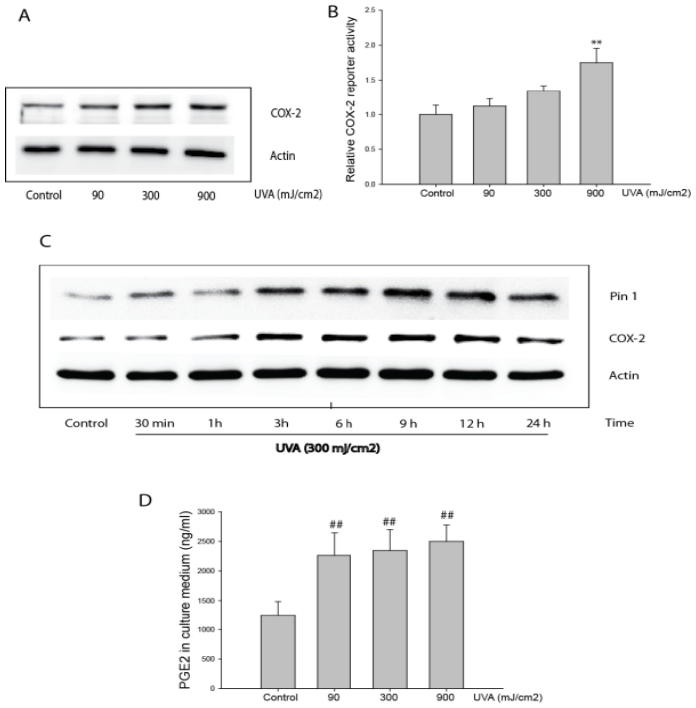

FIGURE 1. UVA-stimulated COX-2 activation in JB6 Cl41 epidermal cells. (A) Immunoblot analysis of COX-2 in JB6 C141 epidermal cells. JB6 C141 cells were exposed to UVA (90–900 mJ/cm^2) and the total cell lysates were obtained 24 h after UVA irradiation. (B) Induction of luciferase activity in by UVA exposure in JB6 Cl41 cells. JB6 cells transiently transfected with COX-2 minimal reporter gene. A dual luciferase reporter assay was performed on the lysed cells co-transfected with the COX-2-Luc (firefly luciferase) and phRL-SV (hRenilla luciferase) (a ratio of 200:1) 18 h after exposure to UVA (30–300 mJ/cm2). The data represents the mean ± SD of six separate samples (significant compared to the control, $^{**}p < 0.01$). (C) Time-dependent Pin1 and COX-2 expression after UVA exposure. Western blot analyses of Pin1 and COX-2 in the total cell lysates which were obtained from JB6 C141 cells exposed to UVA (300 mJ/cm2) (30 min-24 h).

Pin1 and COX-2 was immunoblotted with the respective antibody. (D) UVA-stimulated PGE2 production in culture medium. JB6 cells were cultured in 12-well plate for 24h, and then irradiated to UVA (90-900 mJ/cm^2). After that, cells were incubated for 24h and PGE2 concentration PGE**2** concentrations in culture supernatants determined by EIA against a PGE**2** standard at the given times after irradiation. The data represents the mean ± SD of three separate samples (significant compared to the control, $^{\#\#}p < 0.01$).

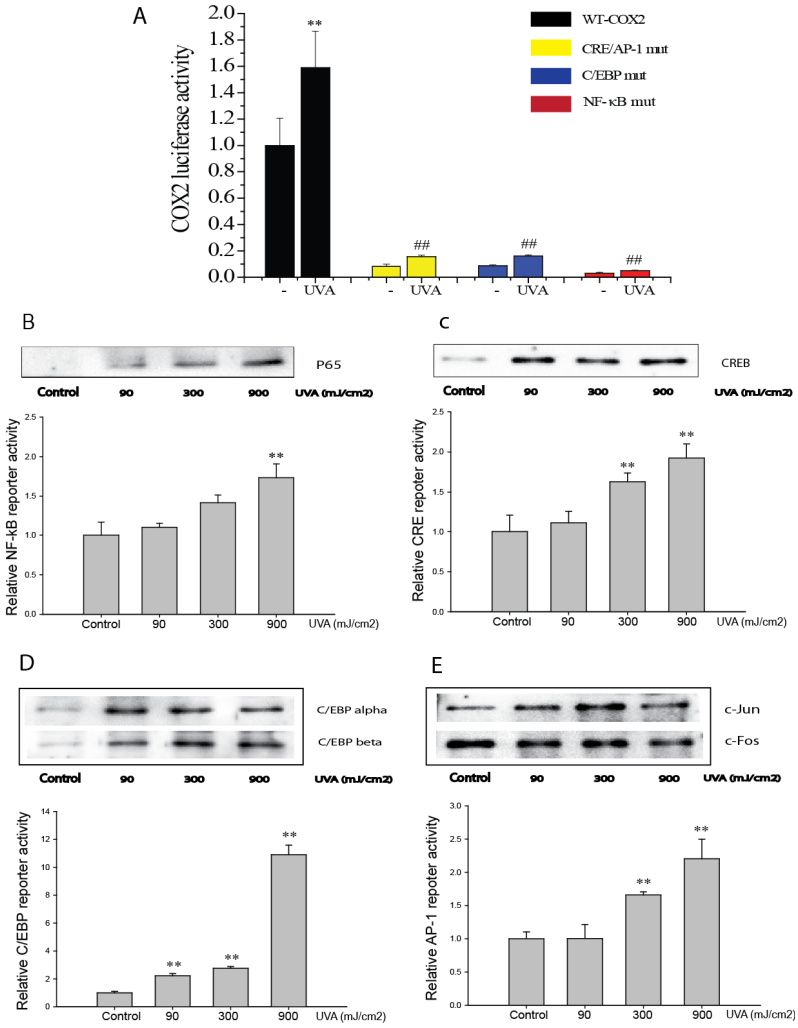

FIGURE 2. Induction of proinflammatory mediators in UVA-irradiated JB6 Cl4 cells. (A), Induction of luciferase activity by UVA-exposed JB6 cells. Reporter activities in JB6 and UVA-exposed JB6 cells transiently transfected with pGL-COX-2-574, NF-κB mutant, C/EBP mutant, or CRE/AP-1 mutant construct were confirmed using a luminometer. After 6 h of transfection, cells were incubated in seum-free medium and then irradiated to UVA. Reporter gene activations were expressed as changes relative to human *Renilla* luciferase activity. The results shown represent the means ± SD of four separate samples (significant as compared with the pGL-COX-2-574-tranfected JB6 cells, **, $p < 0.01$; significant as compared with the pGL-COX-2-574-tranfected UVA-expose JB6 cells, ##, $p < 0.01$). The results were confirmed by two separate experiments. (B) NF-κB activation in UVA-exposed JB6. *Upper panel*, Nuclear levels of p65. Each lane represents different sample. *Lower panel*, NF-κB reporter gene analysis. JB6 cells were transfected with pNF-κB-Luc plasmid and reporter gene analysis was performed, as described in the legend of *A*. Data represent the means ±SD of four separate samples (significant as compared with control cells; **, $p < 0.01$). The results were confirmed by two separate experiments. (C) Nuclear levels of CREB. Each lane represents different sample. *Lower panel*, CRE reporter gene analysis. JB6 cells were transfected with pCRE-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 2*A*. Data represent the means ± SD of four separate samples (significant as compared with control; **, $p < 0.01$). The results were confirmed by two separate experiments. (D), C/EBP activation in UVA-exposed JB6. *Upper panel*, Nuclear levels of C/EBPα and C/EBPβ. *Lower panel*, C/EBP reporter gene analysis. JB6 cells were transfected with pC/EBP-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 2*A*. Data represent the means ± SD of 4 separate samples (significant as compared with JB6 cells; **, $p < 0.01$). The results were confirmed by two separate experiments. *(D)*, AP-1 activation in UVA-treated JB6. *Upper panel*, Nuclear levels of c-Jun, c-Fos. *Lower panel*, AP-1 reporter gene analysis. JB6 cells were transfected with pAP-1-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 2*A*. Data represent the means ± SD of four separate samples (significant as compared with control; **, $p < 0.01$). The results were confirmed by two separate experiments.

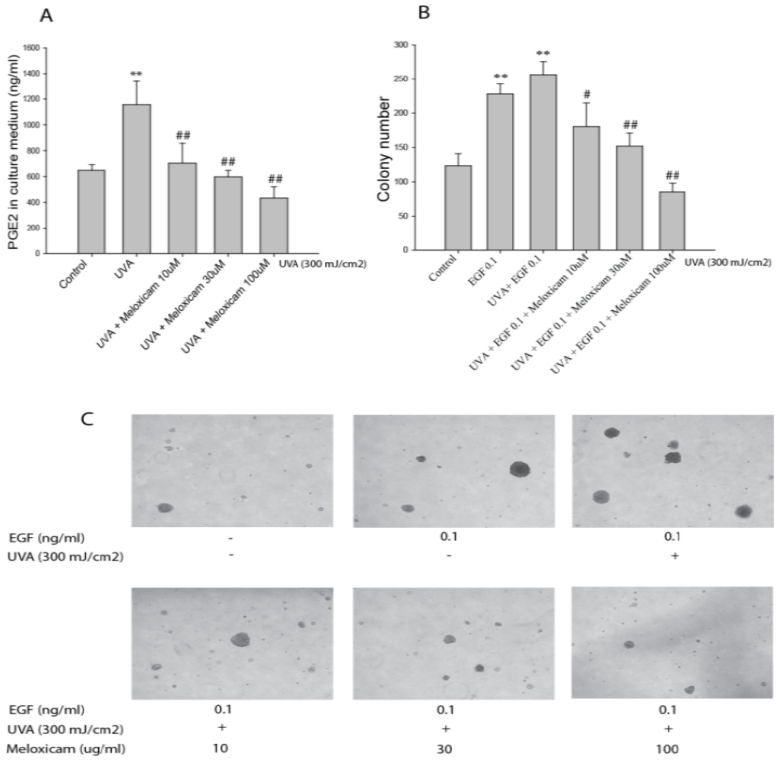

FIGURE 3. Effect of meloxicam, a COX inhibitor, on UVA-irradiated JB6 epidermal cells. (A) The effect of varying doses of meloxicam on PGE2 production in the culture medium. Cells were pre-irradiated to UVA (300 mJ/cm^2) and then exposed to meloxicam at varying concentration for 18h. Levels of PGE2 were determined by enzyme immunoassay kit (EIA) against a PGE**2** standard at the given times after irradiation. The data represents the mean ± SD of three separate samples (significant compared to control; **, $p < 0.01$, significant compared to UVA-exposed group, ##p < 0.01). (B) the quantitative colony numbers in each group (n = 6) (significant compared to the untreated control, **p < 0.01; significant compared to UVA-exposed group, #p< 0.05, ##p < 0.01). (C) representative figures of colony formation assay. JB6 C141 cells exposed to UVA(300 mJ/cm2) were incubated for 14 days with or without meloxicam and 0.1 ng/ml EGF in soft agar plates.

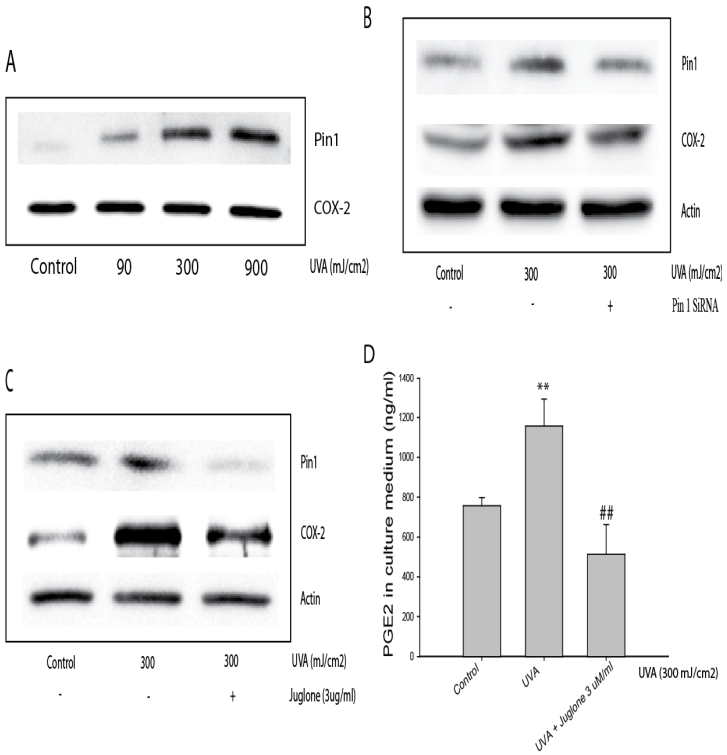

FIGURE 4: Role of Pin1 in the UVA-induced COX-2 activation in JB6 cells. (A) Western blot analysis of Pin1 in UVA-exposed JB6 C141 cells. Total cell lysates were obtained from JB6 C141 cells exposed to UVA (90–900 mJ/cm2) for 24 h. (B) Effect of Pin1 siRNA (*60pmol*) on COX-2 expression. JB6 C141 cells were transfected with Pin1 siRNA (60 pmol) or control siRNA (60 pmol) and then incubated in serum-free medium for 18 h. Total cell lysates extracts were obtained 6h after UVA exposure. (C) Effect of Juglone (3μM) on COX-2 expression. JB6 cells were treated with Juglone in serum-free medium for 18h. Total cell lysates extracts were obtained 6h after UVA exposure. (D) Effect of Pin1 inhibitor, Juglone (3μM/ml) on PGE2 production in the culture medium. Cells were pre-treated with Juglone to UVA (300 mJ/cm^2) and then exposed Juglone for 18h. Levels of PGE2 were determined by enzyme immunoassay kit (EIA) against a PGE**2** standard at the given times after irradiation. The data represents the mean ± SD of three separate samples (significant compared to control; **, $p < 0.01$, significant compared to UVA-exposed group, ##$p < 0.01$).

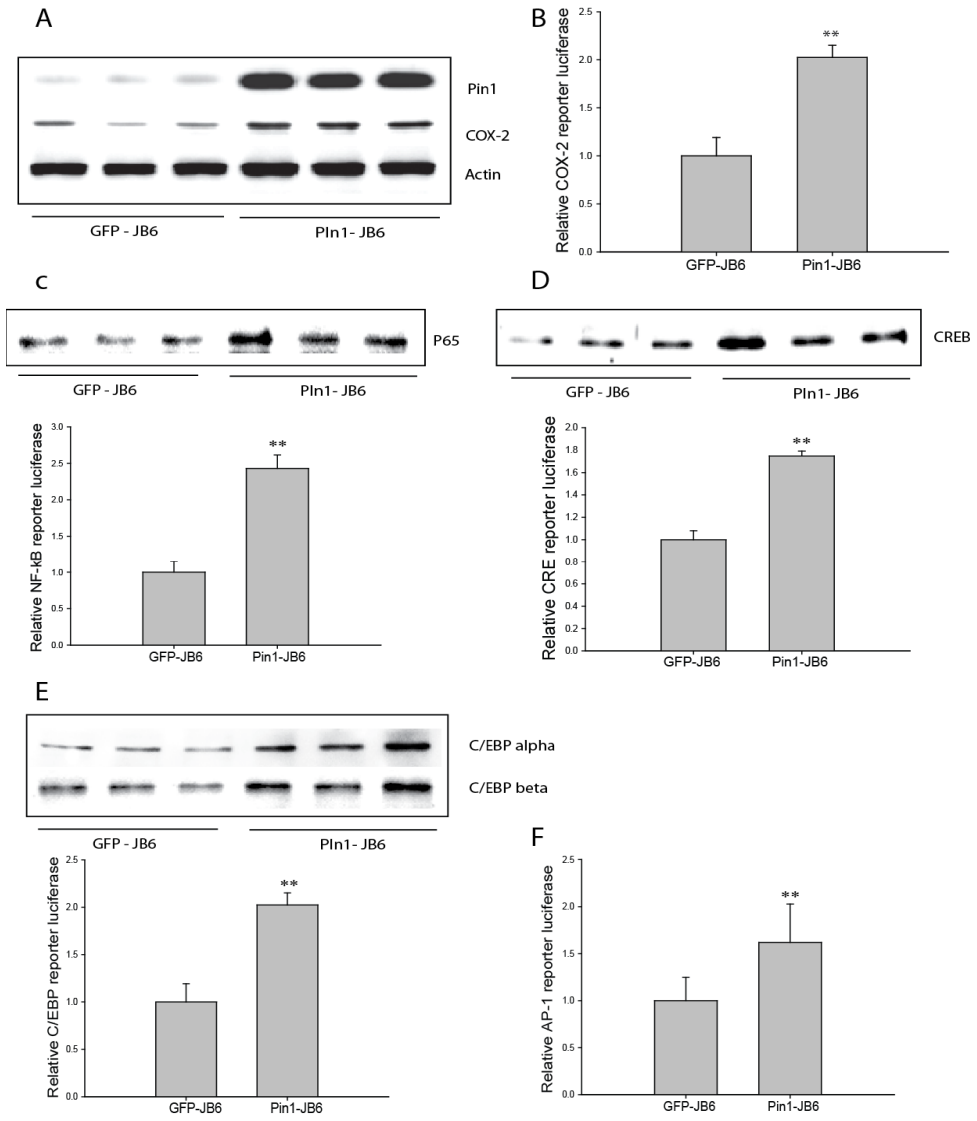

FIGURE 5: Induction of proinflammatory mediators in Pin1-overexpressing JB6 Cl41 cells. (A) COX-2 induction in Pin1-overexpressed JB6 Cl41 cells. A representative immunoblot shows COX-2 and Pin1 proteins in both GFP-JB6 and Pin1-JB6 cells. Each lane was loaded with 20 μg of protein. Equal protein loadings were verified using actin as an internal standard. Each lane represents different sample. The results were confirmed by two separate experiments. (B) COX-2 reporter gene analysis. Pin1-JB6 and GFP-JB6 cells were transfected with pCOX-2-Luc plasmid and reporter gene analysis was performed, as described in the legend of 2*A*. Data represent the

means ±SD of four separate samples (significant as compared with control cells; **, $p < 0.01$). The results were confirmed by two separate experiments. (C) NF-κB activation in Pin1-JB6. *Upper panel*, Nuclear levels of P65. Each lane represents different sample. *Lower panel*, NF-κB reporter gene analysis. Pin1-JB6 and GFP-JB6 cells were transfected with pNF-κB-Luc plasmid and reporter gene analysis was performed, as described in the legend of 2*A*. Data represent the means ±SD of four separate samples (significant as compared with control cells; **, $p < 0.01$). The results were confirmed by two separate experiments. (D) Nuclear levels of CREB. Each lane represents different sample. *Lower panel*, CRE reporter gene analysis. Pin1-JB6 and GFP-JB6 cells were transfected with pCRE-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 2*A*. Data represent the means ± SD of four separate samples (significant as compared with control; **, $p < 0.01$). The results were confirmed by two separate experiments. (E), C/EBP activation in UVA-exposed JB6. *Upper panel*, Nuclear levels of C/EBPα and C/EBPβ. *Lower panel*, C/EBP reporter gene analysis. Pin1-JB6 and GFP-JB6 cells were transfected with pC/EBP-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 2*A*. Data represent the means ± SD of 4 separate samples (significant as compared with JB6 cells; **, $p < 0.01$). The results were confirmed by two separate experiments. *(D)*, AP-1 activation in Pin1-JB6. AP-1 reporter gene analysis. Pin-JB6 and GFP cells were transfected with pAP-1-Luc plasmid, and reporter gene analysis was performed, as described in the legend of Fig. 2*A*. Data represent the means ± SD of four separate samples (significant as compared with control; **, $p < 0.01$). The results were confirmed by two separate experiments.

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Korea, November, 2011

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논문제목	한글 : 마우스 표피세포에서 자외선 A 조사에 의한 cyclooxygenase-2 유도에서 Pin1 활성화의 역할 영어 : Role of Pin1 on UVA-induced cyclooxygenase-2 induction in mouse epidermal cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2011 년 10 월 10 일

저작자: 부이 뚜 퀴엔 (서명 또는 인)

조선대학교 총장 귀하