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The study of Ku80 in the protection of corneal cells from UV-irradiation

조선대학교 대학원
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각막세포에서 자외선조사에 의한 세포 보호효과에 관여하는
Ku80에 관한 연구

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지도교수 유 호 진

이 논문을 이학박사 학위신청논문으로 제출함.

2005 년 10 월 일

조선대학교 대학원

생물신소재 학과

윤 영

윤 영의 박사학위논문을 인준함

위원장	조선 대학교	교수	장 인 엽
위 원	조선 대학교	교수	전 제 열
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위 원	조선 대학교	교수	유 호 진

2005 년 12 월 일

조선대학교 대학원

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각막세포에서 자외선조사에 의한 세포 보호효과에 관여하는 Ku80에 관한 연구

윤 영

(지도교수: 유 호 진)

조선대학교 대학원 생물신소재학과

세포 내외의 유해인자에 의해 DNA 가 손상되면 세포내부에 존재하는 신호전달체계에 의하여 DNA 복제가 정지된 후 손상된 DNA 는 DNA repair system 인 base excision repair, nucleotide excision repair 그리고 mismatch repair 에 의하여 repair 가 이루어진다고 알려져 있다. 이런 손상된 유전자는 유전자손상 복구 시스템은 정교한 시스템에 의하여 조절되지만 아직 그 정확한 조절 기작은 알려져 있지 않다. 세포 신호 전달에 관여하는 Ras 는 방사선에 의한 세포 내성에 관여하는 것으로 알려져 있다. 따라서 본 연구에서는 각막세포에 흔히 손상을 주는 자외선을 보호하는 기작을 규명하고자 각막세포에 Ras 를 발현 시켜서 자외선에 의한 각막세포의 보호 효과 및 그 기작을 관찰 하였다.

본 연구 결과 ras 가 발현된 각막세포가 자외선에 의한 세포 손상이 억제된다는 사실을 확인하였다. 자외선은 유전자 이중 절단이 증가하여 세포를 죽음에 이르게 한다. 본 연구에서는 ras 가 발현된 각막세포에서 유전자 이중 절단을 복구시키는 Ku80 의 발현이 증가된다는 사실을 northern blot 과 western blot 으로 확인하였다. 또한 이중 절단된 올리고를 기질화하여 측정 한 결과 ras 발현 세포에서

대조군 세포에 비하여 현저하게 유전자 이중 나선 부위에 단백질 결합이 증가하는 현상을 관찰하였다. 더욱이 자외선 조사 후 남아 있는 유전자 이중 절단을 측정한 결과 ras 발현 각막 세포에서 현저하게 유전자 이중 절단이 감소한다는 사실을 규명하였다. Ku80 은 세포의 DNA-Pk 활성을 증가하는 것으로 알려져 있기 때문에 ras 발현세포가 ku80 을 증가시켜 DNA-PK 활성을 증가시키는지를 관찰하기 위하여 대조군과 ras 발현 세포에서 DNA-PK 활성을 측정한 결과 예상대로 ras 발현세포에서 현저하게 DNA-PK 활성이 증가함을 관찰하였다.

마지막으로 siRNA 를 이용하여 ras 발현 세포에서 ku80 을 제거한 경우에는 ras 가 발현되더라도 자외선에 의한 각막 보호효과가 사라진다는 사실을 관찰 하였다.

이와 같은 실험결과를 종합하면, ras 발현 각막세포는 유전자 복구 단백질인 Ku80 의 발현을 증가시켜 자외선에 의한 각막세포 손상을 보호하는 것으로 사료된다.

ABSTRACT

The Ras activation contributes to UV resistance but the mechanism is unclear. This paper shows that the expression of the dominant-positive H-Ras increased the Ku80 level, which is one of the key enzymes involved in repairing double-stranded DNA breaks (DSBs). After exposing the cells to ionizing radiation and analyzing them using an electrophoretic mobility-shift assay (EMSA) and pulsed-field gel electrophoresis, it was found that activated H-Ras expression in corneal cells increases the DNA binding activity of Ku80 and increases the DSB repair activity. Ku80 small interfering RNA expression was shown to reduce the oncogenic H-Ras-mediated increase in the DSBs and suppress the oncogenic H-Ras-mediated resistance of the cells toUV irradiation, while Ku80 overexpression in the corneal cells significantly increased the UV resistance. These results suggest that the Ku80 expression induced by oncogenic H-Ras appears to play important role in protecting cells against UV irradiation.

I. INTRODUCTION

Radiation therapy is used as a curative treatment for cancer. However, the radiation responses of tumors differ according to the histology, doubling time, repair capacity and other factors (1). Therefore, understanding the properties of tumor cells that increase or decrease their responsiveness to radiation is the key to improving radiation therapy. Previous studies have shown that oncogenic mutations in H-Ras, which frequently occur in many types of cancer, can contribute to an increased radiation survival rate in transformed cells. It was initially shown that the resistance of NIH 3T3 cells to radiation could be enhanced by the expression of an activated H-*Ras* gene (2). This was later confirmed independently by several other groups using rodent cells, including REF fibroblasts and rat rhabdomyosarcomas (3-6), and human tumor cells, including an EJ Ras-transformed bladder carcinoma, DLD-1 colon carcinoma, and HT1080 fibrosarcoma (7, 8). In agreement with such Ras-mediated UV resistance, several groups have reported that the blocking of Ras activity leads to an increase in radiosensitivity. For example, the expression of an antisense vector to Ras as well as the transfection of cells with an adenoviral vector encoding a single-chain antibody fragment against Ras lead to radiosensitization via the inhibition of Ras action (9, 10). Similarly, blocking Ras activity using pharmacological inhibitors

causes increased radiosensitivity (11, 12).

The major oncogenic signal from Ras uses the serine threonine kinase Raf as the effector (13). The activated Ras complexes with and promotes Raf phosphorylation. Active Raf is the first in a cascade of other kinases, including MEK, which phosphorylates and activates the mitogen activated protein kinase, ERK. While the Ras/Raf/MEK/ERK cascade plays a key role in proliferative signaling, there is also evidence suggesting that Ras activation can activate MEK kinase (MEKK) and phosphatidylinositol 3-OH kinase (PI3K) (14). MEKK is a critical component of the stress-associated protein kinase including the Jun N-terminal kinase JNK (SAPK). This cascade is activated by stress (15). PI3K activation leads to the production of phosphorylated phosphatidyl-inositides with regulatory functions on the kinases, PDK and AKT/PKB, which has been shown to be involved in the survival response (16). Recently, several groups have focused on the down-stream mediators for the Ras-induced UV resistance of cancer cells and reported that the PI3K, Raf and epidermal growth factor receptor (EGFR) signal pathways contributes to the enhanced UV resistance (17-21). Therefore, there is considerable evidence suggesting a causal relationship between the Ras proteins and UV-irradiation resistance. However, the molecular mechanisms underlying this effect are unclear.

In the present study, we sought to determine the downstream target genes regulated by activated H-Ras, particularly those that might also be involved in the UV-irradiation resistance, using ponasterone A regulatable oncogenic H-Ras-expressing corneal cells. The results show that the oncogenic H-Ras-inducible protein Ku80 can function directly as a survival effector on the oncogenic H-Ras expressing cells exposed to UV irradiation.

II. MATERIALS AND METHODS

Cell culture and DNA constructs. The corneal cells were maintained in Earle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/ml and 100 μg of streptomycin/ml (Life Technologies, Inc.). The cells were maintained in 5% CO_2/95% air at 37 °C in a humidified incubator. The constructs of the dominant positive V12-H-Ras are described elsewhere (22). The murine Ku80 cDNA, were amplified by RT-PCR using the Ku80 oligo primer (5`-ATGGCGTGGTCGG TAAATAAGGC-3`, 5`-CTATATCATGTCCAGTAAATCA-3`). pIND was supplied by Invitrogen (Carlsbad, CA).

Microarray Analysis The total RNA was isolated using a TriReagent (Sigma) and further purified with RNAEasy (Qiagen) according the manufacturer's instructions. Hybridization was performed with the cDNA from the oncogenic H-Ras expressing cells labeled with Cy5 and those from the control transfected samples labeled with Cy3. Scanning was carried out using a GenePix 4000A scanner (Axon Instruments, Inc., Foster City, CA), and image acquisition was performed using Axon GenePix image software. Analysis of the gene expression data was performed using the GeneSpring software (SiliconGenetics Inc., Redwood City, CA) and PathwayAssist (Ariadne Genomics, Rockville,

MD).

Statistical Analysis of Microarray Intensity-dependent normalization was also applied, where the ratio was reduced to the residual of the Lowess fit of the intensity versus the ratio curve. Statistical analysis was performed using a Student t-test with a p-value of 0.05 with the additional criteria of the Ras expressing cells being either 1.5-fold higher or lower than the control transfected cells. The genes that met these parameters were classified by a molecular function using the annotations from Silicon Genetics or Ariadne Genomics.

Northern blotting. The total RNA was prepared using Trizol (Gibco-BRL), separated by electrophoresis and transferred to a nitrocellulose filter in 20× SSC, and then baked at 80°C for 2 h. The filters were hybridized using a p^{32}-labeled mouse Ku80 cDNA probe. After hybridization, the same membrane was re-probed with a p^{32}-labeled β-actin cDNA probe. Hybridization were carried out in 50 % formamide, 10 % dextran sulfate, 1% SDS, 1M NaCl at 42 °C for 16 h, followed by two 10 min washes at room temperature with 2x SSC and one 30 min wash at 65 °C in 2x SSC-1% SDS.

DNA-PK assays. The DNA-PK pull-down assays were performed as described previously (23). Briefly, the whole cell extract was incubated with

pre-swollen dsDNA-cellulose (Sigma). The DNA cellulose was washed twice in a buffer and the samples were divided into three aliquots. 0.5 µl [gamma]-^{32}P]ATP (300 Ci/mmol) was added, and the kinase assays were conducted in the presence of 4 nmol of the peptide (0.2 mM) in a total volume of 20 µl for 10 min at 30°C. The reactions were quenched by adding an equal volume of 30% acetic acid and analyzed by spotting onto phosphocellulose paper, washing and subjecting them to liquid scintillation counting. The amino-acid sequences of the modified p53 amino-terminal substrate (wild-type) and mutant p53 peptides were EPPLSQEAFALLKK and EPPLSEQAFALLKK, respectively. All the assays were carried out several times using at least three different extract preparations.

Cell-survival assays. The exponentially growing cells were irradiated with UV. The cells (5,000–15,000 per well) were plated in 6-well Falcon plates and incubated for 2–3 weeks. After staining with methylene blue, colonies of more than 50 cells were counted under magnification. The surviving fraction was calculated as follow: number of colonies formed /(number of cells plated x plating efficiency). Each point on the survival curve represents the mean surviving fraction from at least three dishes.

Pulsed-field gel electrophoresis. Cells were irradiated at a dose of 40 Gy and then cultured at 37 °C. Thirty to 180 min later, the cells were harvested. Samples

were resuspended at 10^7 cells ml^{-1} in 1.0% low-melting-point agarose, and cast into an agarose plug (80 μl). Clamped homogenous electric field (CHEF) gel electrophoresis (CHEF-DRII, Bio-Rad) was used to separate intact and repaired double-stranded DNA (100V, pulsed from 200-1800s for 96 h). The fraction of DNA migrating from the plug into the lane (% DNA extracted) was measured using a UV-transilluminator (312 nM) and image analysis using commercially available software. The fraction of the remaining double-strand DNA breaks was determined as the integrated density value (IDV) of the unrepaired DNA in the lane/total DNA in the lane plus the DNA in the well (24).

Western blotting. The cells were washed with PBS, and lysed at 0 °C for 30 min in a lysis buffer (20 mM Hepes, PH 7.4, 2 mM EGTA, 50 mM-glycerol phosphate, 1% triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μ/ml aprotinin, 1 mM Na_3VO_4, and 5 mM NaF). The protein concentration was determined using a Bio-Rad dye-binding microassay (Bio-Rad, Hercules, CA, USA). Twenty μg of the protein per lane was electrophoresed on the 10% SDS polyacrylamide gels. The proteins were blotted onto the Hybon ECL membranes (Amersham-Phamacia, Biotech) and immunoblotting was performed using the anti-Ku80 and α-tubulin (Santa Cruz Biotech, CA, USA), and H-Ras antibodies (BD Biosciences, San

Diego, CA). The blotted proteins were detected using an enhanced chemiluminescence detect system (iNtRON, Biotech., Seoul, Korea).

Immunofluorescence. The paraformaldehyde-fixed cells were incubated with rabbit anti-mouse ku80 antibodies (Santa Cruz Biotechnology). Staining was visualized by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibodies (Vector, USA). The immunofluorescence images for the Ku80 proteins were obtained using FV300 laser microscopy (Olympus, Japan) at an excitation wavelength appropriate for FITC (488nm).

DNA end-binding assay. DNA end-binding assays were carried out according to the method described elsewhere (25). Briefly, the cell lysates were prepared from approximately 10^7 cells by lysis in a Nonidet P40 (NP40) lysis buffer (50 mM Tris, pH 8.0, 0.5% NP40, 150 mM NaCl, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin) on ice for 15 min. The NaCl concentration was adjusted to 500 mM by adding NaCl. The lysates were then pre-cleared by adjusting to 6% polyethylene glycol 8000 for 10 min on ice, and microcentrifugation for 15 min at 4°C, yielding extracts of ~5 mg/ml. The double-stranded, 56-bp DNA (5`-GATCAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCT CACTGAGGCC-3`) was end-labeled with T4 polynucleotide kinase in the

presence of [γ-^{32}P], and then incubated with the complementary oligonucleotide. The radiolabeled fragments were electrophoresed through 5% polyacrylamide gels and subsequently purified. For DNA end binding, 1-2 μg of the protein extract was mixed with 0.175 ng of the radiolabeled probe, 1 μg of the covalently closed circular plasmid DNA, in 1× binding buffer (20 mM Tris-Cl, pH 7.5,10 mM EDTA, 10% glycerol), 150 mM NaCl, in a volume of 10μl at room temperature for 10 min. The DNA end binding reactions were separated on 5% polyacrylamide gels in 1× TGE (50 mM Tris, 380 mM glycine, pH 8.5,10 mM EDTA). Anti-Ku80 antibodies were used for the supershifting experiments

SiRNAS. The sequences of the 21-nucleotide (nt) sense and antisense RNA with a 2-nt overhang composed of TT(DNA) are as follows: Ku80-siRNA1, 5`-ACAAAAUCCAGCCAAGUUCdTdT-3` for the Ku80 gene (nt 285-305), Ku80-siRNA2, 5`-ACUGAAGUUUCCAAAGAGGdTdT-3` for the Ku80 gene (nt 905-925) and LacZ siRNA, 5`-CGUACGCGGAAUACUUCGAdTdT-3` for the LacZ gene. These siRNAs were prepared by a transcription-based method using a Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. The cells were transfected with the siRNA duplexes using Oligofectamine (Invitrogen).

III. RESULTS

Oncogenic H-Ras confers UV resistance to corneal cells. Oncogenic H-Ras has been shown to confer UV resistance to the majority of cell types examined (2-12). In order to identify the potential oncogenic H-Ras target genes that might be involved in the oncogenic H-Ras-mediated increased UV resistance, the effect of oncogenic H-Ras on the UV-induced cytotoxicity was initially re-evaluated in the corneal cells. Therefore, the dominant active V12-H-Ras was subcloned into the vector pIND and formed pIND-Ras in order to control the generation of oncogenic H-Ras within these cells. Following the transfection and double selection using G418 (selection for pIND-Ras) and zeocin (selection for pVg-RXR) for five weeks, nine clones were isolated and the oncogenic H-Ras expression that could be turned on or off was analyzed using ponasterone A. Western blot analysis revealed that treating the corneal clone-7 with ponasterone A for 24 h resulted in the efficient induction of oncogenic H-Ras expression in a dose- and time-dependent manner (data not shown and see Fig. 2B). In order to examine the effect of the oncogenic H-Ras on the UV-induced cytotoxicity, the cells were subjected to a range of UV doses. The fraction of cells that survived the exposure to UV doses ranging from 200-800 cGy showed that the corneal clone-7 cells, which generated the oncogenic H-Ras as a result of the ponasterone A

treatment, were better protected from UV irradiation than the untreated cells (Fig 1). In the control experiments, both the empty vector pIND transfected corneal (corneal-P) cells treated with or without ponasterone A and the corneal clone-7 cells in the absence of ponasterone A showed a similar low level of protection.

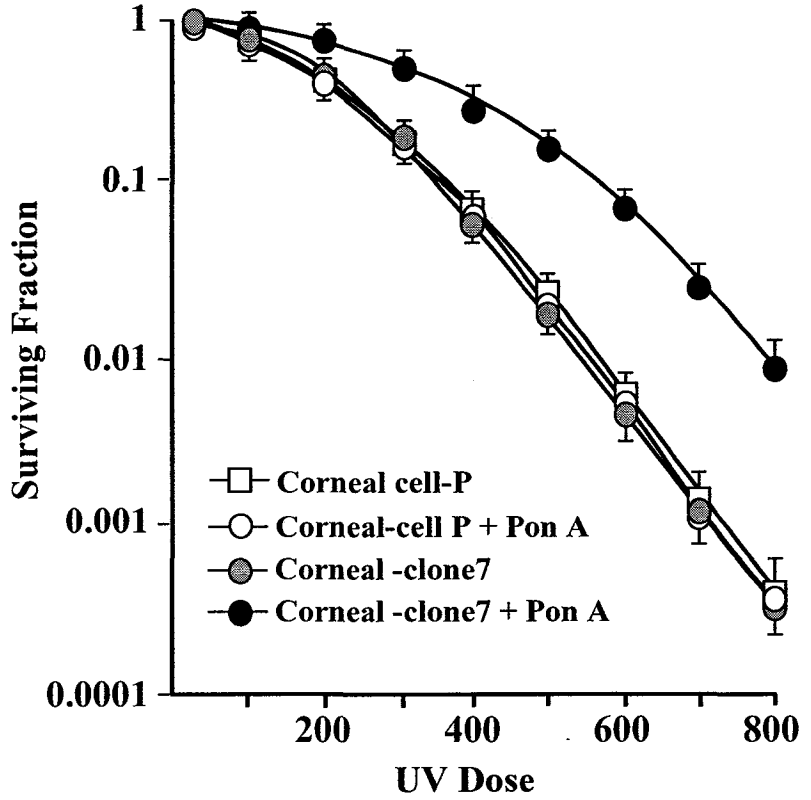


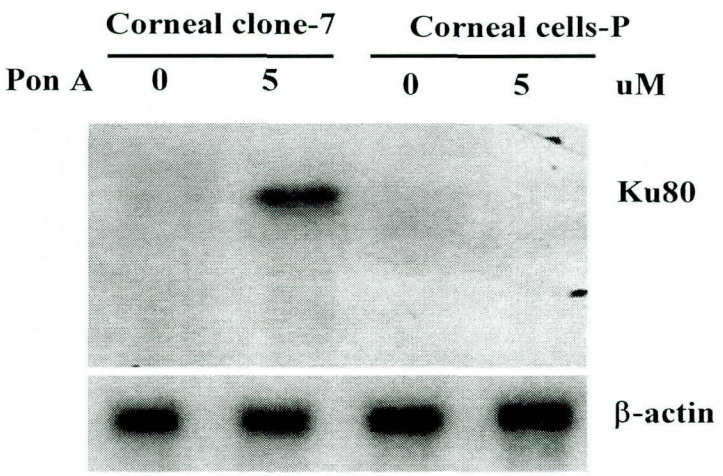
Fig. 1. UV-irradiation survival in oncogenic H-Ras expressing corneal cells. Survival rates of the empty vector pIND transfected corneal cells (corneal-P) and corneal clon-7 cells two weeks after UV irradiation. Open square, control corneal-P cells; open circles, control corneal-P cells treated with 5 μM ponasterone A; gray circles, untreated corneal clone-7 cells; black circles, oncogenic H-Ras expressing cells treated with 5 μM ponasterone A. The values represent the mean ± s.d. of three to four separate experiments.

Oncogenic H-Ras mediates an increase in Ku 80 expression. This study used a DNA chip array to compare the genes expressed in the presence or absence of ponasterone A. Two different populations of RNA were isolated, one from the corneal clone-7 cells, and the other from similar cells generating the oncogenic H-Ras after being treated with 5 μM ponasterone A for 24 h. The cDNA probes, prepared from the total RNA isolated from these cells, were labeled and for hybridization with a mouse cDNA array containing the 7.4 K genes. (GenePlorer TwinChip Mouse-7.4K, Digital Genomics). Among the upregulated genes detected, the transcript for Ku80 was found to increase in response to the oncogenic H-Ras induction. Northern blots were carried out using a probe for Ku80 to confirm that these changes in the hybridization signal on the DNA chip expression array correspond to changes in the mRNA abundance. As shown in Fig. 2A, 24 h after the 5 μM ponasterone A treatment in the corneal clone-7 cells, the level of the Ku80 transcript increased significantly. The level of Ku80 mRNA in the control cell line, corneal-P, remained unchanged after being treated with 5 μM ponasterone A. Experiments using shorter induction times (2 h or 6 h) also failed to produce any significant increase in the levels of Ku80 mRNA (data not shown).

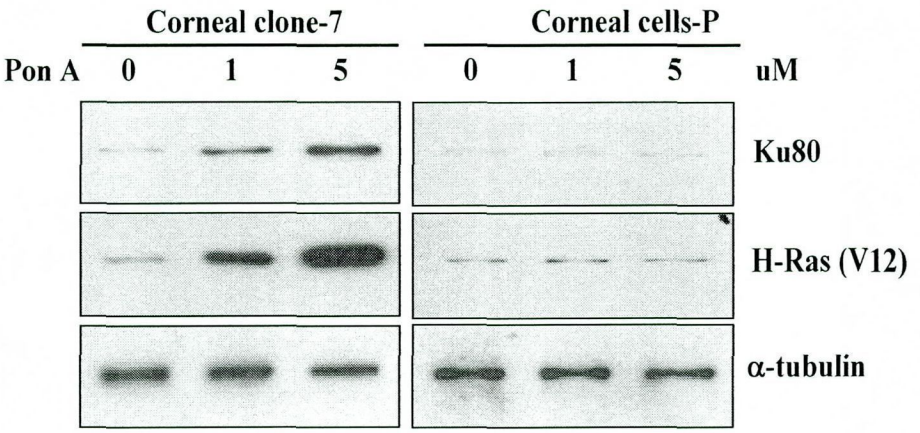
Western blot analysis was performed to determine if the increase in the Ku80 mRNA level corresponded to an increase in the Ku80 protein level.

SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) was used to separate the whole-cell extracts of the protein from the untreated cells, as well as the protein from two independent cells generating oncogenic H-Ras after being treated with 1 μM or 5 μM ponasterone A. Western blot analysis with Ku80 antibodies revealed that the level of the Ku80 protein was higher in the corneal clone-7 cells in response to ponasterone A. The largest increase in the Ku80 level was observed after a treatment with 5 μM ponasterone A for 24h (Fig. 2B). Immunohistochemical analysis showed that Ku80 level was significantly higher in the ponasterone A treated corneal clone 7 cells only (Fig. 2C). This demonstrates that Ku80 is up-regulated via the oncogenic H-Ras.

A


Corneal clone-7
Corneal cells-P
Pon A
0
5
0
5
uM
Ku80
β-actin

B


Corneal clone-7
Corneal cells-P
Pon A
0
1
5
0
1
5
uM
Ku80
H-Ras (V12)
α-tubulin

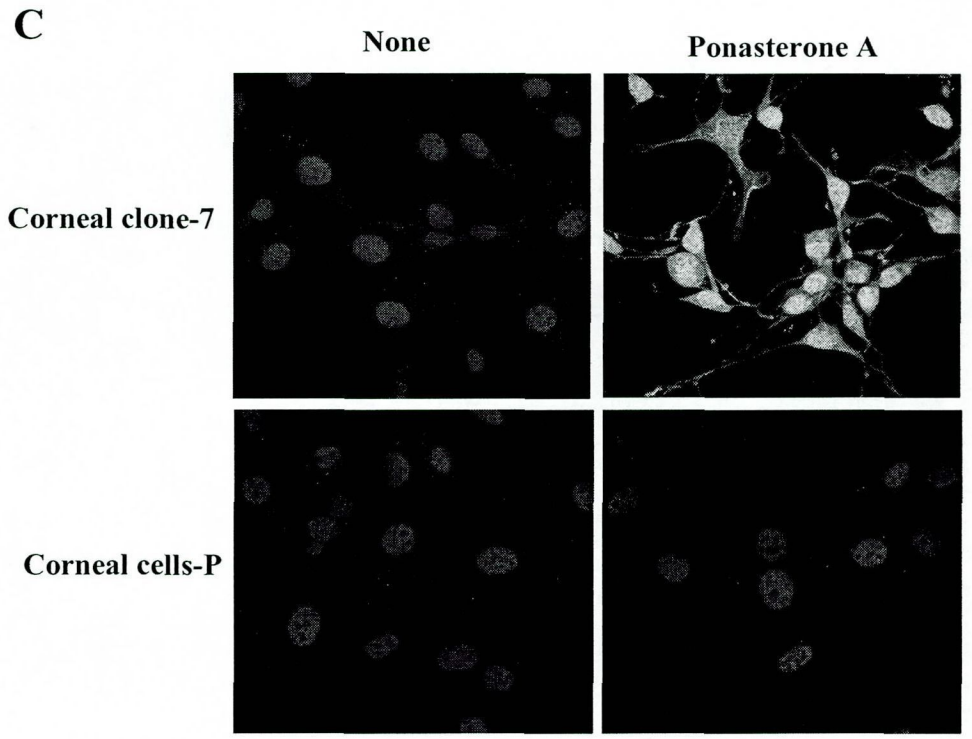


Fig. 2. Oncogeic H-Ras-induced Ku80 mRNA and protein expression. *A,* The empty vector pIND transfected corneal cells (corneal-P) and corneal clone-7 cells were treated with or without 5 μM ponasterone A for 24 h. Northern blots against the total RNA for the indicated proteins in corneal and corneal clone-7 cells grown in the absence or presence of 5 μM ponasterone A. *B,* The corneal-P and corneal clone-7 cells were treated with or without (indicated concentration)

ponasterone A for 24 h. Western blot experiments were performed with the antibodies to Ku80 and Ras and an antibody to α-tubulin as a control for an equal loading. *C,* The corneal-P and corneal clone-7 cells were treated with or without 5 μM ponasterone A for 24 h. The cells were then immunostained with the polyclonal antibody for Ku80 and observed by confocal microscopy.

Expression oncogenic H-Ras enhances DSBs repair in corneal cells.

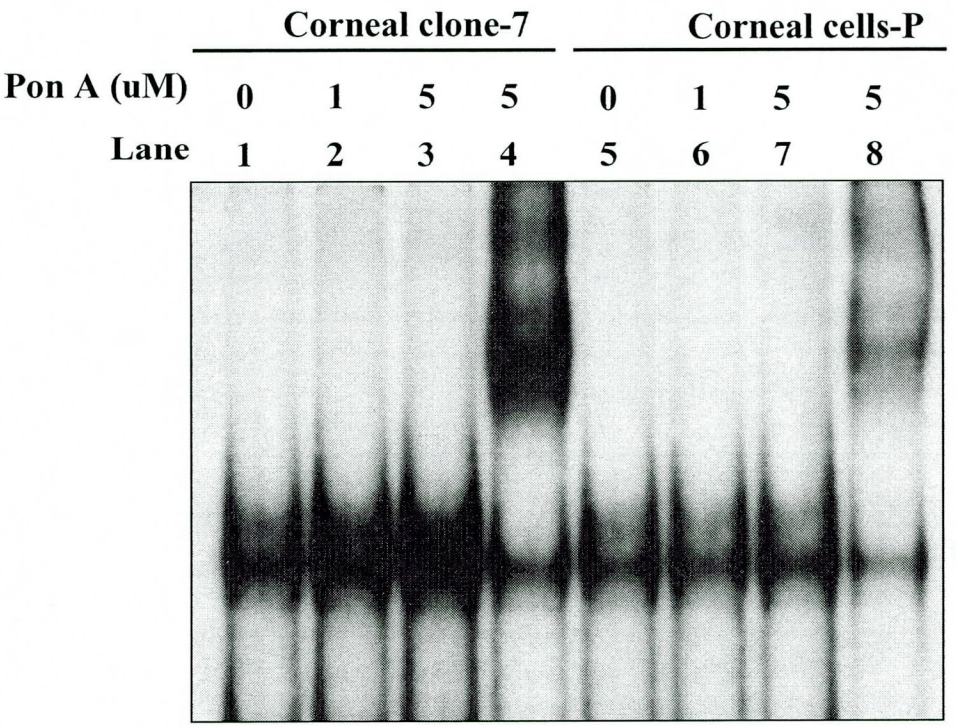
The DNA-binding activity of Ku80 was examined in order to determine the functional significance of the oncogenic H-Ras-mediated increase in Ku80 expression. Because Ku is known to have the major DNA end-binding activity in the whole cell extracts (24), the total cell extracts were prepared and examined by an electrophoresis mobility shift assay (EMSA) to determine the double strand DNA end-binding activity. As shown in Fig. 3A, the DNA binding activity of Ku80 increased as the ponasterone A concentration was increased. The specificity of the Ku80-dependent DNA end binding-activity demonstrated that the supershifted protein-DNA complexes could be observed after adding the anti-Ku80 antibodies to the DNA binding reaction. In contrast to the oncogenic H-Ras expressing cells, the DNA binding of Ku80 was unchanged in the parental corneal cells after the ponasterone A treatment.

This study next used pulsed-field gel electrophoresis to examine the ability of the oncogenic H-Ras-generating cells to repair the DSBs in their DNA, followingUV irradiation at 40 Gy. DSBs repair in the ponasterone A-treated, oncogenic H-Ras-generating corneal clone-7 cells were significantly enhanced (Fig. 3B). After irradiation, the percentage of DSBs remaining decreased rapidly up to 1 h, demonstrating the fast repair in the oncogenic H-Ras expressing cells,

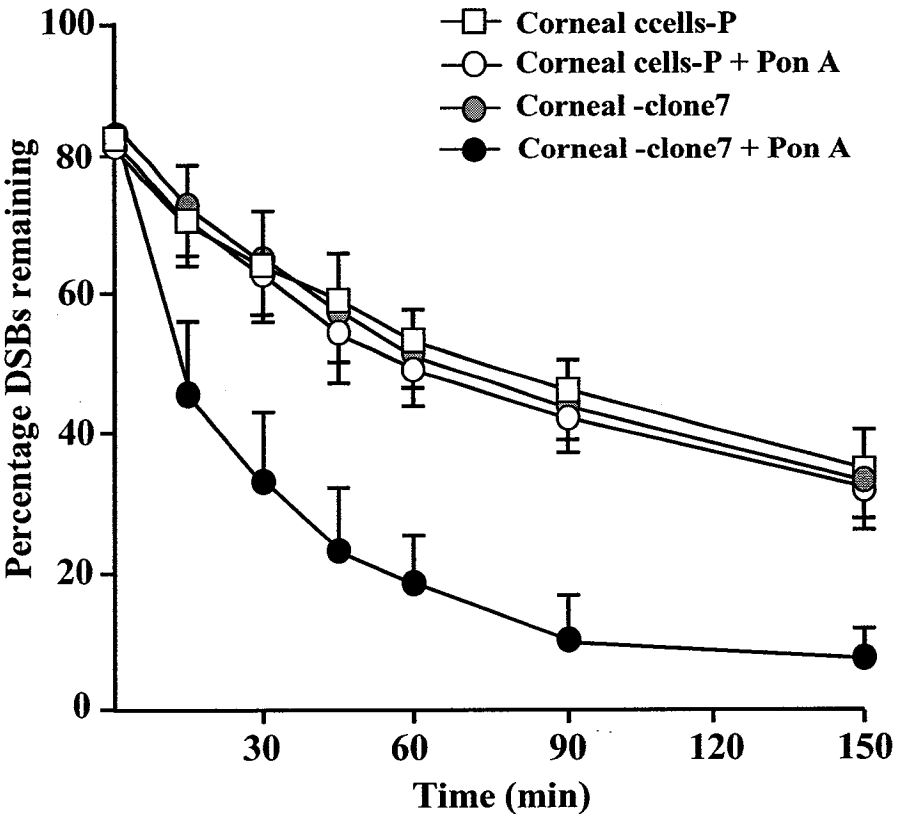
and after a 90-min repair period, these cells showed only 12 ±8 % DSBs. In contrast, both corneal-P cells treated with ponasterone A and corneal clone-7 cells in the absence of ponasterone A revealed similar low levels of DSBs repair. After a 90-min repair period, these cells showed 48 ±7 ~ 52 ± 9 % DSBs.

Previous work has shown that Ku80 plays an important role in activating DNA-PK both *in vitro* and *in vivo* (25, 26). Therefore, a DNA-PK 'pulldown' peptide assay was used to examine whether or not the up-regulation of the Ku80 levels induced by the oncogenic H-Ras affected the DNA-PK activity. In order to address this issue, the corneal clon-7 cells were treated with 1 μM and 5 μM ponasterone A and the DNA-PK activity was subsequently measured. As shown in Fig. 3C, the DNA-PK activity was significantly increased 2.2 ± 0.31 and 4.2 ± 0.57 times in the extracts from cells generating the oncogenic H-Ras after the 1 μM and 5 μM ponasterone A treatment, respectively, compared with the extracts from the untreated control cells. Overall, the up-regulation of Ku80 might contribute to the enhancement of the DSBs repair induced by oncogenic H-Ras in the corneal cells.

A


Corneal clone-7
Corneal cells-P
Pon A (uM)
0 1 5 5 0 1 5 5
Lane
1 2 3 4 5 6 7 8

B


Corneal ccells-P
Corneal cells-P + Pon A
Corneal -clone7
Corneal -clone7 + Pon A
Percentage DSBs remaining
100
80
60
40
20
0
30
60
90
120
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Time (min)

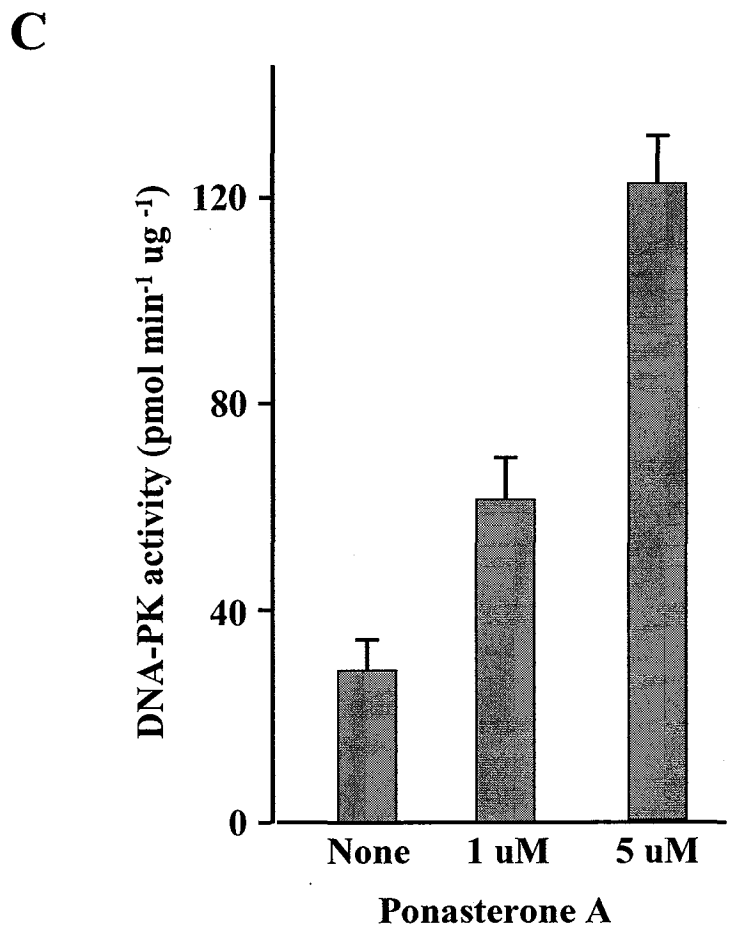


Fig. 3. Oncogenic H-Ras-mediated increase DNA double strand breaks repair. *A,* DNA end-binding activity of Ku80 in oncogenic H-Ras expressing corneal cells, as evaluated by EMSA. Lane 1, untreated corneal clnoe-7 cells; lane 2, corneal clone-7 cells treated with 1 μM ponasterone A for 24 h; lane 3, corneal clone-7 cells treated with 5 μM ponasterone A for 24 h; lane 4, corneal clone-7

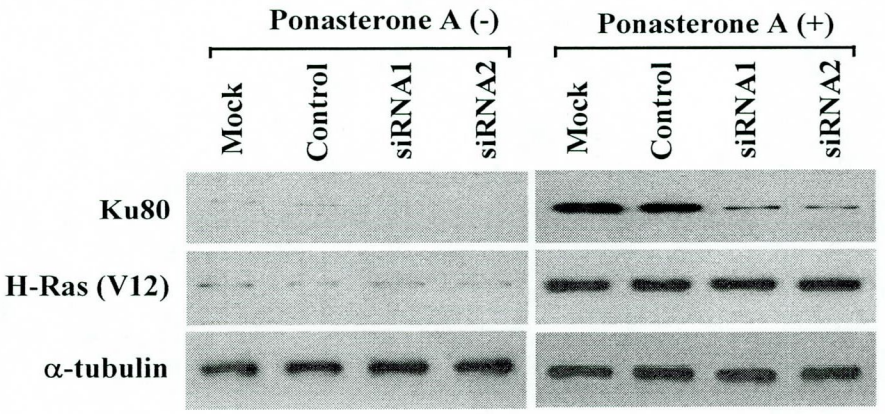
cells treated with 5 μM ponasterone A for 24 h + Ku80 antibody; Lane 5, control corneal-P cells; lane 6, corneal-P cells treated with 1 μM ponasterone A for 24 h; lane 7, corneal-P cells treated with 5 μM ponasterone A for 24 h; lane 8, corneal-P cells treated with 5 μM ponasterone A for 24 h + Ku80 antibodies. For the supershifts, the specific antibodies to Ku80 were added to the reaction mixture and incubated for 30 min prior to separating the DNA-protein complexes (lane 4 and 8). *B,* DNA double-strand breaks (DSBs) remaining in the empty vector pIND transfected corneal cells (corneal-P) and corneal clone-7 cells at the indicated times after a UV-irradiation. Open square, control corneal-P cells untreated with 5 μM ponasterone A; Open circles, control corneal-P cells treated with 5 μM ponasterone A; gray circles, untreated corneal clone-7 cells; black circles, oncogenic H-Ras expressing cells treated with 5 μM ponasterone A. The values represent the mean ± s.d. of three to four separate experiments. *C,* DNA–PK activity in the nuclear extracts from cells grown in the absence (None) or presence (indicated concentration) of ponasterone A. The values represent the means ±s.d. of three separate experiments.

Down-regulation of Ku80 suppresses the oncogenic H-Ras -induced DSBs repair. In order to determine if Ku80 is indeed involved in the oncogenic H-Ras-mediated increase in the DSBs repair capacity, small interfering RNAs (siRNAs) in the form of two independent, non-overlapping, 21-base pair RNA duplexes, which target Ku80, were used in an attempt to inhibit its expression. The oncogenic H-Ras expressing corneal cells and parental corneal cells were transfected with the mock, control siRNA oligonucleotide or the Ku80 specific siRNA oligonucleotides. The cells were harvested 48 h after transfection, and their protein expression levels were determined. Western blot analysis revealed that the Ku80-specific siRNA oligonucleotide levels had decreased by more than 80 % in terms of their overall Ku80 protein expression level compared with the mock or control siRNA-transfected cells (Fig. 4A). By 96-h after transfection, the Ku80 protein levels increased back to the levels comparable with the mock- and control siRNA-transfected cells (data not shown). This suggests that the Ku80-siRNAs are highly specific and efficient in Ku80 gene silencing in the oncogenic H-Ras expressing NIH 3T3 clone-7 cells.

The DSBs repair capacity after Ku80-siRNA transfection was examined in order to determine if Ku80 is involved in the oncogenic H-Ras-mediated increase in the DSBs repair. The results showed that the oncogenic H-Ras expressing cells

with reduced Ku-80 levels had significantly lower levels of DSBs repair activity when compared with the mock- or control siRNA-transfected cells (Fig. 4B), suggesting that Ku80 expression is important for the oncogenic H-Ras-mediated increase in the DSBs repair activity.

A


Ponasterone A (-)
Ponasterone A (+)
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siRNA1
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Ku80
H-Ras (V12)
α-tubulin

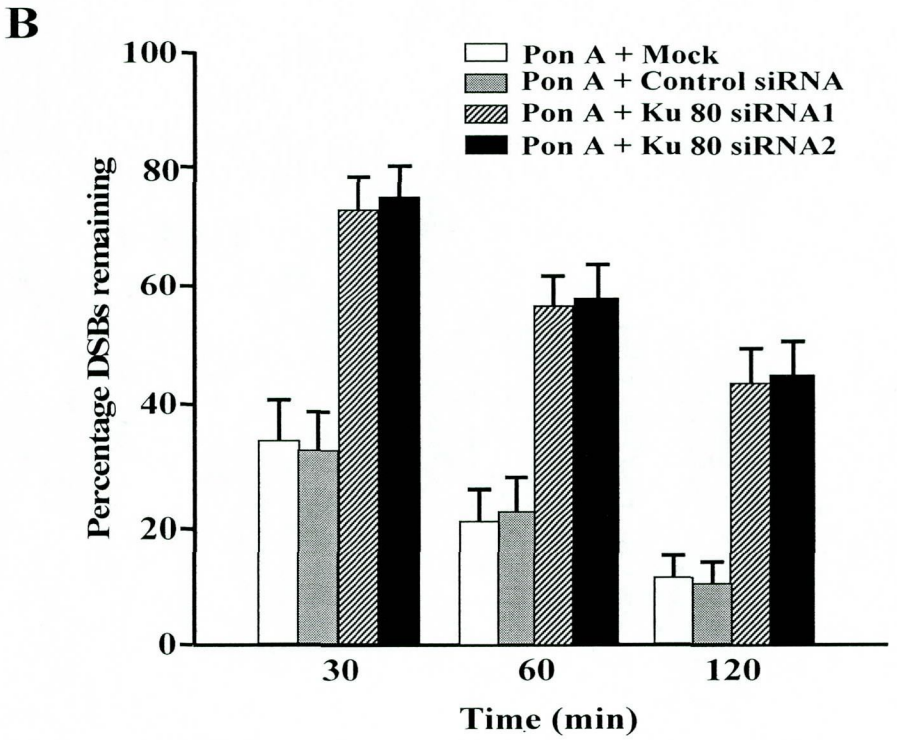


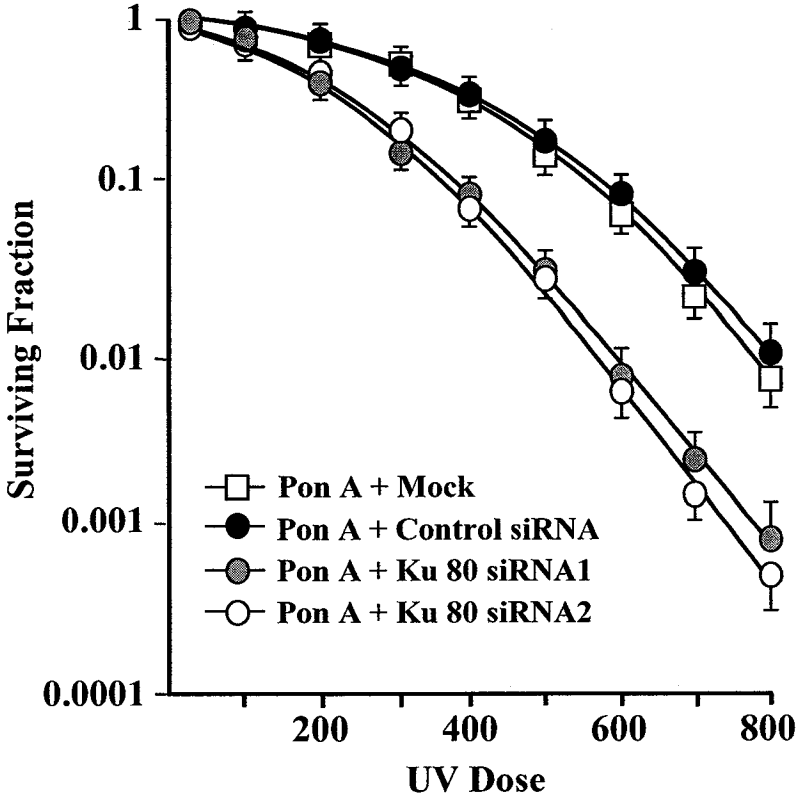
Fig. 4. Requirement of Ku80 for the oncogenic H-Ras-mediated increase in the DSBs repair in corneal cells. *A,* The mock-, control siRNA- or Ku80 siRNAs-transfected cells were incubated with (+) or without (–) 5 μM ponasterone A. 48 h after transfection, the total cell extracts were analyzed by Western blotting using the anti-Ku80 and anti-Ras antibodies. For the control experiment to have an equal loading, the membranes were re-probed with the anti-α-tubulin antibody. *B,* The mock-, control siRNA- or Ku80 siRNAs-treated corneal clone-7 cells were incubated with or without 5 μM ponasterone A (Pon

A) for 24 h. DNA double-strand breaks (DSBs) remaining in corneal clone-7 cells at the indicated times after irradiation with the 40 GyUV. The values represent the mean ± s.d. of three to four separate experiments.

Ku80 plays an essential role in oncogenic H-Ras-mediated cell survival against UV irradiation. In order to determine if this increase in Ku80 expression contributes to the UV resistance in the oncogenic H-Ras transfected cells, the oncogenic H-Ras expressing cells and parental corneal cells were transfected with the mock, control siRNA oligonucleotides or Ku80-specific siRNA oligonucleotides. Twenty four hours after transfection, the cells were irradiated with various of UV doses, and the cellular sensitivity was determined by a clonogenic survival assay. The oncogenic H-Ras expressing cells treated with the Ku80 siRNA oligonucleotides exhibited hypersensitivity to the lethal effects of UV irradiation compared with the mock or control siRNA-transfected cells (Fig. 5A). In the control experiments, the corneal clone-7 cells in the absence of ponasterone A showed a similar low level of protection (Fig. 5B).

In order to further confirm that Ku80 expression is important for the protection against UV irradiation in corneal cells, the murine ku80 was subcloned into the vector pcDNA3, to form pcDNA3-Ku80. This construct was transfected into the corneal cells. Nine stable transfected cell lines were established after selection using G418 for five weeks. Western blot analysis revealed several clones with a high Ku80 expression level (Fig. 5C). In order to test the functional significance of Ku80 expression, the corneal cloned cells were irradiated with

various UV doses and the cellular sensitivity was determined by a clonogenic survival assay. The cells transfected with Ku80 were more resistant to UV irradiation compared with the empty vector (pcDNA3)-transfected cells (Fig. 5D), which suggests that Ku80 expression contributes to the viability of corneal cells in response to UV irradiation.


1
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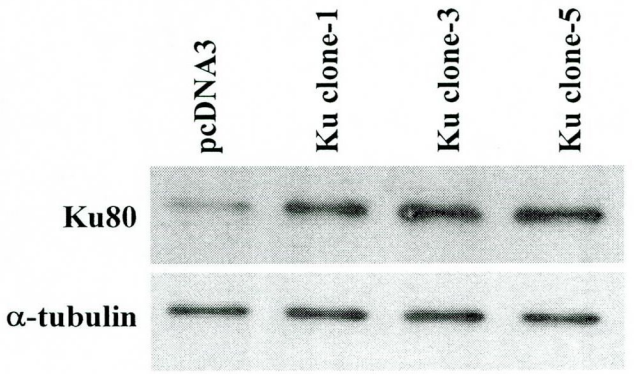
Pon A + Control siRNA
Pon A + Ku 80 siRNA1
Pon A + Ku 80 siRNA2

C



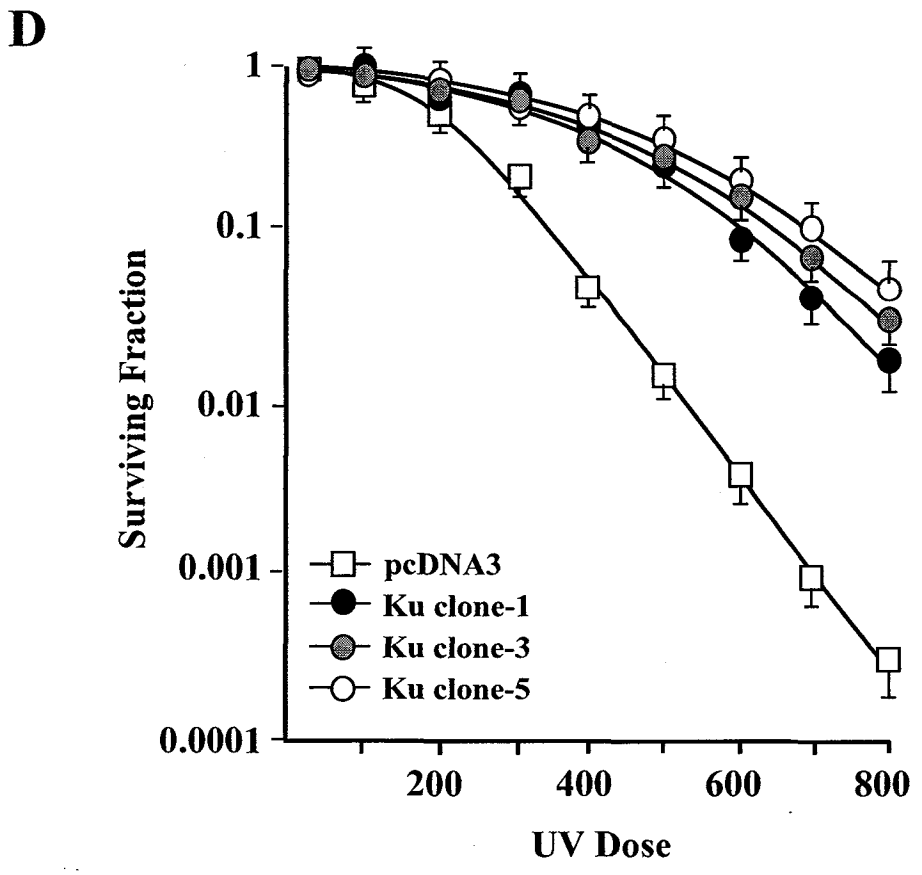


Fig. 5. Ku80 contributes to the oncogenic H-Ras-induced UV resistance. Reduced Ku80 expression results in UV irradiation sensitivity in oncogenic H-Ras expressing corneal cells. The mock-, control siRNA- or Ku80 siRNAs-transfected cells were incubated with (A) or without (B) 5 μM ponasterone A (Pon A) for 24 h. Subsequently, the cells were then treated with the indicated doses of UV

irradiation, and the cell viability was determined by a clonogenic survival assay. The values represent the means ±s.d. from four to five separate experiments. Detection by western blotting of Ku80 expression in three individual corneal + Ku80 clones (Ku-clone-1, Ku-clone-3 and Ku-clone-5) and one corneal + pcDNA3 clone (pcDNA3) (C). Three Ku80 expressing corneal clones (Ku-clone-1, Ku-clone-3 and Ku-clone-5) and one control clone (pcDNA3) were irradiated with various UV doses, and the cell viability was determined by a clonogenic survival assay (D). The values represent the means ±s.d. of four to three separated experiments.

IV. DISCUSSION

In the present study, we demonstrated that Ku80, which is a mammalian DNA repair gene, whose gene product has been shown to play an important role in repairing double strand breaks (DSBs) (27, 28), is the ultimate downstream target of oncogenic H-Ras in corneal cells. Using pulsed-field gel electrophoresis and cell survival assay, we have shown that the transient transfection of the activated H-Ras expressing cells with Ku80 siRNA causes the cells to reduce the oncogenic H-Ras-mediated increase in the DSBs repair activity and become highly sensitive to UV irradiation compared with the mock- and control siRNA-transfected cells. Subsequent studies revealed that the Ku80 expression in corneal cells leads to increased UV resistance.

Recently, several lines of evidence have suggested that activated Ras may be associated with the regulation of the DNA repair activity. For example, transformations by an activated Ras of the human epithelial HBL 100 cells resulted in less formation of cisplatin-induced interstrand cross-links as well as an increase in the DNA repair synthesis (29). Similarly, oncogenic Ras-transfected Syrian hamster Osaka-Kanazawa (SHOK) cells exhibited an increase resistance to cisplatin as well as a decrease in the intracellular platinum binding to DNA (30). In addition, the expression of the erbB-2 proto-oncogene, which encodes a 185

kDa transmembrane glycoprotein (p185) with a tyrosine kinase activity homologous to the EGF receptor, led to the direct regulation of the DNA repair mechanism, and the Ras-coupled pathway is important for modulating the DNA repair induced by erbB-2 (31). Using the host cell reactivation of the reporter gene expression from the UV-damaged plasmid and the unscheduled DNA synthesis (UDS) following the UV treatment of the cells, we previously demonstrated that activated H-Ras expression in corneal cells increased the DNA repair activity (22).

In order to address the question as to what kind of DNA repair protein might to be involved in the oncogenic H-Ras-mediated increase in the DNA repair activity, particularly those that might be also involved in the radiation resistance, we analyzed the effect of oncogenic H-Ras on the on gene expression, and have shown that oncogenic H-Ras mediates an increase in levels of Ku80 mRNA and Ku80 protein, which is a key component of the repair apparatus for DSBs in DNA. The exposure of mammalian cells to ionizing radiation induces lesions in the chromosomal DNA such as strand scissions, single-strand breaks, double strand breaks (DSBs), and base cross-links (32). Among the various forms of DNA damage produced by ionizing radiation, DSBs if not repaired, appear to be responsible for most of the radiation-induced cell death in yeast and mammalian

cells, because the DSBs disrupt the integrity of the genome (33, 34). Homologous recombination and non-homologous end-joining are the two principal pathways that mediate the repair of DSBs in eukaryotic organisms (35, 36). In yeast, homologous recombination is the major mechanism for DSB repair whereas, in mammalian cells, the predominant DSB repair system is the non-homologous end-joining pathway. One of the main participants in this pathway is the DNA-PK complex, which is composed of a catalytic subunit, DNA-PKcs, and a regulatory subunit, Ku70 (70kDa) and Ku80 (86kDa) (37-39). The Ku proteins were originally identified as autoantigens in patients with autoimmune disorders (40), and were found to bind tightly to the DNA ends in a manner independent of the structure of the end. Following the generation of DSBs, the Ku70-Ku80 complex binds to the free DNA ends and subsequently recruits and activates the DNA-PKcs at the site of DSBs (41-43). In addition to the regulatory function of the Ku80 protein in DNA-PK, Ku80 also has independent DNA repair functions, such as single-stranded DNA-dependent ATPase activity and the binding and repair of broken single-stranded nicks, gaps in the DNA, and a single-stranded to double-stranded transition in DNA (44). Using EMSA and pulsed-field gel electrophoresis after exposing the cells to ionizing radiation, it was found that the activated H-Ras expression in corneal cells increases the DNA binding activity of Ku80 and increases the DSB repair activity (Fig. 3). This suggests that Ku80 is a

downstream target protein of the oncogenic H-Ras, which at higher levels might contribute to UV resistance in oncogenic H-Ras transfected cells. However, several studies have suggested that Ras expression does not affect the DSB repair (45-47). This discrepancy with our results and with the findings of previous studies could possibly be because of: (*i*) cell type specificity; (*ii*) different expression level of oncogenic H-Ras; (*iii*) different status of the v-myc oncogene and p53 antioncogene, which may affect the DSB repair activity. Thus, this issue still needs to be studied further to assess the changes of DSB repair with oncogenic Ras in different cells.

Deficiencies in the DNA-PKcs activity are responsible for the lack of appropriate responses to the DSBs observed in the radiosensitive mammalian xrs-6 cell line and *scid* (severe combined immunodeficiency) mouse strain (23, 48, 49). The mutant phenotypes displayed by these cell lines and mice indicate that a loss of the DNA-PKcs catalytic activity results in defective DSB rejoining and an inability to facilitate V(D)J recombination. One of the phenotypic hallmarks of *scid* cells is the extreme radiosensitivity, indicating the requirement of DNA-PK activity in responding appropriately to any genome damage (50). Similarly, the cells deficient in the Ku80 protein are hypersensitive to ionizing radiation and were deficient in V(D)J recombination, which is a process that requires the

specific formation and rejoining of double-strand breaks (51-53). Moreover, when the Chinese hamster ovary cells lacking the functional Ku80 were transfected with the human chromosomal fragment coding for Ku80, V(D)J recombination and radiation sensitivity were restored to the normal levels (54). Therefore, the Ku80 proteins perform important role(s) in non-homologous end joining, which is the primary mode of DSB repair in mammalian cells, and control of UV resistance. In order to determine if the oncogenic H-Ras-mediated increase in the Ku80 expression contributes to the UV resistance, the oncogenic H-Ras expressing cells were transfected with the Ku80-specific siRNA, which targets the Ku80 and inhibits its expression. It was found that the transfection of cells with the Ku80-specific siRNA resulted in a decrease in the DSB repair activity following UV irradiation, compared with the mock- and control siRNA-transfected cells (Fig. 4). In addition, it was also found that the Ku80-targeted siRNA oligonucleotides caused the oncogenic H-Ras expressing corneal cells to be highly sensitive to UV irradiation, and that Ku80 expression caused the corneal cells to increase the viability, following UV irradiation (Fig. 5). Therefore, oncogenic H-Ras-mediated upregulation of Ku80 is involved in UV resistance, when oncogenic H-Ras is expressed.

Recent research has suggested a linkage between Ras-mediated UV resistance

and PI3K. For example, expression of active PI3K in cells with wild-type Ras results in enhanced radiation resistance, and this UV resistance can be inhibited by PI3K inhibitor (17). In addition, Grana *et al.* (19) have shown that the activated Ras transformed RIE-1 epithelial cells (RIE-Ras) exhibit resistant to radiation, and blocking PI3K activity with the inhibitor LY294002 sensitize RIE-Ras cells to radiation. Similarly, it has been suggested that EGRF-Ras-PI3K pathway might play an important role in mediating radiation resistance (18;20). The Ku80 promoter contains Sp1 binding sites. Although, little is known about what transcription factors actually participate in the Ku80 regulation, prior study suggests that a Sp1 transcription factor contributes to the Ku 80 expression (55). Sp1 is important for the expression of many cellular genes, particularly housekeeping genes. However, more recently Sp1 sites have been found to mediate transcription in response to diverse stimuli including oncogenes such as Ras and growth factors and cytokines (56). Regulation of Sp1-dependent transcription may be conveyed by changes in DNA binding activity, by association with other transcription factors, by changes in Sp1 abundance or in transactivation activity owing to biochemical modification, such as phosphorylation (56). Overexpression of Ras has been shown to mediate Sp1-dependent transcriptional activation, involving PI 3-kinase as important intermediary signaling molecules (57). PI3K contributes to the phosphorylation of Sp1, and Sp1 is involved in the

regulation of gene expression by the PI3K/Akt pathway (58, 59). Thus, we speculate that this might occur via phosphorylation of Sp1 leading to its increased binding to and transactivation of the Ku80 promoter. Further experiments are clearly needed to evaluate the effect of Ras-PI3K pathway on the Ku80 expression in the UV resistance of oncogenic Ras expressing corneal cells.

In summary, the sensitivity of cells to death by ionizing radiation is a critical determinant of the probability of a cure in patients receiving radiotherapy for cancer. One factor known to increase the survival of tumor cells after radiation is in the presence of activated oncogenes. Therefore, there is considerable interest in determining which genes mediate the altered UV resistance in tumor cells. This study found that Ku80 is induced by oncogenic H-Ras expression. The results suggest that Ku80 contributes to the oncogenic H-Ras-mediated increase in the capacity of corneal cells to repair DSBs and to afford protection against ionizing radiation. Under normal conditions, the action of the Ras and the other members of the Ras pathway were strictly regulated during the cell cycle and under different growth conditions (60). However, in a tumor cell, the oncogenic activation of *ras* is a consequence of point mutations that either impair the GTPase activity or enhance the GTP binding affinity, resulting in a highly active proliferative signal (61). Furthermore, *ras* mutations are found in a wide variety of

human cancers with the highest incidence being observed in ~30% of all human tumors (62). Therefore, the elevated Ku80 levels induced by Ras activation might confer cancer cell resistance to UV-irradiation. These findings, from a pathway involving H-Ras and Ku80, might be relevant to the development of new therapeutic approaches, in terms of protective agents in corneal cells against UV-irradiation (63, 64).

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