The Roles of p53, DNA Repair, and Oxidative Stress in Ultraviolet C Induction of Proliferating Cell Nuclear Antigen Expression

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ABSTRACT: The expression of proliferating cell nuclear antigen (PCNA) promoter was moderately induced in UV-irradiated, quiescent human and rodent cells. The induction was independent of tumor suppressor gene *p53*, because the PCNA expression was UV-inducible in the subclones of human fibroblasts in which the activity of p53 was abrogated by human papilloma virus E6. Furthermore, the induction did not depend on DNA repair, since PCNA was UV inducible in UVL-10 and *xrs*-6 cells, in which nucleotide excision repair and double-stranded repair, respectively, are largely compromised. However, the induction was inhibited by antioxidant *N*-acetylcysteine. The role of oxidative stress observed here is consistent with the previous finding that the proximal AP-1 site is critical to the UV inducibility of PCNA promoter.

KEYWORDS: UV induction; PCNA expression; p53; DNA repair; *N*-acetylcysteine; human fibroblasts

INTRODUCTION

Proliferating cell nuclear antigen (PCNA), also known as an auxiliary factor of DNA polymerase δ, is required in both DNA synthesis and DNA excision repair in eucaryotes (reviewed in Paunesku *et al.*[1]). UV irradiation is a common genotoxic agent that produces mutagenic lesions in DNA that are primarily repaired by nucleotide excision repair. Previous investigators have found that UV irradiation causes an increased nuclear PCNA staining in non-S-phase or quiescent cells. This process is not inhibited by cycloheximide or aphidicolin and has been thought to be a redistribution of preexisting PCNA to the sites of DNA damage.[2] In addition to this early response, an induction of PCNA expression has been observed in the UV-irradiated human cells.[3] A modest and transient increase of PCNA mRNA has been detected in quiescent human cells within 2 h after irradiation. We have found that the activity of rat PCNA promoter is UV inducible and UV inducibility requires a proximal AP-1 site between nucleotides −64 and −58.[4] The UV inducibility of rat PCNA promoter

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is p53 independent despite the role of p53 in maintaining genomic stability as a checkpoint control.

In this report, we are concerned with the mechanisms that regulate the UV induction of PCNA expression. We examined the role of p53 in the UV inducibility of PCNA expression by using a system in which cellular p53 activity was abrogated by human papilloma virus type 16 E6.[5] We also studied the role of DNA repair in the UV inducibility of PCNA expression by using variants of CHO.K1 cells where the DNA repair is defective.[6,7] In addition, we tested the role of oxidative stress by studying the effect of the antioxidant *N*-acetylcysteine (NAC) in the UV inducibility of PCNA. Our study suggests that oxidative stress, but not DNA checkpoint control, is essential to the UV induction of PCNA gene expression.

MATERIALS AND METHODS

Cell Lines

CHO.K1 cells derived from Chinese hamster ovary were obtained from ATCC. The UVL-10 and *xrs*-6 cells, variants of CHO.K1, were originally from Drs. Gerald M. Adair and Penny A. Jeggo, respectively. These cell lines were routinely cultured in α-MEM/F-10 (1:1) medium with 2.5% FCS and 2.5% bovine serum or McCoys' 5A medium with 5% FCS. Human diploid fibroblasts (HF) derived from neonatal foreskin and their retrovirus-infected subclones (E6-HF, see below), at lower than 25 passages, were grown in DMEM medium containing 10% fetal calf serum.

Construction of E6-Containing Human Fibroblasts

The retrovirus vector pLXSN16 and its packaging cell line NIH3T3/36 generating HPV 16 E6 recombinant retroviruses were used.[5] For infection of human fibroblasts, 1 mL of the amphotropic viral supernatant was combined with 3 mL of medium in the absence of polybrene and then added to subconfluent HF cells in a 100-mm dish for a one-day incubation. Subsequently, 4 mL of fresh medium was added and incubated for one day. Cells were screened by G418 (400 μg/mL) containing medium for at least 14 days, and the stable clones were pooled together and referred to as E6-HF cells.

UV Irradiation

Cells were washed with phosphate-buffered saline and, with the covers off, placed in marked areas in the tissue culture hood, which had been precalibrated for the required dose of UV using the germicidal lamp (254 nm) with the aid of a UV radiometer (UVP, San Gabriel, CA). The media that were removed before irradiation were added again after UV exposure, and the cells were harvested for analysis at certain intervals. Control cells received mock treatment without UV exposure.

Western Blot Analysis

Western blot analysis of PCNA was performed according to the standard protocol. Aliquots of crude extract with 50–80 μg and 180 μg were used for PCNA and

p53 detection and for $p21^{WAF1}$ expression on 12.5% or 10% SDS-PAGE, respectively. Protein blot was prepared by transferring proteins in gel to PVDF membrane. Antibodies for PCNA (Ab-1; Oncogene Science), p53 (Ab-6; Oncogene Science), and $p21^{WAF1}$ (C24420; Transduction Lab) were used. To detect the Ab:Ag complexes, an ECL detection kit (Amersham) was used according to the manufacturere's instructions.

RT-PCR

Total RNA was isolated according to the instructions of REzol (PROtech technologies, Inc.). The quantitative RT-PCR of PCNA mRNA was performed according to the manufacturer's instructions with 1 μg of total RNA, the specific primers, and the reagents supplied by the Promega company. The set of oligonucleotide primers for PCNA consisted of forward primer, 5′-TGGCGTGAACCTCACCAG-3′, and reverse primer, 5′-GGAGACAGTGAAGTGGCTTTTG-3′. The primer set for GAPDH was forward primer, 5′-ACCACAGTCCATGCCATCAC-3′, and reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′.

RESULTS

UV Induction of PCNA Expression and the Role of p53 in the Induction

As reported previously, PCNA gene expression can be induced in quiescent human cells. In this study, the cells were made quiescent by growing to confluence and being serum starved for two days before the UV irradiation. The induction of PCNA following the UV treatment was monitored by Western analysis. A modest but reproducible increase of PCNA level was observed (FIG. 1B, see HF). Previously, we showed that the UV inducibility of the rat PCNA promoter is independent on p53. To study the role of p53 in the UV-induced PCNA expression, an approach to abrogate the function of cellular p53 by E6 protein of human papilloma virus type 16 (HPV16)[5] was adopted. The HPV16 E6-containing human fibroblasts were constructed as described in the MATERIALS AND METHODS section. The establishment of E6-containing cells (E6-HF) was confirmed by examining the induction of p53 and $p21^{WAF1}$ after X-ray irradiation in the parental and HF-E6 cells (see FIG. 1A). Because $p21^{WAF1}$ is a downstream target of p53, its induction by DNA damage was used as a functional assay of p53. In contrast to those in HF cells, little or no expression of p53 and p21 was detected in HF-E6 cells (FIG. 1A). However, the UV induction of PCNA was observed in HF-E6 cells despite the lower basal level of PCNA in the cells (FIG. 1B, HF-E6). These data suggest that function of p53 is dispensable in the UV induction of PCNA.

The Role of DNA Repair in the UV Induction of PCNA

Although the UV induction of PCNA expression might be for the purpose of DNA repair, it is of interest whether UV induction of PCNA expression is DNA repair dependent, like serum induction of PCNA is DNA synthesis independent.[8] To address the question, we studied the UV inducibility of PCNA in UVL-10 cells and *xrs*-6 cells in which the ERCC1 and DNA-PK, respectively, are defective.[6,7] Both

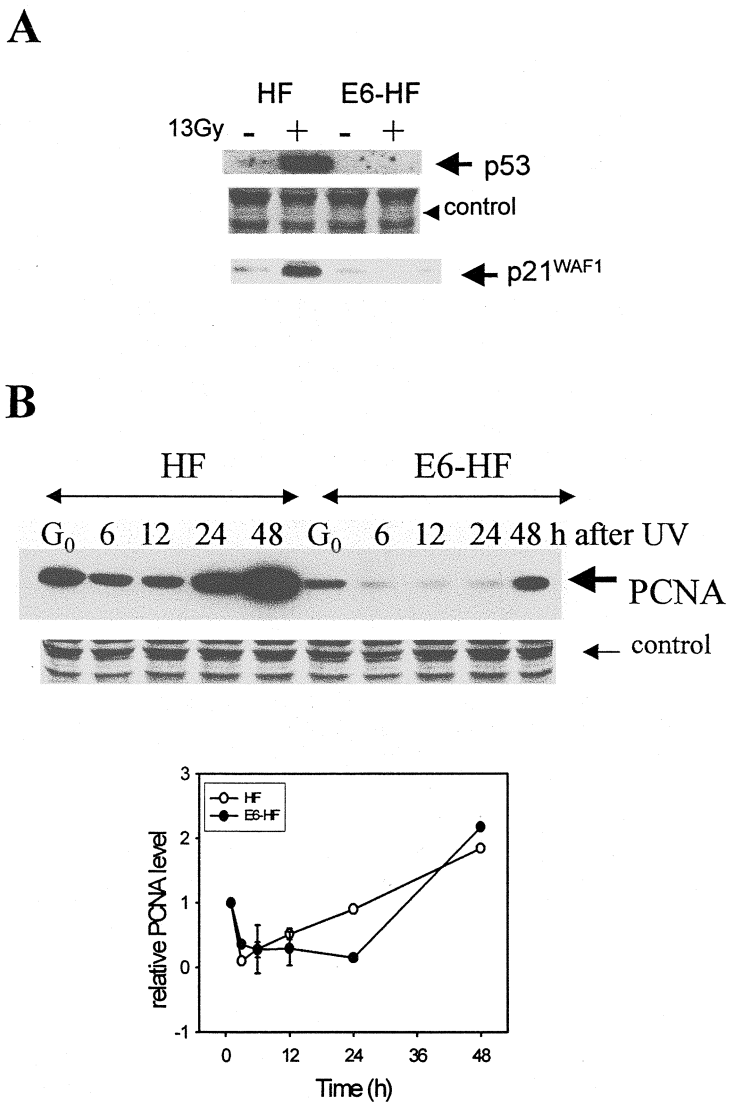

FIGURE 1. p53-independent UV inducibility of PCNA expression. **(A)** Establishment of HF-E6 cells. Western analysis of p53 and/or $p21^{WAF1}$ expression in HF cells and stable subclones of E6-HF following irradiation with 0 (–) or 13 (+) Gy X-rays. Cells were harvested for the Western analysis of p53 and p21 2 hr post irradiation. Samples were loaded in equal amounts as shown in Ponceau S staining. **(B)** Cells were grown to confluence and then serum starved for two days before UV irradiation (25 J/m^2). The cells were subsequently recovered in serum-free medium for 48 h before cell harvest for Western analysis of PCNA. Protein samples were loaded in equal amounts as shown by Ponceau S staining of the immunoblots. The cumulative results were plotted as the relative PCNA levels versus time.

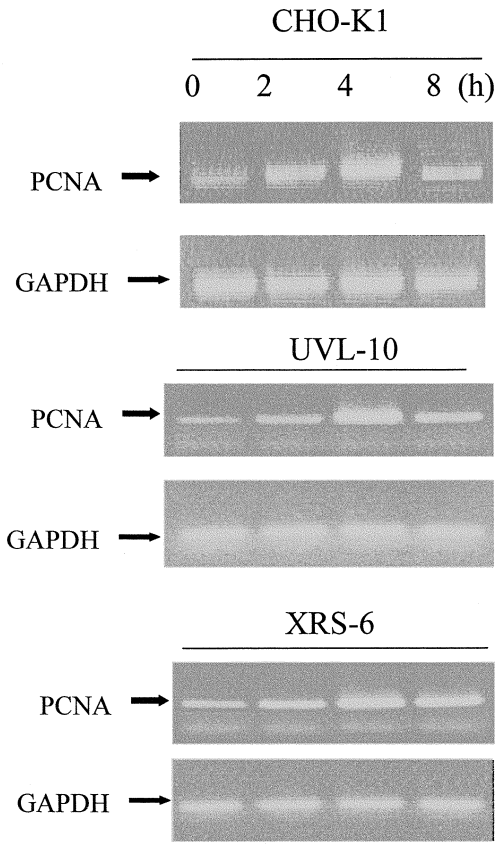

FIGURE 2. Quantitative RT-PCR of PCNA mRNA for studying the role of DNA repair in UV inducibility of PCNA expression. Cells (CHO.K1, UVL-10, and *xrs*-6) were made quiescent before UV irradiation (25 J/m^2) and were cultured in serum-free medium for the indicated interval (0–8 h) before the analysis of PCNA mRNA by RT-PCR procedure. Levels of GAPDH were used as the loading controls.

UVL-10 and *xrs*-6 are variants of CHO.K1 (Chinese hamster ovary) cells. ERCC1 is essential for nucleotide excision repair, while DNA-PK is involved in repair of DNA double-strand breaks and possibly in nucleotide excision repair as well. For this study, the cells were serum-starved for two days before the UV irradiation, and the UV induction of PCNA was monitored by RT-PCR as shown in FIGURE 2. The increase of PCNA gene expression was modest but repeatedly seen in CHO.K1 cells and its variants, suggesting that UV induction of PCNA does not depend on DNA repair. Consistent with these results, it was found that the UV induction of PCNA mRNA was not inhibited by DNA synthesis inhibitors such as aphidicolin (data not shown).

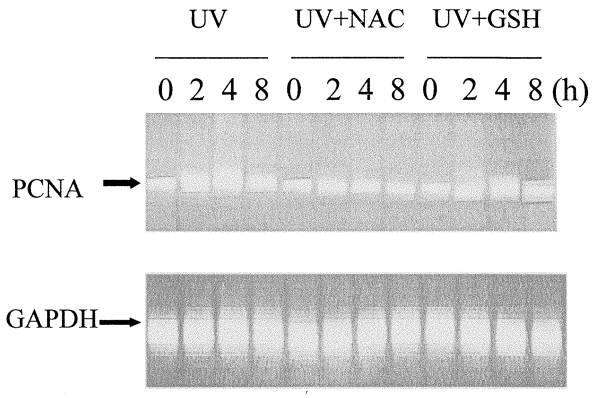

FIGURE 3. Quantitative RT-PCR of PCNA mRNA for studying the role of oxidative stress in UV inducibility of PCNA expression. As described in previous legends, CHO.K1 cells were serum-starved for quiescence before UV irradiation (25 J/m^2) and were then recovered in serum-free but 20 mM NAC- or GSH-containing medium (5 mM). At the indicated intervals (0–8 h), cells were harvested for studying the PCNA mRNA levels by the RT-PCR procedure.

The Role of Oxidative Stress in UV Induction of PCNA

Our previous study on PCNA promoter has indicated that AP-1 is essential in UV induction of PCNA. As NAC is commonly used to suppress the UV-induced activation of AP-1 transcription factor, we examined the effect of NAC on the UV inducibility of PCNA, indicating that NAC could suppress the UV induction of PCNA (FIG. 3). Interestingly, a less inhibitory effect on the UV induction of PCNA expression was seen with glutathione, suggesting that effect of NAC was probably not exerted via its conversion to glutathione.

DISCUSSION

UV Inducibility of PCNA

We have shown here that the PCNA in human or rodent cells is UV inducible (FIG. 1). A modest but reproducible and significant increase of PCNA at the protein or mRNA level was detected in the UV-irradiated cells. It should be noted that this induction is distinct from the UV induction of nuclear PCNA staining observed previously.[2] The latter is the increase of nuclear PCNA staining in UV-irradiated quiescent cells. This increase of PCNA staining is independent of *de novo* protein synthesis and has been thought to be a redistribution of preexisting PCNA to the sites of DNA damage. This induction takes place within *minutes*. In contrast, the induction described in this paper occurred in *hours* after UV irradiation and should be sensitive to protein synthesis inhibitor. Taken together, we think that UV irradiation

triggers an immediate response in cells by migrating the preexisting PCNA to the sites of DNA damage, while inducing a transient increase of PCNA expression as a delayed response. The induction of PCNA gene expression is probably to make up for the loss of the protein due to the UV damage.

The Role of p53

Our study indicates that p53 is dispensable in UV induction of PCNA in human cells (FIG. 1), although E6 may affect other cellular components besides p53 that might affect the expression of PCNA. Although human PCNA promoter contains a p53 binding element,[9] the promoter activity is modulated by p53, according to the *in vitro* studies,[10] in a concentration-dependent manner: that is, lower levels of p53 activate the promoter, whereas higher levels inhibit the promoter. Direct evidence between p53 and UV induction of endogenous PCNA remains elusive. Further investigations with a different approach—for example, negative-dominant p53 mutants—are necessary for clarification.

The Role of DNA Repair

Our study indicates that PCNA remained UV inducible in cells where DNA repair was deficient (FIG. 2) or DNA synthesis was blocked. Interestingly, previous investigators have shown an increased nuclear PCNA staining in UV-irradiated quiescent human fibroblasts in the presence of aphilicodin, an inhibitor of DNA polymerase α and δ.[2] These observations suggest that signals that lead to the redistribution of preexisting PCNA to the sites of DNA damage or the induction of PCNA gene expression do not involve the DNA repair complexes.

The Role of Oxidative Stress

Our study indicates that UV induction of PCNA is inhibited by antioxidant NAC, and the inhibition was probably not via glutathione. NAC was able to inhibit the rat PCNA promoter activity (data not shown). Because the AP-1 site is essential to the UV inducibility of the PCNA promoter, we suspected that NAC inhibited UV induction of PCNA via its modulation of AP-1 activity.

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