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Active oxygen species mediate the solar ultraviolet radiation-dependent increase in the tumour suppressor protein p53 in human skin fibroblasts

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Abstract Active oxygen species mediate many of the biological consequences of exposing cultured human skin cells to solar ultraviolet (UV) radiation (290–380 nm). A critical step in the escape from the carcinogenic potential of UV radiation is mediated by the protein p53. P53 activates growth arrest, allowing for DNA repair, and apoptosis, which removes damaged cells. Here I show that p53 in cultured human skin fibroblasts is elevated after treatment with hydrogen peroxide, an oxidant produced in cells during exposure to solar UV radiation. Simulated solar UV radiation increased p53, and agents that scavenge active oxygen species, *N*-acetylcysteine, ascorbate and α-tocopherol, inhibited the increase. The generation of DNA single strand breaks has been proposed to be an important step in the pathway leading to the increase in p53 initiated by a variety of cytotoxic agents. In this study I show that compounds that allow the accumulation of DNA single strand breaks, ara c and hydroxyurea, enhanced the UVC radiation (254 nm)-dependent increase in p53, but had no effect on the solar UV radiation-dependent increase. Thus, while DNA single strand breaks are involved in the UVC radiation-dependent increase in p53, the increase caused by solar UV radiation occurs by an alternative mechanism involving active oxygen species.

Key words: Active oxygen species; UV radiation; p53; Antioxidants; Signal transduction

1. Introduction

Ultraviolet (UV) radiation (190–380 nm) is toxic to living organisms. DNA strongly absorbs radiation in the UVC and B radiation ranges (190–320 nm) and modifications to DNA are the life threatening lesions that occur on exposure of cells to these wavelengths [1]. It is fortunate for terrestrial life that, while the sun produces radiation over the entire UV range, the earth's atmosphere allows only wavelengths greater than 290 nm to reach the surface. The UV radiation that is relevant to life on earth is arbitrarily divided into the UVB (290–320 nm) and UVA (320–380 nm) ranges.

Exposure of cultured mammalian cells [2–6], or human skin [7,8], to UV radiation in either the C, B or A ranges elevates the transcription factor protein 53 (p53) in the nuclei. However, it is not known whether p53 is elevated in cultured human skin cells by radiation containing UVA and B in proportions that approximate those found in the solar radiation reaching the earth's surface. Genes that are transcriptionally activated by p53 include those whose protein products are involved in growth arrest and apoptosis [9,10]. These two processes have been proposed as important steps by which cells with potentially malignant modifications stop dividing and are removed [11]. Many of the agents that increase p53, including UVC and UVB radiation, also damage DNA. This damage, particularly where it involves single strand breaks, has been proposed as an important step in the signal transduction pathway leading to an increase in p53 [3,4]. However, a recent report by Renzing et al. [2] described that the UVC radiation-dependent increase in p53 in murine fibroblasts occurred independently of DNA damage, and was inhibited by the antioxidant *N*-acetylcysteine.

Hydrogen peroxide is generated in cultured human skin cells during UVA [12] and UVB [13] irradiation, and other active oxygen species, such as singlet oxygen, mediate many of the biological effects of solar UV radiation. Damage to membranes and killing of cultured human skin fibroblasts by UVA radiation occur via active oxygen species and protection is provided by a range of antioxidants [12,14,15]. Solar UV radiation depletes antioxidants in murine skin in situ [16], and killing of cultured human skin cells can be significantly enhanced if the intracellular antioxidant glutathione is depleted prior to irradiation [14]. Reactions of active oxygen species with cell membranes have been implicated in the solar UV radiation-dependent activation of the transcription factor NF κB [15], and singlet oxygen mediates UVA radiation-dependent cell killing [17], and activation of haemoxygenase and collagenase transcription [18,19].

In this study I examine whether hydrogen peroxide and UV radiation, containing UVA and UVB in proportions that approximate terrestrial solar radiation, can increase p53 in cultured human skin fibroblasts. I also examine the role active oxygen species and DNA single strand breaks have in the solar UV radiation-initiated signal transduction pathway leading to an increase in p53.

2. Methods

2.1. Cell culture

Human skin fibroblasts were derived from a foreskin removed during routine circumcision. Cells were cultured in Minimum Essential Medium with Earle's salts, supplemented with glutamine, penicillin, streptomycin, sodium bicarbonate and 15% foetal bovine serum (all from Life Technologies, Auckland, New Zealand). The cells were cultured as adherent monolayers in 9 cm diameter petrie dishes (Nunc, Roskilde, Denmark) and used in passages 7–18.

2.2. Cell treatments

2.2.1. Irradiation. When monolayers of cells reached approximately 75% confluency they were separated from their growth media, washed with phosphate buffered saline (PBS, 10 mM, pH 7.4) and UV irradiated in Hanks buffer (PBS containing calcium (100 mM), magnesium (50 mM) and glucose (100 mg ml^{-1})). The media was retained and added back to cells after irradiation. Solar UV radiation was simulated by a bank of four 100 W 'Cleo' fluorescent tubes (Philips,

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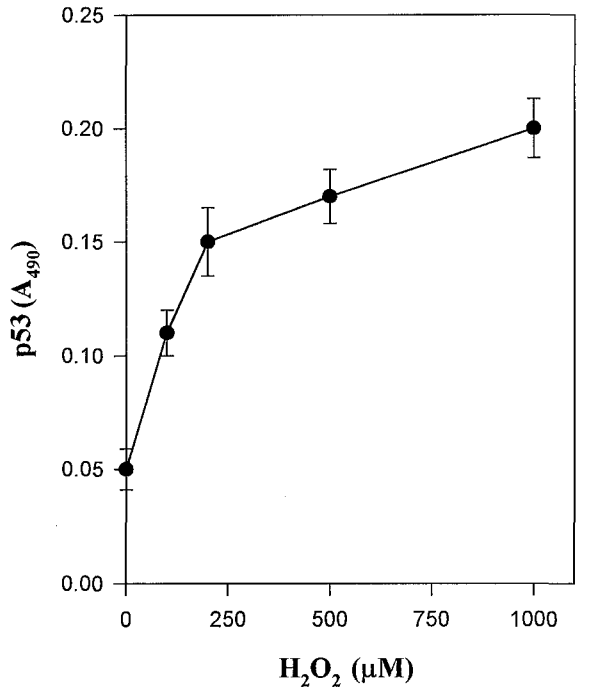

Fig. 1. The effect of hydrogen peroxide on p53 in human skin fibroblasts. Cells were treated for 30 min with hydrogen peroxide in Hanks buffer at the concentration shown and reincubated in media. Nuclear extracts of cells were made 24 h later and p53 was measured via ELISA. Each point represents the mean and standard deviation for 3 or 4 determinations.

Holland) which produced radiation from 290–420 nm at a dose of 0.5 W m^{-2} at 312 nm and 19 W m^{-2} at 365 nm as measured with a VLX-3W radiometer (Vilber Lourmat, Marne la Vallee, France). The proportion of radiation at 312 nm (UVB) compared to 365 nm (UVA) was similar to that occurring in sunlight at Christchurch, New Zealand (sea level, 43° South) at 10am on a clear day in October (spring).

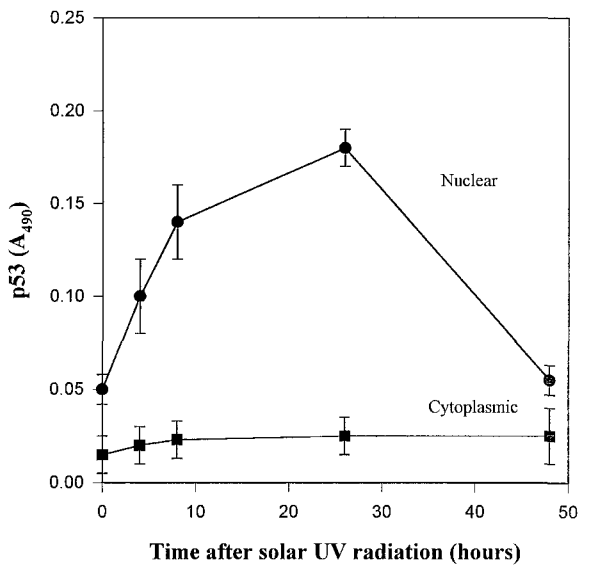

Fig. 2. Time course of the change in p53 in nuclear and cytoplasmic extracts of human skin fibroblasts exposed to simulated solar UV radiation. Cells were exposed to 70 kJ m^{-2} of simulated solar UV radiation (1.8 kJ m^{-2} at 312 nm and 68 kJ m^{-2} at 365 nm) and extracts were made at the times shown. Each point represents the mean and standard deviation for 3 determinations.

UVC radiation was produced by a germicidal lamp producing 0.086 W m^{-2} at 254 nm.

2.2.2. Biochemical. All biochemicals were from Sigma (St Louis, USA) unless otherwise stated. The DNA repair inhibitors ara c (1-[β-D-arabinofuranosyl]cytosine, 20 μM) and hydroxyurea (2 mM) were added to the media together immediately after irradiation and were present until the cells were harvested. *N*-Acetylcysteine and ascorbate were prepared in Hanks buffer. The pH of the *N*-acetylcysteine was adjusted to 7.4 with sodium hydroxide. α-Tocopherol was prepared in ethanol. These were added to the media of cultured cells either 4 h prior to irradiation and added back to the cells with the media following irradiation, or added to the media immediately after irradiation. Actinomycin D (5 μg ml^{-1}) was added directly to the media. Cells were treated with hydrogen peroxide in Hanks buffer for 30 min then washed thoroughly with PBS. Reserved media was added back to cells for continued incubation.

2.3. Cell extracts

Monolayers of cells were washed twice in PBS, harvested with a rubber policeman and centrifuged at 100×*g*. The cell pellets were lysed for 15 min at 4°C in HEPES buffer (10 mM, pH 7.9) containing Complete protease inhibitor (1.6 mg ml^{-1}) (Boehringer Mannheim, Mannheim, Germany), dithiothreitol (1 mM) and NP-40 (0.5%) and nuclei were pelleted at 14000×*g* for 20 min. The resulting supernatant (cytoplasmic extract) was stored at −80°C. The pelleted nuclei were lysed by resuspending for 30 min at 4°C in HEPES buffer (20 mM, pH 7.9), containing Complete protease inhibitor (1.6 mg ml^{-1}), dithiothreitol (1 mM) and sodium chloride (100 mM). The lysed nuclear suspension was centrifuged at 14000×*g* for 20 min at 4°C and the resulting supernatant (nuclear extract) was stored at −80°C. Protein content of the extracts was determined using the method of Bradford [20].

2.4. Enzyme linked immunosorbant assay

The immunosorbant method of Vojtesek et al. [21] was used to measure p53 in human skin fibroblast extracts. Briefly, 96 well plates (Nunc, Roskilde, Denmark) were coated with a monoclonal antibody to human recombinant p53 recognising both wild type and mutant p53 (DO7 from Novacastra Laboratories, Newcastle upon Tyne, UK, diluted 1:500 in PBS), and then with bovine serum albumin. Cell extracts containing 20 μg of protein were added to each well. The

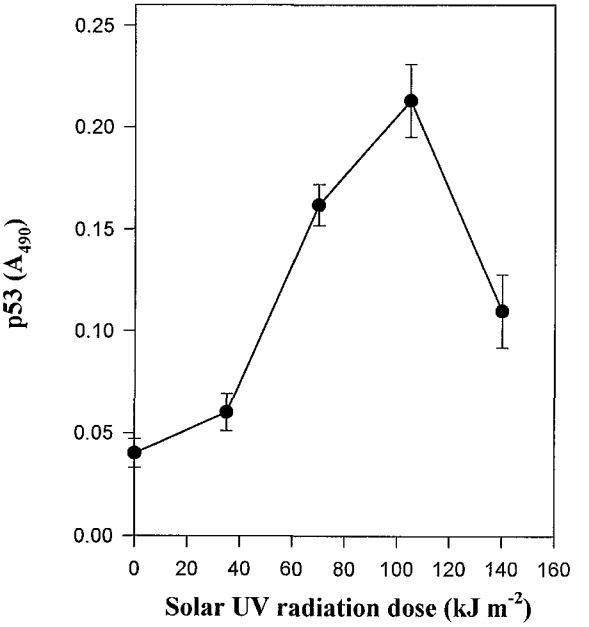

Fig. 3. The effect of simulated solar UV radiation dose on p53 in human skin fibroblasts. P53 in nuclear extracts of human skin fibroblasts was measured by ELISA 24 h after exposure to UV radiation. The dose shown is the total at 312 nm and 365 nm. Each point represents the mean and standard deviation of 4 determinations.

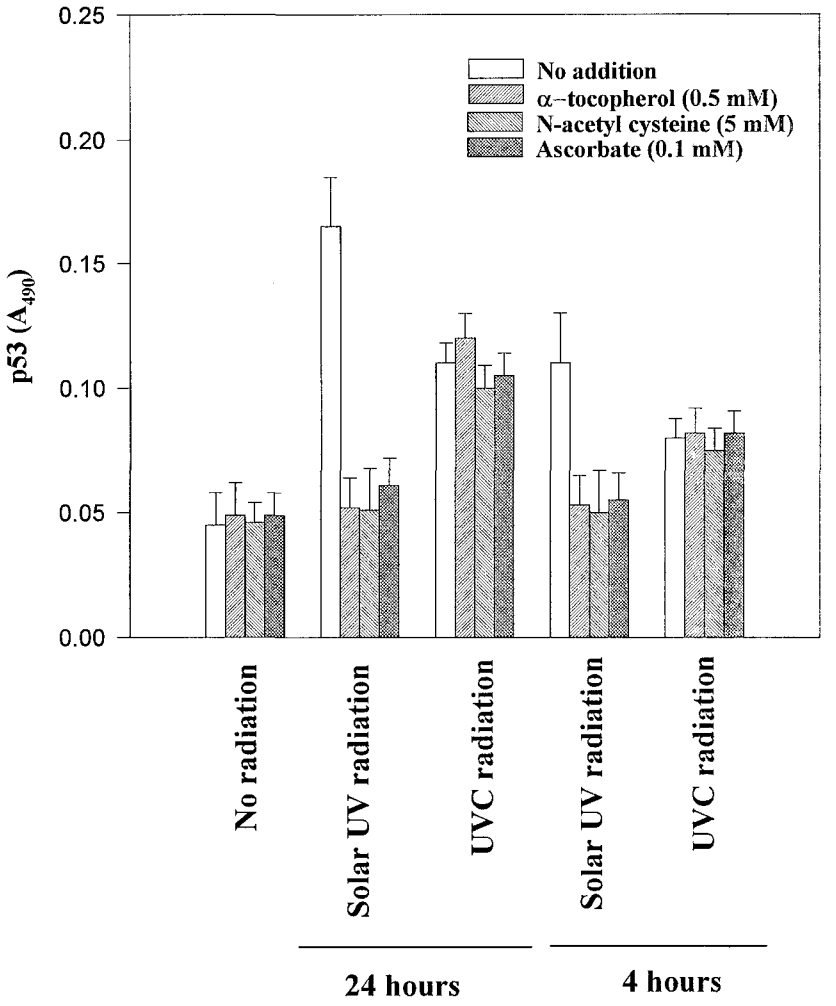

Fig. 4. The effect of antioxidants on the UV radiation-dependent increase in p53 in human skin fibroblasts. Cells were treated with the antioxidants *N*-acetylcysteine, α-tocopherol or ascorbate for 4 h prior to irradiation. Irradiation was either with simulated solar UV radiation (70 kJ m^{-2}, 1.8 kJ m^{-2} at 312 nm and 68 kJ m^{-2} at 365 nm), or UVC radiation (15 J m^{-2} at 254 nm). P53 in nuclear extracts of cells was measured by ELISA 24 or 4 h after irradiation. The results shown are the mean and standard deviation for 4 determinations.

polyclonal p53 antibody, CM1 (Novacastra Laboratories), diluted 1:1000 in 1% bovine serum albumin, was used as a secondary antibody followed by a peroxidase-conjugated anti rabbit IgG antibody. *o*-Phenylenediamine was used as a peroxidase substrate, and the absorbance was measured by an ELISA plate reader at 490 nm. Recombinant human p53 (Oncogene Science, Cambridge, USA) was used as a positive control for the ELISA.

3. Results and discussion

Many of the effects of solar UV radiation on cultured cells occur via active oxygen species. Hydrogen peroxide is one of the active oxygen species that is generated in cultured human skin cells during solar UV radiation [12,13]. It was added to fibroblasts to determine if it could alter the amount of p53 in the cells. Hydrogen peroxide increased p53 in nuclear extracts in a dose-dependent manner, with concentrations as low as 100 μM being effective (Fig. 1). Cells were exposed to simulated solar UV radiation to examine if radiation containing UVA and UVB at proportions that approximate those found in solar radiation could increase p53. A moderate dose of simulated solar UV radiation (70 kJ m^{-2}, 1.8 kJ m^{-2} at 312 nm and 68 kJ m^{-2} at 365 nm) elevated p53 in nuclear extracts of skin fibroblasts 3–4 fold over unirradiated levels as determined by enzyme linked immunosorbant assay (Table 1). This radiation dose is equivalent to approximately 60 min of solar UV radiation at sea level at 10.00 h on a clear day in spring at Christchurch, New Zealand. Positive results for recombinant p53, or for nuclear extracts of fibroblasts treated with actinomycin D and UVC radiation, both of which are known to increase p53 [3,4], confirmed the specificity of the assay (Table 1). P53 in nuclear extracts of cells was maximal 8–24 h after exposure to 70 kJ m^{-2} of solar UV radiation and returned to baseline 48 h after exposure (Fig. 2). To confirm that the site

Table 1
P53 in nuclear extracts of human skin fibroblasts 24 h after treatment with agents as shown

	A_{490}
Untreated	0.04 ± 0.01
Simulated solar UV radiation (70 kJ m^{-2})	0.15 ± 0.03
UVC radiation, 254 nm (15 J m^{-2})	0.11 ± 0.02
Actinomycin D (5 μg ml^{-1})	0.31 ± 0.05
Recombinant p53 (10 ng)	0.28 ± 0.02

Extracts contained 20 μg of protein. Absorbance values shown are the mean ± standard deviation of 4 measurements obtained with the ELISA method described in Section 2. The absorbance of a sample of purified recombinant human p53 is also shown.

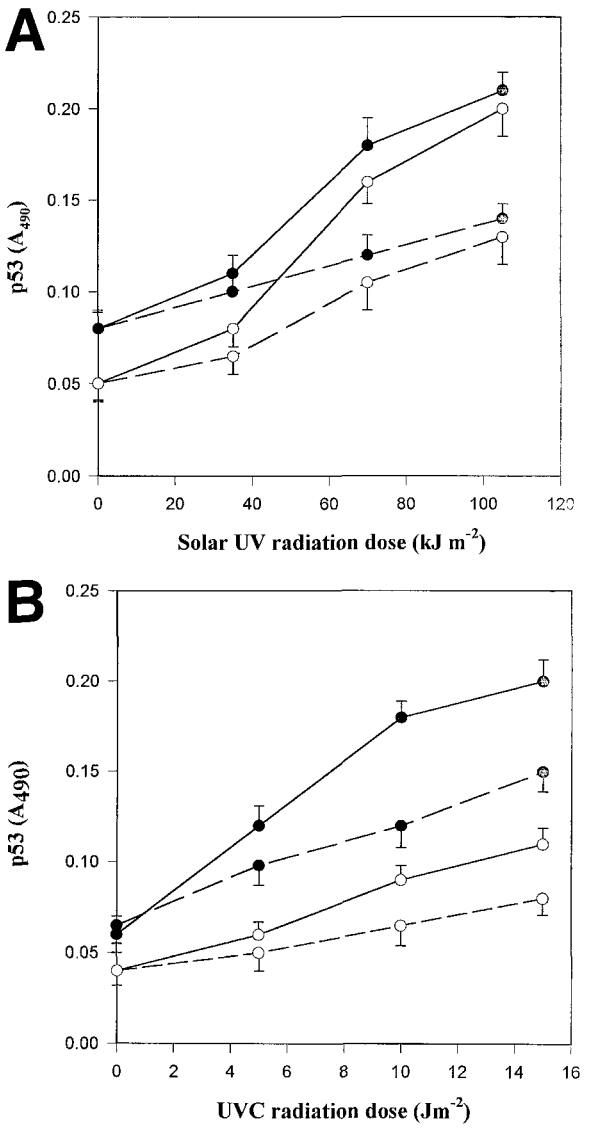

Fig. 5. (A) The effect of DNA repair inhibitors on the simulated solar UV radiation-dependent increase in p53 in human skin fibroblasts. The radiation dose shown is the total at 312 and 365 nm. P53 was measured by ELISA in nuclear extracts of cells irradiated then treated with (closed symbols) or without (open symbols) the DNA repair inhibitors hydroxyurea (2 mM) and ara c (20 μM). Extracts were made 24 h (solid line) or 4 h (dashed line) after irradiation. Values shown are the mean and standard deviation for 4 determinations. (B) The effect of DNA repair inhibitors on the UVC radiation-dependent increase in p53 in human skin fibroblasts. The radiation dose shown was measured at 254 nm. P53 was measured by ELISA in nuclear extracts of cells irradiated then treated with (closed symbols) or without (open symbols) the DNA repair inhibitors hydroxyurea (2 mM) and ara c (20 μM). Extracts were made 24 h (solid line) or 4 h (dashed line) after irradiation. Values shown are the mean and standard deviation for 4 determinations.

of accumulation of p53 after UV radiation was the nucleus, cytoplasmic extracts were also analysed. As shown in Fig. 2, solar UV radiation had no effect on the p53 concentration in cytoplasmic extracts of human skin fibroblasts measured over a 48 h period. Simulated solar UV radiation elevated p53 in a dose-dependent manner to a maximum at approximately 105 kJ m^{-2} (2.7 kJ m^{-2} at 312 nm and 102.6 kJ m^{-2} at 365 nm) (Fig. 3). At higher doses, the increase in p53 declined. This study has determined that radiation containing UVB and UVA at proportions that approximate those found in solar radiation, and hydrogen peroxide, an oxidant generated by solar UV radiation, can increase p53 in human skin fibroblasts.

To determine the role of active oxygen species in the solar UV radiation-dependent increase in p53, skin fibroblasts were treated with the *N*-acetylcysteine, α-tocopherol or ascorbate, prior to irradiation. These antioxidants were chosen because of their ability to inhibit the effects of solar UV radiation on cultured cells, at the concentrations selected. *N*-Acetylcysteine is taken up by cells and increases the intracellular antioxidant glutathione [22]. In cultured human skin fibroblasts glutathione is depleted during exposure to solar UV radiation [23], implicating it as an important compound in the protection of cells from active oxygen species generated by UV radiation. *N*-Acetylcysteine has been shown to inhibit the UVC radiation-dependent activation of p53 and other transcription factors [2,24,25]. α-Tocopherol is a lipid soluble antioxidant that is particularly good at inhibiting reactions of active oxygen species in cell membranes. It has been shown to inhibit solar UV radiation-dependent oxidation of membranes of cultured fibroblasts, and activation of NF κB [15]. In addition to regenerating oxidised α-tocopherol, ascorbate reacts with a wide range of active oxygen species [26]. All three antioxidants, added individually, markedly inhibited the increase in p53 measured either 4 or 24 h after irradiation (Fig. 4). α-Tocopherol absorbs radiation in the UVB region (absorption maximum at 292 nm). To ensure that the inhibition of the increase in p53 was due to the antioxidant activity of α-tocopherol and not its UV absorption, the radiation absorbed by a monolayer of fibroblasts treated with α-tocopherol (0.5 mM) was measured. At 312 nm the incident radiation was 0.5 W m^{-2} and the transmitted radiation was 0.45 W m^{-2}. This absorption would not account for the almost complete inhibition of the increase in p53 by α-tocopherol. None of the antioxidants inhibited the increase in p53 when added immediately after solar UV irradiation (data not shown), indicating that the generation of active oxygen species during irradiation was responsible.

To examine the role of active oxygen species in the UVC radiation-dependent increase in p53, human skin fibroblasts were treated with the same panel of antioxidants that were effective for solar UV radiation. In contrast, the increase in p53 after UVC radiation was not affected by antioxidants (Fig. 4), even when these antioxidants were used at 4-fold higher concentrations (data not shown). It is apparent that active oxygen species do not mediate the UVC radiation-dependent increase in p53 in cultured human skin fibroblasts however, inhibition of a UVC radiation-dependent increase in p53 by *N*-acetylcysteine in a sub-population of murine fibroblasts has been reported by Renzing et al. [2]. It is apparent that for human skin fibroblasts there are two radiation-dependent signal transduction pathways leading to an increase in p53, the solar UV radiation-initiated pathway mediated by active oxygen species.

Solar radiation at wavelengths that reach the earth's surface generates DNA single strand breaks [27,28]. Active oxygen species may be involved in this process [29]. To determine the role of single strand breaks in the solar UV radiation-dependent increase in p53, fibroblasts were treated with a combination of ara c and hydroxyurea immediately after irradiation, to inhibit the nucleotide excision repair process.

Treatment with hydroxyurea and ara c at the concentrations used would cause the accumulation of single strand breaks [30], and would be expected to augment the radiation-dependent increase in p53 if this type of DNA damage is involved. These two compounds increased the basal level of p53 in nuclei of unirradiated cells, however they did not affect the increase caused by solar UV radiation at either 4 or 24 h (Fig. 5A). Thus, single strand breaks do not mediate the solar UV radiation-dependent increase in p53. In contrast, hydroxyurea and ara c substantially enhanced the UVC radiation-dependent increase in p53 measured at either 4 or 24 h (Fig. 5B). This indicates that the two compounds were enhancing UV radiation-induced single strand breaks in the cells used in this study, and that single strand breaks are important in the UVC radiation-dependent increase in p53. Two observations from this study indicate that p53 in human skin fibroblasts can be increased by agents that primarily modify DNA. Firstly, p53 was increased in unirradiated fibroblasts treated with hydroxyurea and ara c (Fig. 5A and B). Secondly, actinomycin D, which intercalates into DNA, and UVC radiation, which causes a variety of DNA modifications including base dimerisation and strand breaks, increased p53 in the fibroblasts (Table 1). The considerable enhancement of the UVC radiation dose-dependent increase in p53 in cells treated with hydroxyurea and ara c confirmed that p53 in cultured human skin fibroblasts was able to be elevated by a DNA damage-dependent pathway as has been shown for other cells [3,4].

This study implicates active oxygen species as important intermediates in the solar UV radiation-dependent increase in p53 in human skin fibroblasts. P53 is a mediator of growth arrest and apoptosis, processes that permit repair or removal of damaged cells. Thus a consequence of the active oxygen species generated by solar UV radiation would be the initiation of a signal transduction pathway that would ultimately prevent the perpetuation of cell damage into subsequent cell generations.

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