BYSTANDER EFFECTS AND DNA REPAIR FOLLOWING UV EXPOSURE

AN EXAMINATION OF UV-INDUCED BYSTANDER EFFECTS AND THE REPAIR OF A UV-DAMAGED REPORTER GENE IN HUMAN CELLS

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ABSTRACT

We have used a non-replicating recombinant adenovirus, AdCA17*lacZ*, which expresses the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus immediate early (HCMV-IE) promoter, to examine host cell reactivation (HCR) of a UVC-damaged reporter gene in human fibroblasts. Since β-gal expression in mammalian cells is expected to occur following transcription of a lesion-free *lacZ* reporter gene, the removal of UV-induced DNA lesions and subsequent β-gal expression is a measure of the DNA repair capacity of the infected cell. In the present work, we have examined HCR and UV-enhanced HCR of the UV-damaged reporter construct, AdCA17*lacZ*, in normal and nucleotide excision repair (NER) deficient fibroblasts. Xeroderma pigmentosum (XP) group C and E fibroblasts are deficient in the global genome pathway (GGR) of NER, while Cockayne syndrome (CS) fibroblasts are deficient in the transcription-coupled repair pathway (TCR) of NER and XP group A fibroblasts are deficient in both TCR and GGR. HCR of the UV-damaged reporter gene activity was significantly reduced in XP-A, XP-C, XP-E and CS-B cell strains relative to that in normal human fibroblasts, indicating that both TCR and GGR contribute to expression of the UV-damaged reporter gene in human cells. Pre-treatment of human fibroblasts with UVC (12 J/m^2) immediately prior to infection results in enhanced HCR of the UV-damaged reporter gene. UV-enhanced HCR of the UV-damaged reporter gene was detected in normal fibroblasts at 12 hr post-infection and in CS-B, XP-C and XP-E fibroblasts, but not XP-A fibroblasts at 24 and 40 hr post-infection. These results indicate that both TCR and GGR are UV-inducible in human cells.

We have examined the colony survival of various human cells following exposure to UVC and UVA radiation. HaCAT, an immortalized human keratinocyte cell line, demonstrated increased resistance to UVC exposure compared to glioma (U373, T98G) and colon carcinoma (HT29) cell lines, while exhibiting increased sensitivity to UVA radiation and H_2O_2 treatment. This increased sensitivity towards both UVA and H_2O_2 treatment suggests that HaCAT cells have a reduced capacity to repair oxidative DNA damage. In addition, we show that p53-null keratinocytes (HPV-G) have reduced clonogenic survival compared to p53-mutant keratinocytes (HaCAT) following H_2O_2 treatment, consistent with an involvement of p53 in the survival of keratinocytes following H_2O_2 treatment. We also examined whether the interaction of H_2O_2 with growth media resulted in chemical by-products that were toxic to cells. Even though the H_2O_2 levels in the media were reduced following 24 hr incubation, similar clonogenic survival curves of HaCAT cells were observed following treatment of cells with immediately-prepared or 24hr-incubated H_2O_2-containing media solutions. Thus, extended incubation (24 hr) of H_2O_2 with media did not alter its cytotoxicity towards HaCAT cells, indicating that no detectable levels of toxic chemical by-products were produced.

Ultraviolet irradiation of cells can have both cytotoxic and/or mutagenic consequences, resulting in increased levels of cell death or the induction of a state of genomic instability that persists for several cell generations after irradiation. However, the extent of the effects induced in non-irradiated cells by UV-irradiated cells has not been fully investigated. Using the medium transfer technique, we have tried to address

whether UV irradiation of cells can induce biological effects in non-irradiated cells. Medium obtained from UVA, but not UVC, irradiation of various human cell lines in phosphate-buffered saline (PBS) solution was capable of reducing the relative clonogenic survival and colony size of non-irradiated HaCAT cells. In the absence of cells, UVA-irradiation of PBS reduced the clonogenic survival, but not the colony size, of various non-irradiated human cells. These results indicate a cytotoxicity of UVA-irradiated PBS towards non-irradiated cells. Hydrogen peroxide, a reactive oxygen species (ROS) generated following UVA irradiation, was measured following UVA treatment (or mock-treatment) of PBS (in the presence or absence of cells) either immediately or 24 hr post-irradiation. Hydrogen peroxide levels increased immediately following UVA irradiation, suggesting that it may contribute to the reduced survival of non-irradiated human cells. However, human glioma T98G and U373 cells produced elevated H_2O_2 levels in mock-irradiated conditions at 24 hr post-incubation, while demonstrating different sensitivities towards treatment with medium containing UVA-irradiated PBS. These results suggest that UVA-induced H_2O_2 is not responsible for the reduction in clonogenic survival of non-irradiated human cells. We also examined colony number and size at different times following the addition of media containing UVA-irradiated PBS and media from UVA-irradiated cell cultures. Increasing the time before scoring for colonies resulted in an increased clonogenic survival and a decrease in relative colony size for HaCAT cells. These preliminary results suggest that the relative survival and relative colony size of non-irradiated cells are inter-related, and indicate that treating non-irradiated HaCAT cells with medium from UVA-irradiated cells or medium containing UVA-irradiated PBS

reduced the growth rate of HaCAT colonies. Therefore, in order to properly evaluate clonogenic survival, a time-dependent examination of relative survival and relative colony size should be conducted in order to address whether the treatment results in an inhibition of cell growth and/or true cell killing.

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LIST OF ABBREVIATIONS

6-4 PP	pyrimidine-pyrimidone (6-4) photoproduct
8-oxoG	8-oxoguanine (or 7,8-dihydro-8-oxoguanine)
ATP	adenosine triphosphate
Ad	adenovirus
α-MEM	α-minimal essential medium
β-gal	β-galactosidase
BER	base excision repair
CPD	cyclobutane pyrimidine dimer
CPRG	chlorophenol red β-D-galactopyranoside
CS	Cockayne syndrome
ddH_2O	distilled/deionized water
DNA	deoxyribonucleic acid
D_{37}	dose/fluence that gives a survival fraction of 0.37 (e^{-1})
FBS	fetal bovine serum
GGR	global genome repair
GJIC	gap junction intercellular communication
Gy	Gray
H_2O_2	hydrogen peroxide
HCMV-IE	human cytomegalovirus immediate early promoter
HCR	host cell reactivation
hHRAD23B	human homologue of the S. cerevisiae protein 23
HPV	human papillomavirus
hr	hour
ICCM	irradiated cell conditioned medium
IR	ionizing radiation
LET	linear energy transfer
LFS	Li Fraumeni syndrome
lacZ	gene that encodes for the β-galactosidase enzyme
MCMV-IE	murine cytomegalovirus immediate early promoter
MOI	multiplicity of infection
NER	nucleotide excision repair
PB	phosphate buffer solution
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
pfu	plaque forming units
polβ	DNA polymerase β
RNA	ribonucleic acid
RNAPII	RNA polymerase II
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute cell culture media
SF	survival fraction
TFIIH	transcription factor-II H

TCR	transcription coupled repair
Tg	thymine glycol
TTD	trichothiodystrophy
UV	ultraviolet radiation
XP	xeroderma pigmentosum

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Chapter 1

Introduction

1. Ultraviolet Radiation and Skin Cancer

Cells from living organisms are constantly threatened by the action of various environmental agents, which can interact with and modify their DNA structure. Ultraviolet radiation (UV), a component of sunlight, is one of these agents which poses a health risk to humans.

Ultraviolet radiation is divided into three regions, each of which has distinct biological effects: UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). UVC and the majority of UVB are effectively absorbed by the stratospheric ozone layer, preventing such radiation from reaching the earth's surface. The remaining portion of solar UV that penetrates the ozone layer, in amounts that have biological consequences, consists of UVA and UVB, with UVA constituting the of the solar UV radiation that reaches the earth's surface (reviewed in Matsumura and Ananthaswamy, 2004; Ravanat *et al.*, 2001a). While UVA accounts for 90-95% of the terrestrial UV, it accounts for less than 25% of the skin cancer risk. Although UVB comprises only ~5% of the UV radiation to reach the earth's surface, it is believed to account for the majority of the skin cancer risk in humans (IARC, 1992).

Human skin is a natural barrier against a variety of different types of external stress including UV exposure. However, chronic UV exposure can have detrimental effects to the skin. Therefore, the skin has developed various mechanisms to protect itself from the deleterious effects of UV. The epidermis (outermost layer of skin) is a thin, light-absorbing layer equipped with pigments for UV protection. UVB is largely absorbed by the upper layers of the epidermis, with little reaching the basal epidermal

cells or dermis (Bruls *et al.*, 1984). In contrast, UVA penetrates deeper in the skin, with considerable levels reaching the basal layer of the skin (Lim *et al.*, 2001).

Skin cancer is a common neoplasm caused by repeated sun exposure, especially in fair-skinned individuals world-wide, and is increasing at an alarming rate (4-6% per year) (Gloster and Brodland, 1996). The relaxed attitude towards sun protection and tanning, along with increases in UV fluence at the earth's surface due to stratospheric ozone depletion (de Gruijl *et al.*, 2003), may account for the recent increases in skin cancer prevalence caused by increased cutaneous UV exposure.

2. UV-mediated Photo-excitation of DNA

The majority of adverse effects associated with ultraviolet radiation may be attributed to the cytotoxic and mutagenic effects of UV-induced DNA damage. The biological effects of different UV wavelengths vary across the UV spectrum. Although UVC presents little biological relevance to humans on earth, it is a useful tool in demonstrating DNA damage resulting from direct absorption of photons by nucleotide bases. UVC and UVB radiation (245 to 290 nm) are absorbed maximally by DNA (Tornaletti and Pfeifer, 1996), capable of inducing mutagenic photolesions that cause inappropriate bonding between adjacent pyrimidines. The DNA damage induced by UVB and UVC consist of two classes of pyrimidine photoproducts: cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PP). Although CPDs are produced 3 times more often than 6-4PP, both photoproducts distort the double helical structure of DNA; 6-4PP are more pronounced, causing a 44° bending

among adjacent pyrimidines, while CPDs demonstrate less (7-9°) of a structural distortion (see Figure 1-1) (Kim *et al.*, 1995).

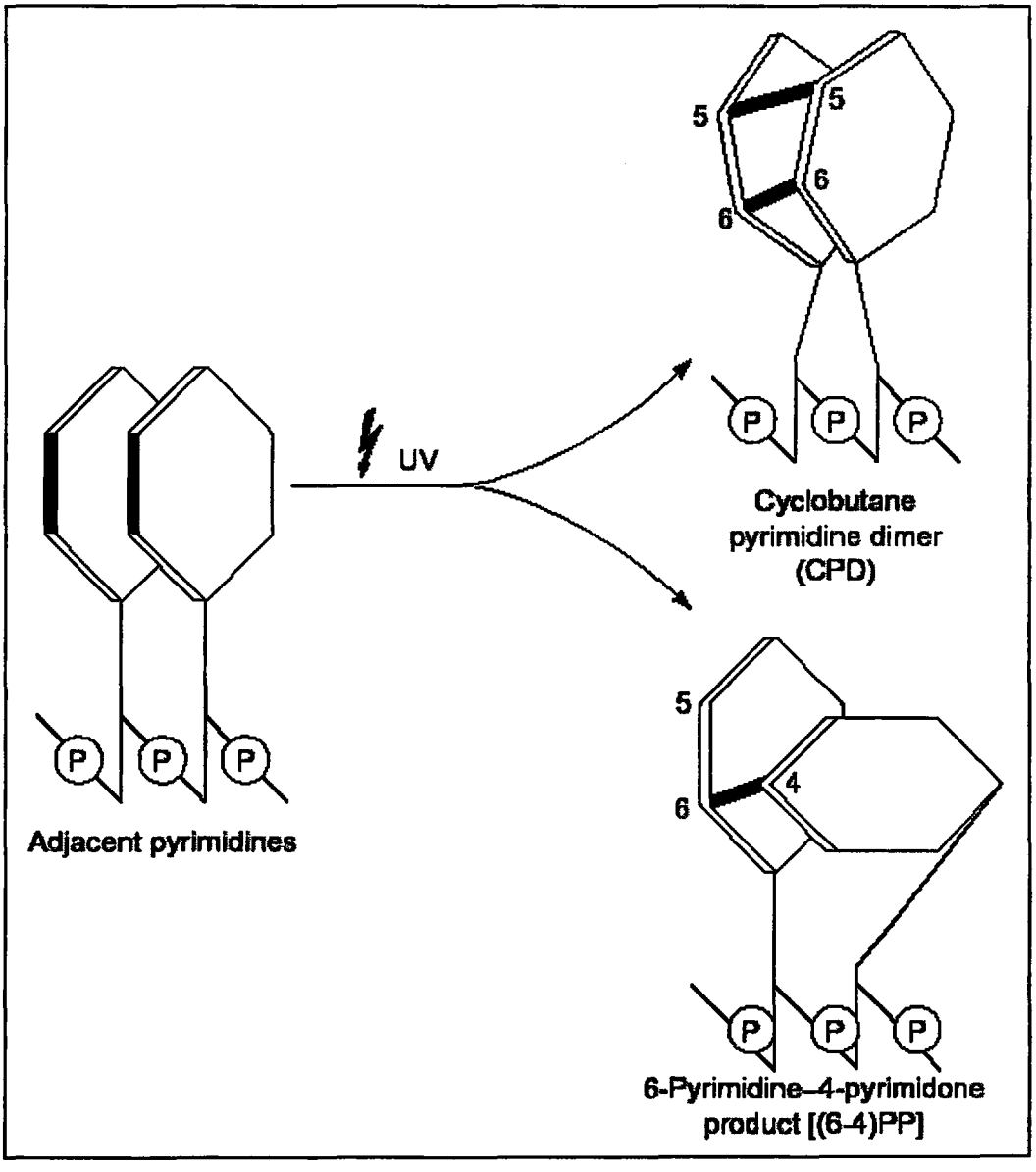

Figure 1-1. Formation of ultraviolet-induced DNA lesions between adjacent pyrimidine residues. The absorption of UV light by DNA results in the production of two major types of lesions that disrupt the DNA double helix structure: cyclobutane pyrimidine dimers (CPDs) and 6-pyrimidine-4-pyrimidone photoproducts (6-4PP) (Adapted from van Steeg and Kraemer, 1999).

The accumulation of DNA helix-distorting (bulky) photolesions can have profound cytotoxic and mutagenic consequences on living organisms, as they can inhibit transcription mediated by RNA polymerase II (RNAPII) (Donahue *et al.*, 1994). If left unrepaired, these UV-induced lesions can also result in DNA mutations; a cytosine to tyrosine transition is a common mutation observed at dipyrimidine sequences containing cytosine residues (Sage, 1993), and are observed in human skin cancers (Sarasin and Giglia-Mari, 2002).

3. UV-mediated Photo-oxidation of DNA

UVA has been considered to be less of a causative factor in skin carcinogenesis than UVB since it is only weakly absorbed by DNA, yet the widespread use of UVA-emitting tanning beds and efficient UVB-blocking (not UVA) sunscreens (Applegate *et al.*, 1997), accompanied by prolonged periods of sunbathing, lead to an increase in the level of UVA exposure, and leading a vital role in UV-induced phototoxicity.

In contrast to the formation of mutagenic pyrimidine photoproducts through direct absorption of UV-B radiation by DNA, UVA radiation produces theses photolesions to a lesser extent due to its poor ability to photoexcite DNA directly (Kvam and Tyrrell, 1997; Kielbassa *et al.*, 1997; Perdiz *et al.*, 2000). Although UVA fluences several orders of magnitude greater than UVB are required to produce equitoxic and equimutagenic events in vitro (Setlow, 1974), UVA is the major UV component of sunlight to reach the earth's surface. Instead, UV irradiation results in oxidative DNA damage mediated by the photo-excitation of endogenous chromophores within cells. Cellular chromophores, such

as tryptophan, riboflavin (Mahns *et al.*, 2003), collagen and elastin (extracellular matrix proteins) (Wondrak *et al.*, 2003) act as photosensitizers, absorbing photons in the UVA region and initiating either Type I and/or Type II photosensitized oxidation reactions (reviewed in Foote, 1991). Absorption of light by the sensitizer produces an excited state, which can react with a substrate or solvent (Type I), resulting in the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) and superoxide anion (O_2^-). Reactive oxygen species may interact with various biological molecules (biomolecules), such as DNA, resulting in oxidative modifications that can be deleterious to a cell (see Figure 1-2).

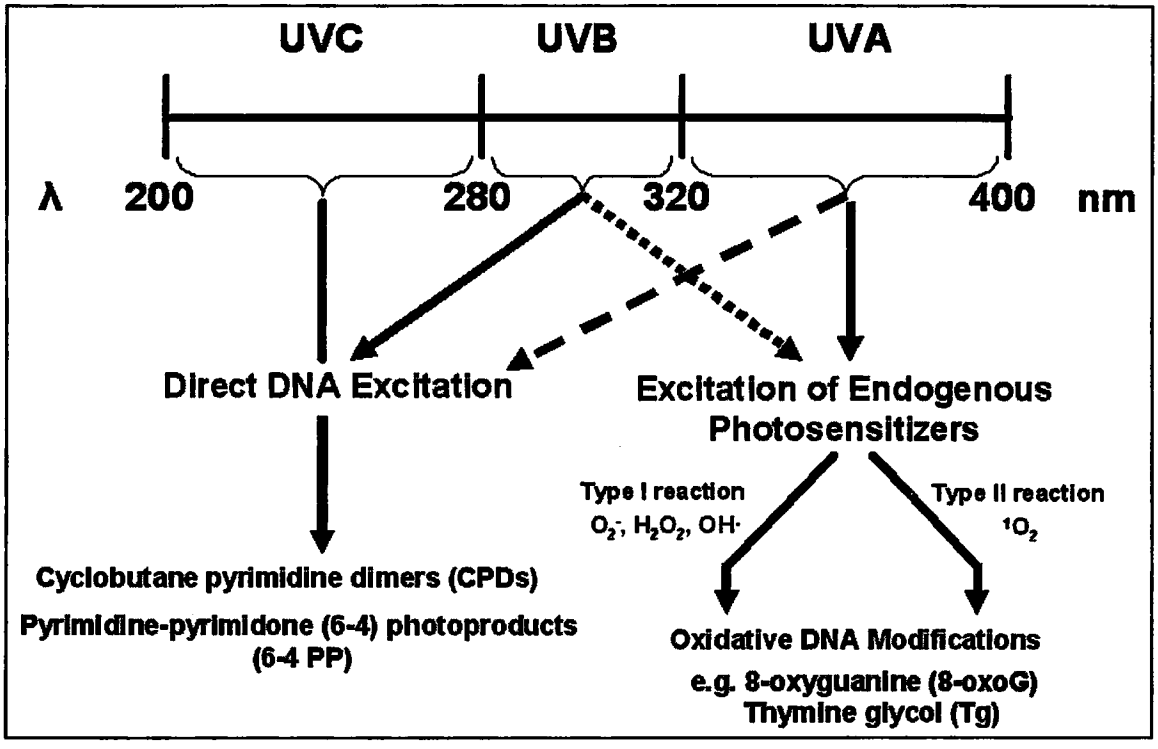

Figure 1-2. UV-wavelength dependent induction of pyrimidine dimers and oxidative DNA modification through direct and indirect mechanisms. Solid lines represent major mechanistic pathways, while dashed/dotted lines represent minor pathways (Adapted from Kielbassa *et al.*, 1997).

Type II photosensitized reactions involve the excited photosensitizer to react with oxygen leading to singlet oxygen ($^1O_2$) production (see Figure 1-2). Singlet oxygen interacts preferentially with guanine residues in DNA, as it displays the lowest ionization potential of the nucleotide bases, yielding 8-oxoguanine (8-oxoG) (Ravanat, *et al.*, 2001b). The formation of 8-oxoG has routinely been used as a marker of oxidative DNA damage.

4. Reactive Oxygen Species (ROS)

4.1 Endogenous and Exogenous Sources

The major source of ROS within the cell is the mitochondrial electron transport chain. Upon consuming oxygen during mitochondrial aerobic respiration, humans generate essential energy in the form of ATP through a series of metabolic reduction reactions. The mitochondrion, the intracellular organelle responsible for energy production, generates ATP by transporting electrons between different molecules that compose the mitochondrial electron transport chain, and eventually reducing O_2 to H_2O. Even under ideal conditions, the electron transport chain is inefficient, resulting in superoxide radical production (1-3%) due to the interaction of oxygen with electrons that escape the electron transport chain (reviewed in Valko *et al.*, 2004).

Exposure of cells to various environmental (exogenous) sources, such as ultraviolet and ionizing radiation, can also lead to ROS formation. Gamma (γ) irradiation of cells has been demonstrated to result in the radiolysis of water, causing $OH\cdot$ radicals to be produced (Table 1; reactions (1) and (2)) (LaVerne, 2000). UVA treatment of cell culture media in the presence of tryptophan and riboflavin, two photosensitizers identified *in vivo*, results in significant H_2O_2 production (Mahns *et al.*, 2003), and it has been suggested that the conversion of H_2O_2 to $OH\cdot$ is responsible for UVA-induced DNA damage (Petersen *et al.*, 2000). The $OH\cdot$ radical is extremely reactive towards biological molecules, generating a multitude of DNA modifications such as base damage, sugar damage, and DNA-protein crosslinks (Jaruga and Dizdaroglu, 1996). While H_2O_2 (and

O_2^-) is relatively unreactive with DNA (Bielski *et al.*, 1985), it is a relatively stable oxidant that should be viewed as a dangerous molecule; its small size and lack of charge allows it to diffuse across biological membranes (Darr and Fridovich, 1994). Irrespective of their origin, reactive oxygen species may interact with various cellular macromolecules, like DNA, leading to oxidative modifications that can have detrimental cellular consequences.

4.2 ROS-mediated Effects on DNA

The formation of DNA lesions caused by oxidizing agents such as UVA, H_2O_2 and ionizing radiation is mediated by the ROS produced within the cell upon exposure to these agents. DNA is a major target for ROS judging from the multitude of different oxidized lesions generated and these lesions have implications in mutagenesis and carcinogenesis. Although $O_2^{\cdot -}$ and H_2O_2 do not demonstrate their oxidative effects on DNA directly, their cytotoxic nature may be due to their ability to generate intracellular OH·. In the presence of transition metals, such as iron and copper, hydrogen peroxide is converted to the superoxide radical (OH·) via the Fenton reaction (see Table 1-1; reaction (3)). Since DNA is a strong chelator of Cu^+ and Fe^{2+} ions, the breakdown of H_2O_2 is likely to occur through a Fenton reaction mechanism (Rodriguez *et al*, 1997; Petersen *et al.*, 2000), implicating both H_2O_2 and OH· in the oxidation of DNA bases.

Two of the most studied oxidative lesions are 8-oxoguanine (8-oxoG) and thymine glycol (Tg), representing the most common forms of oxidative base modifications to purines and pyrimidines, respectively. Oxidation of guanine residues to

produce 8-oxoG has been demonstrated to occur following exposure to H_2O_2 (mediated by OH· radicals) (Riemschneider, 2002), as well as singlet oxygen ($^1O_2$) as previously discussed. This oxidative base lesion is known to be mutagenic, resulting in GC → TA transversions due to adenine residues being misincorporated opposite 8-oxoG (Moriya, 1993). Thymine glycol is produced upon treatment of thymine to oxidizing agents such as H_2O_2, and acts as a replication block to DNA polymerases (McNulty *et al.*, 1998). In order to reduce the mutagenic and cytotoxic potential of both 8-oxoG and Tg, cells possess DNA repair proteins that recognize and remove these oxidative DNA lesions.

4.3 Cellular Defences against Oxidative Damage

The mutagenic and cytotoxic potential of DNA damage is dependent on the repair capacity of the cell. Cells with inadequate repair capacity will experience persistent DNA damage, which can promote genetic mutation or cell death. To alleviate the potential mutagenic and cytotoxic effects of oxidative base modifications such as 8-oxoG and Tg, cells possess DNA glycosylases that recognize and excise the oxidative DNA lesion, which initiates the base excision repair (BER) pathway (Figure 1-3; the BER pathway is further reviewed in Evans *et al.*, 2004).

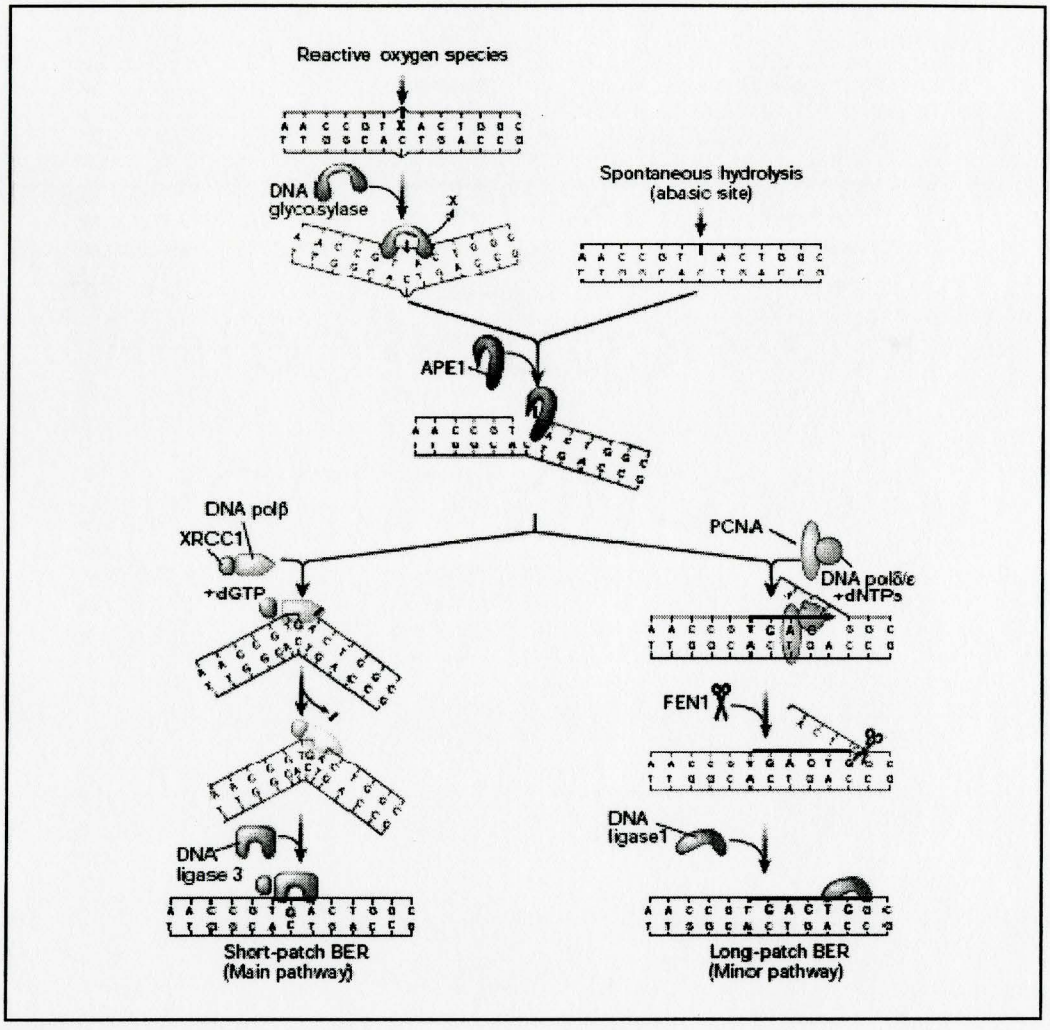

Figure 1-3. Schematic representation of the base excision repair (BER) pathway in human cells. A number of glycosylases, each dealing with a limited, partially overlapping spectrum of oxidative lesions generated by reactive oxygen species, removes the damaged base from the sugar phosphate backbone. The resulting abasic site can also occur spontaneously due to hydrolysis. The base excision repair process is initiated at the abasic site by APE1 endonuclease, which incises the DNA strand. In mammals, short-patch repair requires DNA polymerase β (polβ) to perform a single-nucleotide gap-filling reaction, removing the 5'-terminal baseless sugar residue by its lyase activity. The XRCC1-ligase 3 complex seals the nick. In long-patch BER, repair synthesis (2-10 bases) requires DNA polymerase δ/ε and proliferating cell nuclear antigen (PCNA), while FEN1 endonuclease removes the displaced DNA and ligase 1 seals the nick (Adapted from Hoeijmakers, 2001).

In addition to the oxidative effects on DNA, reactive oxygen species can have a toxic effect on other cellular biomolecules, such as lipids and proteins. In order to prevent the oxidation of these biomolecules and their subsequent repair or replacement, the cell produces antioxidant enzymes that act as a primary defence against ROS. Superoxide dismutase (SOD) and catalase are two antioxidant enzymes present in cells that mutually work to prevent the oxidation of DNA, lipids and proteins. Superoxide dismutase reduces O_2^- to H_2O_2, while catalase catalyzes the decomposition of H_2O_2 to water and oxygen (see Table 1-1; reactions (4) and (5)), thus protecting the cell from the oxidative capabilities of H_2O_2 and OH· radicals (reviewed in Bandyopadhyay *et al.*, 1999).

Table 1-1. Formation and decomposition of reactive oxygen species (ROS) in cells (Adapted from Slupphaug *et al.*, 2003; LaVerne *et al.*, 2000; Bandyopadhyay *et al.*, 1999)

ROS Formation:

Radiolysis of water

$H_2O \rightarrow H_2O^+ + e^-$ (1)

$H_2O^+ + H_2O \rightarrow H_3O + OH^\bullet$ (2)

Fenton Reaction

$Fe^{2+} + H_2O_2 + H^+ \leftrightarrow Fe^{3+} + OH\cdot + H_2O$ (3)

Cellular Defences against ROS:

Superoxide reduces superoxide anion to hydrogen peroxide

$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (4)

Catalase decomposes hydrogen peroxide

$2H_2O_2 \rightarrow 2H_2O + O_2$ (5)

5. Nucleotide Excision Repair (NER)

Nucleotide excision repair (NER) is a pathway that senses and removes lesions that distort the conformation of the DNA double helix, interfere in base-pairing and block DNA duplication and transcription. The most common examples of these lesions are UV-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone protoproducts (6-4PP). The process of NER in eukaryotes can be described in the following basic steps, which involves (i) specific recognition of the DNA lesion, and (ii) recruitment and assembly of the NER machinery which unwind the DNA double helix and excise both 5' and 3' of the lesion, producing an oligonucleotide around 24-30 bases long. This is followed by (iii) the removal of the DNA segment containing the lesion and (iv) re-synthesis (and ligation) of the excised fragment, using the undamaged complementary strand as a template. If left unrepaired, these lesions can interfere in base-pairing, blocking transcription and DNA replication, resulting in the induction of mutations and chromosomal aberrations.

Defects in NER have been associated with three hereditary diseases: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). The clinical features of XP are characterized by sun-sensitivity that leads to progressive degeneration of sun-exposed eye and skin regions, such as excessive freckling, abnormal dryness of the skin (xeroderma), pigmentation abnormalities (pigmentosum) and high incidences of skin cancers (Kraemer *et al.*, 1987). In addition, a small percentage of XP patients demonstrate neurological disorders. Individuals afflicted with CS demonstrate sensitivity to sunlight as well as developmental defects such as mental retardation and

physical abnormalities (bird-like face, long limbs, dwarfism). The clinical features of CS have little in common with XP, as the sun-sensitivity demonstrated in CS patients is manifested as a severe rash, with none of the skin pigmentation changes and skin cancers seen in XP (Nance and Berry, 1992). The clinical characteristics of TTD include symptoms that are uncommon in the other two disorders. The defining feature of TTD is sulphur-deficient brittle hair, while small stature, sensitivity to sunlight, mental retardation and scaly skin are also common TTD manifestations (Itin *et al.*, 2001).

5.1 Sub-pathways of NER

The majority of the NER studies were performed with cells mutated at different steps of the NER pathway, including cells obtained from XP, CS and TTD patients, individuals afflicted with genetic syndromes directly related to DNA repair. The differential repair rate of CPDs in transcribed and non-transcribed DNA lead to the current hypothesis that NER consists of two distinct sub-pathways: transcription-coupled repair (TCR) and global genome repair (GGR). Transcription-coupled repair (TCR) is characterized as the preferential repair of lesions from the transcribed strand of active genes, while global genome repair (GGR) refers to repair throughout the genome, including repair within non-transcribed strands of active genes. In addition, studies have shown that the efficiency of CPD removal from the transcribed strand of the DHFR gene is greater than in upstream sequences or even the non-transcribed strand in cultured hamster and human cells, suggesting that GGR is a slower repair process than TCR (Bohr *et al.*, 1985; Mellon *et al.*, 1986 and 1987).

The characterization of genes that have a function in NER, specifically in either TCR or GGR, was possible from the availability of cells derived from XP and CS patients. Hybrid cells formed from the fusion of skin fibroblasts from different patients would result in the restoration of normal UV-induced repair if the two patients belong to different complementation groups. Such complementation studies revealed that at least seven complementation groups (XP-A through XP-G) exist, each displaying a deficiency in NER (Kleijer *et al.*, 1973). Most XP groups are deficient in both sub-pathways of NER except in the case of XP-C (Venema *et al.*, 1990; Venema *et al.*, 1991) and XP-E (Hwang *et al.*, 1999). Cells from XP-C and XP-E individuals have been characterized with proficient TCR, able to efficiently remove lesions from actively transcribed genes while demonstrating a deficiency in repair of inactive DNA (GGR deficient). In contrast, cells from CS patients are unable to resume DNA and RNA synthesis following the introduction of UV-induced DNA damage. This phenomenon of impaired transcription recovery following UV irradiation has been used to identify two CS complementation groups (CS-A and CS-B) (Mayne and Lehmann, 1982), which exhibit deficiencies in the TCR sub-pathway of NER but retain proficient GGR (van Hoffen *et al.*, 1993). However, there are mutations in XP genes (specifically XP-B, XP-D, and XP-G) which exhibit clinical symptoms of both XP and CS patients (Rapin *et al*, 2000), with cell lines derived from these individuals demonstrating deficiencies in both TCR and GGR of UV-induced DNA damage (see Table 1-2).

Table 1-2. The function(s) of the XP and CS gene products in NER, as determined from NER-deficient cells. The NER pathway consists of two sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). A positive (+) sign indicates proficiency in repair by the given sub-pathway, whereas a minus (-) sign indicates a deficiency (Adapted from Stary and Sarasin, 2002).

Complementation group	TCR	GGR	Function in DNA Repair
XP-A	-	-	damage verification interacts with TFIIH and XPF-ERCC1
XP-B	-	-	3'→5' helicase activity in TFIIH
XP-C	+	-	damage recognition (GGR only)
XP-D	-	-	5'→3' helicase activity in TFIIH
XP-E	+	-	damage recognition (GGR only)
XP-F	-	-	endonuclease (5' incision)
XP-G	-	-	endonuclease (3' incision) stabilizes open complex
CS-A	-	+	TCR / recovery of RNA synthesis
CS-B	-	+	TCR / recovery of RNA synthesis

The differential involvement of XP and CS (complementation) proteins in the NER sub-pathways may be attributed to the differences in DNA lesion recognition by (the proteins involved in) the TCR and GGR pathways. The XPC protein (complexed with hHR23B) is responsible for sensing DNA damage, in particular 6-4PP, in the GGR pathway by recognizing helical distortions throughout the genome (Batty *et al.*, 2000). For some lesions like CPDs, the XPE complex (consisting of the DDB1 and p48 (DDB2) protein subunits) may facilitate in the recognition step (Tang *et al.*, 2000). In the TCR pathway, RNA polymerase II detects the DNA lesion, as a result of stalling of transcriptional elongation of an active gene. The sequential steps following the initial recognition of DNA lesions by the GGR and TCR pathways are similar. The basal transcription factor TFIIH, which contains the XPB and XPD gene products, is recruited to the damage site. The XPB and XPD proteins display 3'-5' and 5'-3' helicase activity, respectively, allowing local unwinding of DNA surrounding the lesion. This enables other NER proteins, such as XPF, XPG, XPA, replication protein (RPA), to accumulate at the lesion site. RPA binds to the undamaged strand, allowing proper positioning of the XPF/ERCC1 complex and XPG endonucleases both 5' and 3' of the lesion, respectively, in order to allow for cleavage and formation of a 24-30 nucleotide excised fragment. The resulting gap is re-synthesized by DNA polymerase δ/ε and ligated by DNA ligase I (see Figure 1-4) (reviewed in Stary and Sarasin, 2002; van Hoffen *et al.*, 2003).

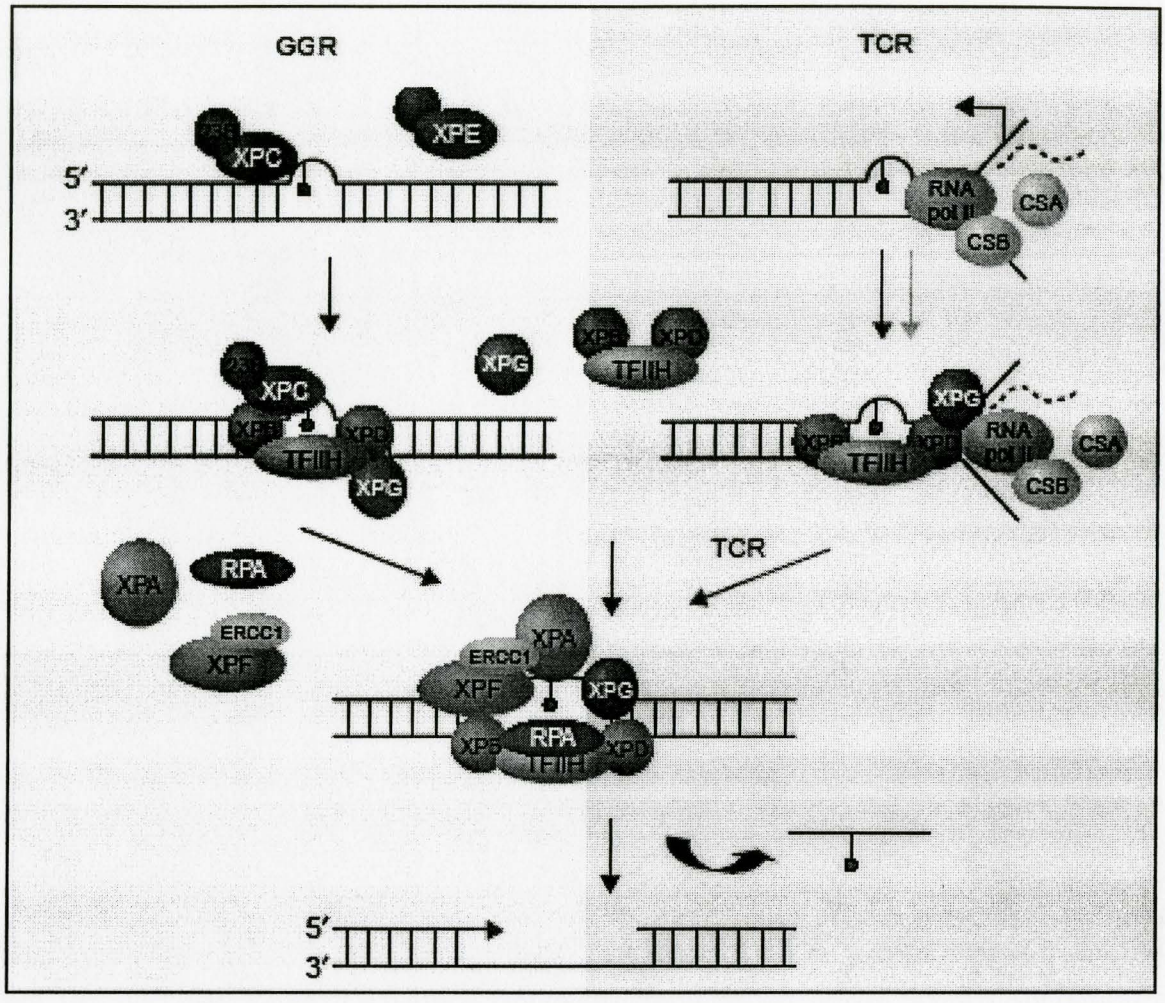

Figure 1-4. Schematic representation of the nucleotide excision repair pathway in human cells. The NER pathway consists of two sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). Depending where the DNA lesions are situated (transcribed *versus* non-transcribed regions), recognition of the lesions will involve NER factors responsible for either GGR or TCR (Adapted from Mitchell *et al.*, 2003).

5.2 Involvement of p53 in NER

The p53 tumour suppressor functions primarily as a transcription factor that regulates its downstream functions by activating or repressing various target genes. The TP53 protein (encoded by the p53 gene) plays a pivotal role in monitoring various biochemical pathways responsible for preventing neoplastic transformation of mammalian cells following various environmental stresses including ultraviolet radiation. This is accomplished by inducing cell cycle arrest, repair of DNA damage or apoptosis. The p53 gene is considered to be the most commonly altered gene in cancer (Hollstein *et al.*, 1991). Loss of p53 function may disrupt a cell's ability to carry out growth arrest, DNA repair or programmed cell death.

To explore the role of p53 in protecting the genome, various studies have investigated the direct effect of p53 on the removal of lesions by the nucleotide excision repair pathway. There is considerable evidence supporting a role for p53 in the GGR sub-pathway of NER. However, the possible role of p53 in TCR remains controversial. Cells derived from patients with the cancer prone Li-Fraumeni syndrome (LFS), which are homozygous for mutations in the p53 gene, are defective in the GGR of CPDs following UVC irradiation, but demonstrated normal TCR when compared with normal cells and heterozygous p53 mutants (Ford and Hanawalt, 1995). Additional studies involving human fibroblasts transformed with the human papillomavirus (HPV) E6 gene, which degrades p53 and limits its activity, exhibit proficient TCR despite demonstrating a deficiency in GGR following UVC treatment (Ford *et al*, 1998).

In addition to its accepted role in GGR, other studies also suggest a role for p53 in TCR. Drobetsky and colleagues demonstrated that Li-Fraumeni and HPV E6-expressing fibroblasts exhibited reduced CPD removal (relative to normal) along both the transcribed and non-transcribed strands of active genes, suggesting that both TCR and GGR are p53-dependent upon UVB exposure (Therrien *et al.*, 1999). In yet another study, the requirement for functional p53 in TCR, but not GGR, has been suggested to depend on the incident UV wavelength. Removal of CPDs along both the transcribed and non-transcribed strands of active genes was less efficient in p53-deficient lymphoblastoid strains following UVB treatment, however, UVC irradiation resulted in less efficient CPD removal only along the non-transcribed strand of the target genes (Mathonnet *et al.*, 2003). In order to resolve the discrepancies between studies, the authors suggested that the involvement of p53 in TCR may be dependent on the incident UV-wavelength.

5.3 p53-dependent transcription and recruitment of NER factors

One potential mechanism for p53-dependent NER is through direct transcriptional regulation of NER genes. In humans, p53 regulates transcription of the DDB2 gene product (p48 subunit of the XPE protein complex) (Hwang *et al.*, 1999), by activating its transcription following p53 binding to the DDB2 promoter (Tan and Chu, 2002). XPC is another GGR-specific gene that has been demonstrated to be regulated by p53 in response to DNA damage. Ford and colleagues demonstrated that mRNA and protein products of the XPC gene were UV-inducible in a dose-dependent manner in p53-

proficient cells, while no induction was observed in p53-deficient cells (Adimoolam and Ford, 2002). Interestingly, p53 has been demonstrated to bind to CSB, which is primarily involved in TCR, suggesting that p53 may also play a role in this repair sub-pathway (Troelsta *et al.*, 1992). Recruitment of NER factors has also been suggested to be p53-dependent. Experiments involving co-localization of NER factors have been shown that XPC and TFIIH were rapidly recruited to DNA damage, however this recruitment occurred less efficiently in LFS (p53-null) cells (Wang *et al.*, 2003). Taken together, these studies demonstrate that in addition to up-regulating the expression of NER factors involved in DNA damage recognition, p53 also plays a role in damage recognition by recruiting these factors to damaged sites.

6. *In vitro* Approach to Studying NER

6.1 The Use of Recombinant Adenoviruses to Examine DNA Repair

Adenoviruses (Ad) are non-enveloped double stranded DNA viruses, with a genome that comprises about 36 kilobases (kb). These viruses infect vertebrates, capable of infecting both replicating and non-dividing (quiescent) cells and causing generally mild diseases of the respiratory and gastrointestinal tracts in humans. Due to its ability to infect a wide variety of cells, it has been used as a vector for transgene (foreign gene) expression in mammalian cells. Non-replicating recombinant Ad vectors lack the E1 region of the adenovirus genome, and contain a transgene inserted in its place. The E1 region is removed from the Ad genome as it is the first to be expressed following

infection and is necessary for the expression of other viral genes, in particular those responsible for replication (reviewed in McConnell and Imperiale, 2004; Cusack, 2005).

A number of Ad constructs containing a reporter gene, such as the bacterial *lacZ* gene (which encodes β-galactosidase) or luciferase gene, have been constructed to be used in reporter gene assays. In order to promote high levels of transgene expression in both human and rodent cells, the human or murine cytomegalovirus immediate early (HCMV-IE or MCMV-IE) gene promoters are inserted upstream of the transgene (Addison *et al.*, 1997). Ad5HCMVsp1*lacZ* (Morsy *et al.*, 1993) and AdCA17*lacZ* (Addison *et al.*, 1997) are two recombinant non-replicating adenoviruses that can efficiently infect and express the reporter gene in human cells. Both constructs contain the *lacZ* gene under the control of the HCMV-IE promoter inserted in the deleted E1 region, except that the orientation of the *lacZ* gene is different. Infection of cells with these Ad constructs following UV treatment can be used to assess a cell's capacity to repair a UV-damaged gene (see Figure 1-5).

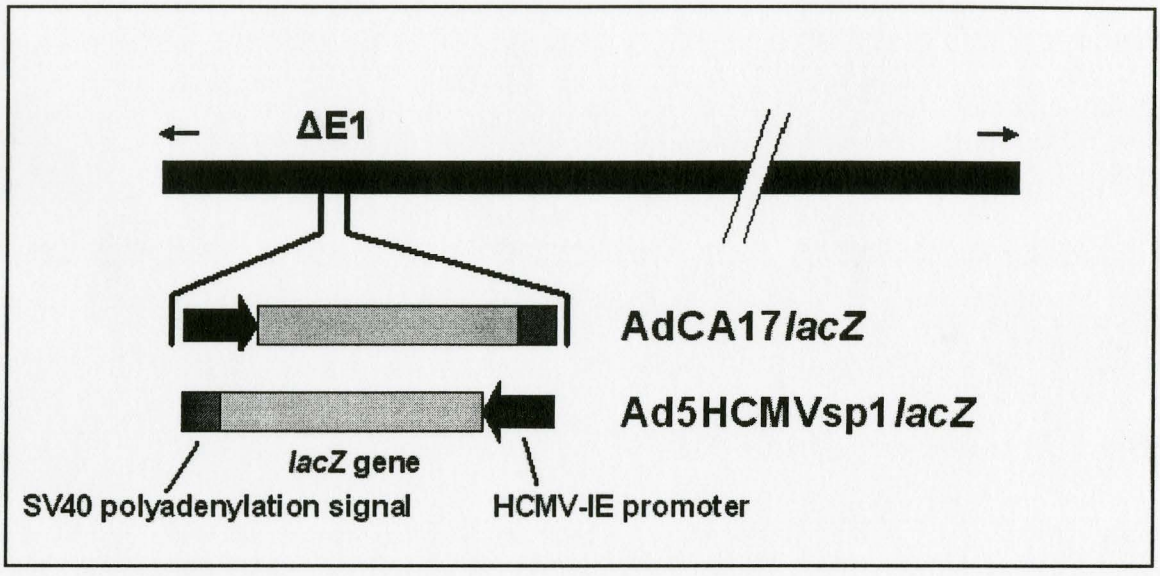

Figure 1-5. Construction of the recombinant AdCA17*lacZ* and Ad5HCMVsp1*lacZ* virus probes. Both constructs are a non-replicating adenovirus which contain the bacterial *lacZ* reporter gene, expressing β-galactosidase (β-gal) under the control of the human cytomegalovirus immediate-early (HCMV-IE) promoter. The transgene is inserted in place of the deleted E1 region of the virus genome, which is responsible for adenovirus replication. The solid arrows represent the inverted terminal repeats of the adenoviral genome. (Adapted from Addison *et al.*, 1997).

6.2 Host Cell Reactivation of a UVC-damaged reporter gene in human cells

The repair capacity of a cell has been assessed by its ability to repair a damaged reporter gene contained within a probe that is used to infect or transfect the host cell, such as non-replicating adenovirus or plasmid constructs. Reactivation of the damaged reporter gene activity by the host cell (also termed host cell reactivation (HCR)) has been used as an *in vitro* experimental model for DNA repair, in particular NER. In previous studies, recombinant non-replicating Ad constructs have been used to introduce UVC-damaged reporter genes into non-treated human cells in order to assess the repair of the

damaged DNA using HCR of the reporter gene activity as an endpoint (McKay *et al.*, 1997a; Francis and Rainbow, 1999). The presence of UV-induced bulky lesions in the template strand of active genes inhibit RNA polymerase II-mediated transcription (Donahue *et al.*, 1994) and the presence of a single CPD is believed to be sufficient to prevent transcription of the reporter gene contained within an Ad construct (Francis and Rainbow, 1999). Therefore, HCR of a reporter gene is thought to reflect the host cell's ability to repair transcription-blocking lesions from the transcribed strand of the reporter gene since proper β-gal expression is expected to only occur from transcription of a lesion-free gene.

Using this experimental approach, non-irradiated and UVC-irradiated Ad5HCMVsp1*lacZ* have been used to infect human fibroblasts in order to study the repair capacity of NER-proficient and deficient cells (Francis and Rainbow, 1999). HCR of β-galactosidase (β-gal) activity was significantly reduced in all XP (XP-A to XP-G) and CS (CS-A and CS-B) fibroblast strains relative to normal human fibroblasts (McKay and Rainbow, 1996; Francis and Rainbow, 1999; Pitsikas *et al.*, 2005). In addition, CS fibroblasts retained a considerable ability (57-90% relative to normal levels) to repair the UV-damaged *lacZ* gene (Francis and Rainbow, 1999), considering that it is characterized as being deficient in the repair of the transcribed strand of active genes. Therefore, these results suggested that both TCR and GGR contributed to the repair of the UVC-damaged reporter gene in human fibroblasts.

6.3 Enhanced host cell reactivation (HCR) of a UVC-damaged reporter gene following UVC pre-treatment of human cells.

In addition to studying HCR of a UVC-damaged reporter gene in non-irradiated cells, the host cells can be treated with a DNA damaging agent, such as UV radiation, prior to adenovirus infection. Using this experimental approach, HCR of a UV-damaged reporter gene has been examined in both UVC-treated and non-irradiated cells. UVC pre-treatment of normal fibroblasts result in enhanced HCR of the UVC-damaged *lacZ* gene. UV-enhanced HCR was also observed in TCR-proficient XPC fibroblast, but was absent or delayed in TCR-deficient XP and CS fibroblasts, suggesting that TCR is involved in the enhanced HCR of the *lacZ* gene contained in Ad5HCMVsp1*lacZ* (Francis and Rainbow, 1999; Pitsikas *et al.*, 2005; Rainbow *et al.*, in press).

7. Radiation-induced Bystander Effects

The interaction of radiation with DNA, either by direct ionization or by the indirect production of reactive oxygen species, is believed to be the mechanism of radiation-induced DNA damage formation (within an irradiated cell). However, several studies have reported that irradiation of cells can induce biological effects in non-irradiated cells in close proximity or in direct contact with the irradiated cells. This causes the non-irradiated cells to behave as though they had been irradiated, demonstrating signs of genetic instability and/or increased cell death. The term designated to describe the induction of a biological effect in non-irradiated cells by a factor(s) generated by irradiated cells is referred to as a radiation-induced bystander effect (see Figure 1-6).

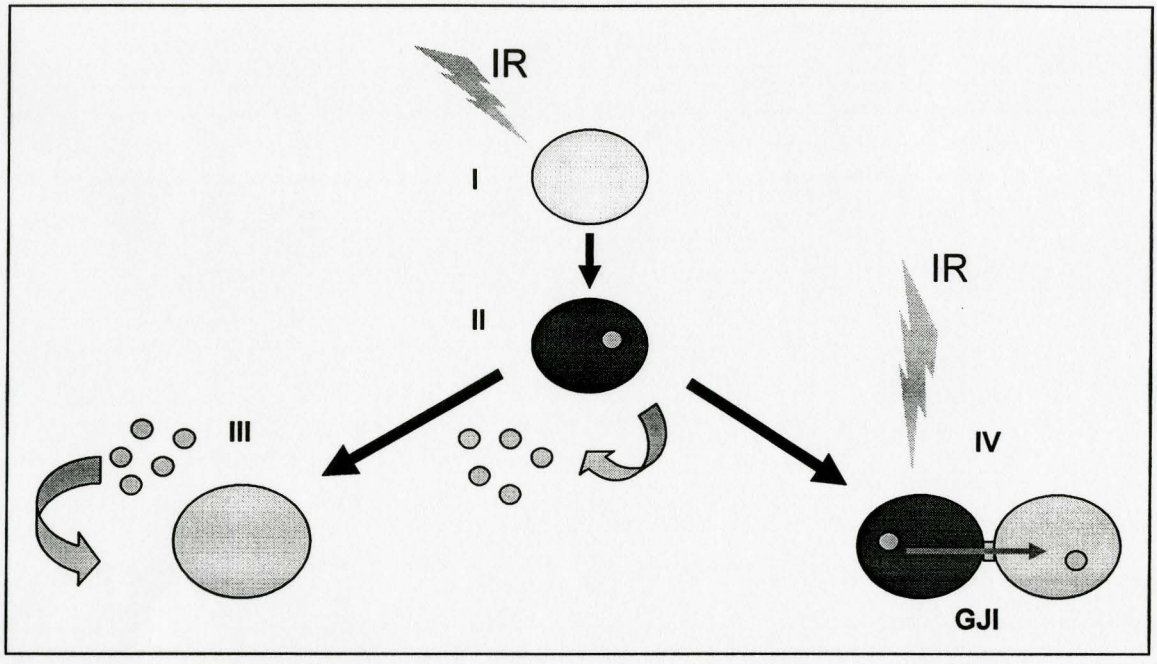

Figure 1-6. Schematic representation of the bystander effect. There are two mechanisms that cause bystander responses in non-irradiated cells, depending on the type of ionizing radiation (IR) applied. (I). Irradiation of the cell induces the production and release of the cytotoxic factor(s) (II) that stimulates responses (eg. cell death) in non-irradiated cells (III, IV). Some studies have suggested that the factor(s) is able to be secreted (III), while others have indicated that these responses are mediated between adjacent cells (IV) via gap junction intercellular communication (GJIC) (Adapted from Mothersill *et al.*, 2004a).

The concept of radiation-induced bystander effects was initially developed from reports demonstrating that serum/plasma harvested from radiotherapy patients was capable of inducing cell death and causing chromosomal damage in unexposed cells grown in culture (Goh and Sumner, 1968; Hollowell and Littlefield, 1968). More recently, Emerit and colleagues have also reported the presence of radiation-induced

chromosome damaging (clastogenic) factors in the plasma of children exposed at Chernobyl (Emerit *et al.*, 1997). These observations changed the conventionally thinking that genetic alterations and radiation-induced cell killing requires direct exposure of cells to radiation, and has lead to a plethora of studies investigating the "bystander effect" phenomenon.

7.1 *In vitro* Studies of Bystander Effects

Studies of radiation-induced bystander effects have followed two main experimental approaches. One approach involves harvesting culture medium from irradiated and control cells and transferring the medium onto non-irradiated cells in order to determine the effect of any radiation-induced medium-borne factors. Experimental evidence supporting bystander responses induced by sparsely ionizing (or low LET) radiation, such as X-rays and gamma rays, has been provided by such studies. Mothersill and Seymour demonstrated that medium from gamma-irradiated epithelial cells could reduce the clonogenic survival of non-irradiated cells. In the absence of cells during irradiation, the irradiated medium did not effect the survival of non-irradiated cells. This effect was dependent on the number of cells present at the time of irradiation and the length of time between irradiation and removal of the conditioned media, but there was no increase in cytotoxicity with dose since a 0.5 Gray (Gy) dose reduced the clonogenic survival to the same extent as 5 Gy (Mothersill and Seymour, 1997). This suggests that the bystander response saturates at very low doses and further irradiation does not increase the strength of the cytotoxic signal in the transferred media.

Although clonogenic death has been extensively used as the measureable endpoint in bystander effect studies, genetic instability has also been implicated as a bystander response in non-irradiated cells. Within the context of radiation-induced bystander effects, genetic instability is described as a hypermutatable state induced in the progeny of cells exposed to the irradiated cell conditioned medium (ICCM). The genetic instability concept was investigated by re-plating the surviving progeny of cells exposed to medium from irradiated cell cultures. The progeny of the cells that survived treatment to irradiated cell conditioned medium (ICCM) demonstrated reduced cloning efficiency in comparison to their mock-treatment controls (Seymour and Mothersill, 2000; Mothersill *et al.*, 2004). This suggested that the bystander effect is transmissible to the progeny of cells exposed to the cytotoxic factor(s) in media generated by irradiated cells, and has supported the hypothesis that genetic instability can be induced by the bystander effect.

In an attempt to demonstrate whether the production of cytotoxic signal by irradiated cells is dependent on the repair capacity of the cell line, various repair-deficient and proficient-equivalent cell lines have been used in bystander experiments. Mothersill and colleagues demonstrated that repair-deficient cells produced an irradiated cell-conditioned media (ICCM) that is more toxic than the ICCM produced by their repair-proficient controls (Mothersill *et al.*, 2004b). This suggests that the repair capabilities of the irradiated (donor) cell lines, or the pathways regulating cellular damage control, are crucial in determining the toxicity of the ICCM on the reporter (recipient) cell line.

Similar to low-LET radiation studies, high-LET radiation has been shown to produce a bystander effect. A second approach to the study of radiation-induced bystander effects involves the focus and delivery of high-energy particles to individual cells using microscope optics, which allows specific cells to be irradiated, while being able to study the biological effects in both the irradiated and non-irradiated cells within the culture. High-LET induced bystander studies by Nagasawa and Little (1992) demonstrated that a larger proportion of cells showed chromosomal damage, as sister chromatid exchanges (SCEs), than were estimated to have been hit by an alpha particle. More specifically, 30% of cells showed an increase in SCEs even though less than 1% of cells were directly traversed by an alpha particle.

While both low and high-LET radiation are capable of inducing bystander effects, experimental evidence suggests that their mechanisms of action may be different. In high-LET bystander effect studies, transfer of the clastogenic factor has been implicated to occur between neighbouring cells via connexin-mediated gap junctions; connexin inhibitors were able to block the effect, while gap junction null cells eliminated any effect (Azzam *et al.*, 1998). In addition, this bystander effect was demonstrated to be dependent on the p53 status (Iyer and Lehnert, 2000), unlike the bystander effect of low-LET radiation that occurs in p53 mutant and human papillomavirus (HPV)-transfected (p53-null) cell lines (Mothersill and Seymour, 1997). In contrast, clonogenic death due to ICCM treatment of non-irradiated cells occurs without any contact with irradiated cells in media transfer experiments and suggests that these bystander responses are independent of gap junction intracellular communication (GJIC) (Mothersill and Seymour, 1998).

Although these findings suggest that the effects of high-LET and low-LET radiations may possibly have different mechanisms of action, they do share similar endpoints of cell death and genetic instability (mutations and/or chromosomal aberrations). The signal(s) responsible for mediating both high- and low-LET bystander effects have yet to be identified.

7.2 Studies of UV-mediated Bystander Effects

Preliminary studies suggest that UV radiation can mediate bystander effects in human and rodent cells. In the past, UVC irradiation of human fibroblast cells has been shown to stimulate the release of factors in the surrounding medium; this medium was capable of mimicking the UVC-induced synthesis of various gene products (Schorpp *et al.*, 1984). More recently, UVA and UVB radiation have been the focus of UV-mediated bystander effect studies. In a study involving UVA treatment of Madin Darby canine kidney cells (MDCK II), the irradiated cultures exhibited an increased occurrence of isolated dead cell clusters rather than random cell death throughout the culture (Dahle *et al.*, 2001). Based on these observations, the authors suggested that these dead cell clusters are the result of the UVA-damaged cells enhancing the predisposition for damage in their neighbouring cells. In another study, Dahle and colleagues suggested that the bystander effect may modulate UVA and UVB-induced genomic instability in rodent cells through gap junction intercellular communication (GJIC), demonstrating an increased mutation frequency among cells allowed to be contact with their progeny (Dahle *et al.*, 2005). More recently, a study demonstrated reduced cell survival of non-

irradiated human keratinocytes upon treatment with conditioned media from UV(A+B)-irradiated cells, and implicated apoptotic cell death due to the expression of certain apoptotic markers (Banarjee *et al.*, 2005). Therefore, although it is a relatively new focus in radiation-induced bystander effect studies, there is evidence to suggest that UV radiation can induce bystander effects.

8. Project Introduction

We have previously reported that UVC pre-treatment of cells was found to enhance the HCR of a UVC damaged non-replicating adenovirus vector, Ad5HCMVsp1*lacZ*, in normal and XP-C fibroblasts, but was absent or delayed in CS-B and XP-A cells (Francis and Rainbow, 1999; Pitsikas *et al.*, 2005; Rainbow *et al.*, in press). AdHCMVsp1*lacZ* and AdCA17*lacZ* are non-replicating adenoviruses, expressing the *lacZ* reporter gene under the control of the HCMV-IE promoter, however the orientation of the *lacZ* gene differs in the two recombinant viruses (Figure 1-5). Using the AdCA17*lacZ* construct, we were interested in examining whether repair of the UV-damaged *lacZ* gene in non-irradiated (and UVC-irradiated) normal and NER-deficient cells was dependent on the orientation of the transgene in these recombinant viruses.

Another goal of this research project was to further examine the effects of UV-irradiation on cells not directly exposed to the UV radiation. By employing the experimental approach of Mothersill and Seymour (1997), we were interested in determining whether UVC and UVA irradiation of cells were capable of producing an ICCM that was cytotoxic to non-irradiated cells. In light of the recent reports suggesting

UV(A+B) irradiation can effect the viability of non-irradiated cells (Banerjee *et al.*, 2005), we have examined the effects of UVC or UVA irradiation of cells on the clonogenic survival of non-irradiated cells.

Finally, we were interested in examining the effect of UVC, UVA and H_2O_2 on the clonogenic survival of cell lines employed in the bystander effect studies. As UVA irradiation of PBS and growth media have been implicated in hydrogen peroxide production (Mahns, *et al.*, 2003), we were interested in determining whether these cell lines demonstrated different survival profiles upon H_2O_2 and UVA treatment. Since UVC irradiation results in different DNA lesions generated than both H_2O_2 and UVA (oxidative DNA damage), we were interested in examining how different environmental agents affect the clonogenic survival of the cell lines examined.

Chapter 2

UV-Enhanced host cell reactivation of a UV-damaged reporter gene suggests that both transcription-coupled repair and global genome repair are UV-inducible in human cells

Abstract

We have used a non-replicating recombinant adenovirus, AdCA17*lacZ*, which expresses the β-galactosidase (β-gal) reporter gene under the control of the human cytomegalovirus immediate early (HCMV-IE) promoter, to examine host-cell reactivation (HCR) of a UVC-damaged reporter gene in human fibroblasts. Since β-gal expression in mammalian cells is expected to occur following transcription of a lesion-free *lacZ* reporter gene, the removal of UV-induced DNA lesions and subsequent β-gal expression is a measure of the DNA repair capacity of the infected cell. In the present work, we have examined HCR and UV-enhanced HCR of the UV-damaged reporter construct, AdCA17*lacZ*, in normal and nucleotide excision repair (NER) deficient fibroblasts. Xeroderma pigmentosum (XP) group C and E fibroblasts are deficient in the global genome repair pathway (GGR) of NER, while Cockayne syndrome (CS) fibroblasts are deficient in the transcription-coupled repair pathway (TCR) of NER and XP group A fibroblasts are deficient in both TCR and GGR. HCR of the UV-damaged reporter gene activity was significantly reduced in XP-A, XP-C, XP-E and CS-B cell strains relative to that in normal human fibroblasts, indicating that both TCR and GGR contribute to expression of the UV-damaged reporter gene in human cells. Pre-treatment of human fibroblasts with UVC (12 J/m^2) immediately prior to infection results in enhanced HCR of the UV-damaged reporter gene. UV-enhanced HCR of the UV-damaged reporter gene activity was detected in normal fibroblasts at 12 hr post-infection and in CS-B, XP-C and XP-E fibroblasts, but not XP-A fibroblasts at 24 and 40 hr post-

infection. These results indicate that both TCR and GGR are UV-inducible in human cells.

Introduction

The integrity of the human genome is constantly being compromised by alterations induced by various endogenous and exogenous agents. Ultraviolet (UV) radiation is one of these agents that is capable of inducing the formation of helix-distorting (bulky) lesions, including cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4 PP), within the genome. These photolesions, formed as a result of inappropriate bonding between adjacent pyrimidines, have been shown to inhibit transcription by causing RNA polymerase II (RNAPII) arrest (Donahue *et al.*, 1994), as well as cause DNA mutations, most notably a cytosine to tyrosine transition (Sage, 1993). However, cells have developed mechanisms to repair a wide variety of bulky adducts, particularly those induced following UV irradiation from the sun.

Nucleotide excision repair (NER) is a DNA repair pathway that senses and removes UV-induced lesions that distort the conformation of the DNA structure. The removal of CPDs from the genome by NER was elucidated to occur at different rates, revealing two distinct, yet interrelated, sub-pathways of NER: transcription-coupled repair (TCR) and global genome repair (GGR). The TCR pathway specifically targets transcription-blocking lesions, resulting in the preferential removal of lesions from the transcribed strand of active genes, whereas the GGR pathway acts to remove lesions from

the non-transcribing strand of active genes and throughout the non-transcribing regions of the entire genome (Bohr *et al.*, 1985; Mellon *et al.*, 1986 and 1987).

To elucidate the mechanism by which the NER pathway responds to UVC-induced DNA damage, we have used human fibroblasts taken from patients that have deficiencies in NER. The hereditary disorders xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are each characterized by deficiencies in NER. While both XP and CS patients demonstrate sensitivity to sunlight, only XP patients are predisposed towards the development of skin cancer. XP is composed of a minimum of seven complementation groups (XP-A to XP–G), with most displaying a deficiency in both sub-pathways of NER. The XP-C and XP-E complementation groups are exceptions as both retain TCR activity, capable of efficiently removing lesions from actively transcribed genes while being deficient in the repair of inactive regions of the genome (GGR-deficient) (Venema *et al.*, 1990; Venema *et al.*, 1991; Hwang *et al.*, 1999). Two complementation groups of CS (CS-A and CS-B) have also been identified, each exhibiting a deficiency in the TCR sub-pathway of NER while demonstrating GGR-proficiency (van Hoffen *et al.*, 1993).

To examine the role of both GGR and TCR in mammalian cells, we have employed a reporter gene assay involving non-replicating recombinant adenovirus (Ad) constructs that contain the bacterial *lacZ* reporter gene and express β-galactosidase (β-gal) under the control of a human cytomegalovirus immediate early (HCMV-IE) promoter. Using such constructs to infect a host cell, the cellular repair capacity can be assessed by its ability to repair the damaged reporter gene, thereby restoring (or

reactivating) the β-gal activity. Reactivation of the damaged reporter gene activity (hence the term, host cell reactivation (HCR)) has been used as an *in vitro* model for DNA repair (see Figure 2-1). By infecting the UV-damaged Ad construct into various NER-deficient and/or proficient cell lines, the ability to express β-gal in the host cell is a reflection of the cell's NER capacity, as β-gal expression is expected to only occur following repair of the transcribed strand of the *lacZ* gene.

The adenovirus vectors Ad5HCMVsp1*lacZ*, AdCA17*lacZ* and AdCA35*lacZ* are three different adenovirus constructs that have been used to study HCR of UV-damaged reporter gene activity in NER-proficient and deficient fibroblast cells (McKay and Rainbow, 1996; McKay *et al.*, 1997, Francis and Rainbow, 1999; Liu and Rainbow, 2004; Pitsikas *et al.*, 2005; this work). The Ad construct, Ad5HCMVsp1*lacZ*, contains the *lacZ* gene under the control of the HCMV-IE promoter, inserted into the deleted E1 region of the adenovirus genome. Like Ad5HCMVsp1*lacZ*, the adenovirus AdCA17*lacZ* bears the *lacZ* gene under the control of the HCMV-IE promoter except that the orientation of the transgene is different in comparison with Ad5HCMVsp1*lacZ*. Since different promoters may have a significant effect on gene expression, AdCA35*lacZ* was constructed to contain the *lacZ* gene under the control of the murine cytomegalovirus immediate early (MCMV-IE) promoter, thus allowing for promoter specific studies (Addison *et al.*, 1997).

Using the Ad5HCMVsp1*lacZ* virus, it has previously been reported that HCR of UV-damaged reporter gene activity is significantly reduced in XP and CS fibroblast strains relative to normal, indicating that TCR and GGR contribute to expression of the

UV-damaged reporter gene in human cells (McKay and Rainbow, 1996; McKay *et al.*, 1997a, Francis and Rainbow, 1999; Pitsikas *et al.*, 2005). It has also been reported that pre-treatment of normal human fibroblasts with low UVC fluences immediately prior to infection with the UVC-irradiated viral construct, Ad5HCMVspl*lacZ*, results in enhanced HCR of the UV-damaged reporter gene suggesting the presence of inducible DNA repair in human cells (McKay *et al.*, 1997a; Francis and Rainbow, 1999). Using a quantitative PCR technique, it has been reported that UVC pre-treatment of normal human fibroblasts results in an enhanced rate of removal of UVC-induced photoproducts from the *lacZ* reporter gene in Ad5HCMVspl*lacZ* following infection of human fibroblasts, with reduced removal in NER deficient cells (Boszko and Rainbow, 2002). These results suggest that the enhanced HCR for expression of the UV-damaged reporter gene reflects the presence of one or more inducible DNA repair pathways in mammalian cells. While TCR and GGR contribute to repair of the UV-damaged reporter gene in untreated normal human fibroblasts, the enhanced HCR observed in UVC pre-treated cells may result from the induction of TCR and/or GGR in the transcribed strand of the reporter gene.

The p53 tumour suppressor gene is associated with cancer prevention, and is the gene most commonly altered in cancer (Hollstein *et al.*, 1991). There is also considerable evidence supporting a functional role for p53 in NER. Hanawalt and colleagues have demonstrated that Li-Fraumeni syndrome (LFS) fibroblasts, which are homozygous for p53 mutations, are deficient in the removal of UV-induced CPDs from genomic DNA, but still demonstrating proficient TCR (Ford and Hanawalt, 1995). Additional studies involving human fibroblasts transformed with the human papillomavirus (HPV) E6 gene,

which degrades p53 and limits its activity, exhibit proficient TCR despite demonstrating a deficiency in GGR following UVC treatment (Ford *et al*, 1998). However, there is also evidence suggesting that the preferential repair of actively transcribing genes is p53-dependent. Drobetsky and colleagues demonstrated that LFS and HPV E6-expressing fibroblasts exhibited reduced CPD removal (relative to normal) along both the transcribed and non-transcribed strands of active genes, suggesting that both TCR and GGR are p53-dependent upon UVB exposure (Therrien *et al.*, 1999). In addition, cells from LFS patients have also been shown to be deficient in UVC-enhanced reactivation of a UVC-damaged reporter gene (McKay *et al.*, 1997a), implicating p53 in the process. These results conflict with the reports that suggest that GGR, and not TCR, is up-regulated in a p53-dependent manner in human cells in response to UV treatment.

It has previously been shown that pre-UVC-treatment immediately prior to infection of normal and XP-C, but not CS-B, cells resulted in enhanced HCR for the Ad5HCMVsp1*lacZ* encoded reporter gene when β-gal activity is scored at 40 hr after infection (Francis and Rainbow, 1999), and UVC-enhanced HCR was only detected in CS-B cells when the time between UVC treatment of cells and Ad5HCMVsp1*lacZ* infection was delayed to between 40 and 60 hr (Pitsikas *et al.*, 2005). This has led to the current model proposed for the mechanism of UV-induced GGR in human cells: UV-induced p53-dependent up-regulation of the DDB2 gene product (p48 subunit of the XPE protein complex) (Hwang *et al.*, 1999) and XPC proteins (Adimoolam and Ford, 2002) is expected to be dependent on TCR, which is required to remove UV-induced lesions from the transcribed strands of the p48 and XPC genes. Therefore, it would be expected that

p53-dependent up-regulation of GGR, which is dependent on TCR, would be delayed in TCR-deficient (GGR-proficient) CS cells.

To determine whether orientation of the transgene may influence its repair in normal and various NER-deficient cell strains, we examined HCR and UVC-enhanced HCR of the UVC-damaged reporter construct, AdCA17*lacZ*, in normal, XP-C, XP-E, XP-A and CS-B human fibroblasts. In the present work, cells were UVC-irradiated or mock-irradiated and then immediately infected with UVC-irradiated or mock-irradiated AdCA17*lacZ*, and subsequently scored for β-gal expression at various times after infection. The results demonstrate that HCR of the UVC-damaged reporter gene activity was reduced in XP-A, XP-C, XP-E and CS-B cell strains at both 24 and 40 hr post-infection, in comparison to normal fibroblasts. In addition, UVC-enhanced HCR of the UVC-damaged reporter gene activity was detected in normal fibroblasts at 12 hr post-infection and in CS-B, XP-C and XP-E fibroblasts, but not XP-A fibroblasts at 24 and 40 hr post-infection.

Materials and Methods

Cell Strains and Virus

The normal human fibroblast strain GM09503 and the repair-deficient primary human fibroblast strains XP12BE (GM05509; XP-A), XP2BE (GM00677; XP-C), XP23PV (GM01389; XP-E), CS1AN (GM00739; CS-B) were obtained from the NIGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden, NJ). All cell cultures were grown at 37°C in a humidified incubator in 5% CO_2 and cultured in

Eagle's α-MEM supplemented with 10% fetal bovine serum and antimycotic/antibiotic (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL).

The viral vector AdCA17*lacZ* is a non-replicating recombinant human adenovirus (Ad) expressing the bacterial *lacZ* gene under the control of the HCMV-IE promoter (-299 to +72 relative to transcription start site) inserted into the E1 deleted region. The deletion in the E1 region of the Ad genome renders the virus unable to replicate in most mammalian cells. AdCA17*lacZ* was obtained from Dr. F.L. Graham (McMaster University, Canada). The viral vector was propagated, collected and titred as described previously (Graham and Prevec, 1991).

Cell Seeding

Confluent flasks of cells were trypsinized, cells were counted with a haemocytometer and seeded in 96 well-plates (Falcon, Lincoln Park, NJ) at a density of 2×10^4 cells/well in supplemented α-MEM. Once seeded, cells were incubated for 24 hr at 37°C in a humidified incubator in 5% CO_2.

UVC Treatment of Cells

After 24 hours of incubation, the media was aspirated from each well, and 40 μl of warmed phosphate-buffered saline (PBS; 9 g/L NaCl, 0.8 g/L Na_2HPO_4, 0.14 g/L KH_2PO_4 [pH 7 – 7.2]) was overlayed on the cells. Cells were UV-irradiated (or mock irradiated) at a fluence of 20 J/m^2 using a General Electric germicidal bulb (emitting

predominantly at 254 nm) at a fluence rate of 1 W/m^2. UVC exposure of 20 J/m^2 was corrected for irradiation in 96-well plates (12 J/m^2) as previously reported (McKay *et al.*, 1997b).

Preparation of UVC Treatment to Virus

An aliquot of 40 µl or 80 µl of AdCA17*lacZ* (1.0 x 10^{10}pfu/ml) was placed in 1.8 ml of ice cold PBS placed in a small (35 x 10 mm) petri dish while being kept on ice, such that the multiplicity of infection (MOI) would be approximately 80 or 160 pfu/cell, respectively. The viral suspension was UV-irradiated (or mock-irradiated) at various UVC fluences using a General Electric germicidal bulb (emitting predominantly at 254 nm) at a fluence rate of 2 W/m^2, while continuously being stirred. To obtain the various UV exposures to virus, 200 µl aliquots of the viral suspension were sequentially removed and added to 1 ml of serum-free α-MEM at various time points, allowing for cumulative exposures of UVC to virus in a single experiment. To completely inactivate the virus and obtain a background level of β-galactosidase (β-gal) activity within the cells, the virus was exposed to a UVC fluence of 8,000 J/m^2.

Viral Infection of Cells

After immediate treatment (or mock treatment) of cells with UVC, cells were infected with 40 µl of UVC-irradiated or non-irradiated viral suspension at a multiplicity of infection (MOI) of approximately 80 or 160 pfu/cell. After infection, cells were incubated for 90 min at 37°C in a humidified incubator containing 5% CO_2. Following

incubation, cells were given an additional 160 µl of warmed supplemented media and further incubated for 24 or 40 hours to allow for reporter gene expression.

Quantitation of β-gal Activity

After the appropriate period of incubation, the media was aspirated from each well and 60 µl of 1 mM chlorophenolred-β-D-galactopyranoside (CPRG; Boehringer-Mannheim, Indianapolis, IN), dissolved in 0.01% Triton X-100, 1 mM $MgCl_2$ and 100 mM phosphate buffer at pH 8.3, was added to each well. The absorbance at 570 nm was determined at several time points after the addition of CPRG solution using a 96-well plate reader (Mandel, BIO-TEK Instruments, EL 340 Bio Kinetics Reader).

Results

HCR of β-gal activity for UVC-irradiated AdCA17*lacZ* is reduced in XP-A, XP-C and CS-B human fibroblasts relative to that in normal fibroblasts.

Normal, XP and CS fibroblast strains were infected with UVC-irradiated and non-irradiated AdCA17*lacZ* at a multiplicity of infection of approximately 80 pfu/cell (MOI 80). Typical curves of relative β-gal activity for UV-irradiated AdCA17*lacZ* in non-irradiated normal, XP-A, XP-C, XP-E and CS-B fibroblasts at 40 hr post-infection are shown in Figure 2-2. It can be seen that HCR was reduced following infection of the XP-A, XP-C, and CS-B fibroblast strains in comparison to the normal fibroblast, which reflects the deficiency of NER in these cell lines. The UVC fluence required to reduce the relative β-gal activity to 37% (D_{37}) was determined for each strain at both 24 and 40

hr post-infection. Mean D_{37} values for each of the strains examined are presented in Table 2-1. These results demonstrate that the HCR of β-gal activity was reduced in all the XP (XP-A, XP-C and XP-E) and CS (CS-B) strains relative to normal human fibroblasts at both 24 and 40 hr post-infection.

As indicated by the relative HCR values in Table 2-2, the CS-B, XP-A and XP-C strains exhibited a significant deficiency in the ability to reactivate the UV-damaged *lacZ* gene activity at 40 hr post-infection in non-irradiated cells ($P < 0.05$ by an independent *t*-test). However, only the CS-B and XP-A fibroblast strains exhibited a significant deficiency at 24 hr following infection. The XP-C fibroblast strain demonstrated a significant deficiency (compared to normal cells) in the ability to reactivate β-galactosidase (β-gal) activity at 40 hr post-infection, while XP-E cells did not demonstrate a significant deficiency at either 24 or 40 hr post-infection.

Detection of enhanced HCR of β-gal activity for UVC-irradiated AdCA17*lacZ* in UVC pre-treated XP-C, XP-E and CS-B cell strains, but not in normal or XP-A cell strains.

HCR of β-gal activity for UV-irradiated AdCA17*lacZ* was also examined in UV-irradiated fibroblasts. Cells were infected with either UVC-irradiated or non-irradiated AdCA17*lacZ* immediately after UVC pre-treatment of cells and scored for β-gal activity 24 and 40 hr later. Representative results for the relative β-gal activity of UV-irradiated AdCA17*lacZ* in non-irradiated *versus* UV-treated fibroblasts are shown in Figures 2-3 and 2-4. D_{37} values for HCR of β-gal activity in non-irradiated and UV-treated cells were used to determine the relative D_{37} value, an indication of the UV-enhanced HCR of the UV-damaged reporter gene activity for a given cell line. The results from multiple

experiments were combined and the average relative D_{37} values in UVC-treated compared to non-irradiated cells at 24 and 40 hr post-infection are shown in Table 2-3. As demonstrated in Table 2-3, pre-treatment of normal fibroblasts with 12 J/m^2 did not result in a significant enhancement in HCR of the UV-damaged *lacZ* gene activity at 24 and 40 hr post-infection. UV-enhanced HCR of the UV-damaged *lacZ* gene activity was also examined in CS-B, XP-C, XP-E and XP-A fibroblast strains, and was detected in CS-B, XP-C and XP-E fibroblasts at 24 and 40 hr post-infection, but not for XP-A fibroblasts. However, the enhancement in HCR of the UV-damaged *lacZ* activity was only significant at 24 hr post-infection for CS-B, and 40 hr post-infection for the XP-C and XP-E cell strains. In addition, the UV-enhanced HCR of the UV-damaged *lacZ* gene activity was not significantly different for CS-B, XP-C or XP-E at both 24 and 40 hr post-infection (Table 2-3).

Detection of UV-enhanced HCR in normal fibroblasts is dependent on the HCR value of the UV-damaged reporter gene in non-irradiated cells.

Since prior UVC irradiation of cells did not enhance the reactivation of the UVC-damaged *lacZ* gene activity in normal (GM09503) fibroblasts within the 24 – 40 hr time range, the relative D_{37} values for the HCR of β-gal activity in UVC-treated compared to non-irradiated cells (i.e. UV-enhanced HCR) were plotted *versus* the basal HCR (D_{37}) value for each individual experiment as shown in Figure 2-5. It can be seen that UV-enhanced HCR of the UV-damaged *lacZ* gene activity was observed in the normal fibroblast strain when the HCR value in the non-treated cells was low, while the relative D_{37} values decreased with increasing HCR value. Based on these results, it was

considered on interest to examine UV-enhanced HCR of the UV-damaged *lacZ* gene in normal fibroblasts at times post-infection, earlier than 24 hours when HCR values in the non-treated cells would be expected to be low. Normal fibroblasts were pre-UVC-irradiated or mock irradiated and subsequently infected with UVC-irradiated AdCA17*lacZ* either immediately or 24 hr later. The infected cells were then scored for β-gal activity at 12 hr post-infection. Representative results are shown in Figure 2-6. It can be seen that normal fibroblasts exhibit UV-enhanced HCR of β-gal activity at 12 hr following an immediate or 24 hr delayed infection of AdCA17*lacZ* following UVC pre-treatment to cells. Pooled results from several experiments show a significant enhancement in HCR was observed following immediate infection of UV-treated cells (Table 2-4).

Discussion

We have used recombinant non-replicating adenovirus constructs to examine HCR of a UV-damaged reporter gene in human and rodent cells as a means to assess the repair capacity of NER-proficient and deficient cells. UV-induced DNA lesions, such as CPDs, are capable of inhibiting transcription and subsequent expression of a reporter gene in cultured human cells (Protic-Sabljic and Kraemer, 1985). This decrease in RNA synthesis is observed in cultured cells following UV irradiation, but recovers quickly in normal cells. However, this recovery in RNA synthesis is reduced or absent in Cockayne syndrome (CS) and xeroderma pigmentosum (XP) cells from different complementation groups (Mayne and Lehmann, 1982), suggesting that efficient cellular repair of active

genes is responsible for a cell's ability to recover RNA synthesis following UV exposure. In mammalian cells, the transcription-coupled repair (TCR) sub-pathway is believed to be responsible for repairing transcription-blocking lesions from active genes, and is demonstrated by the rapid removal of UV-induced CPDs from the transcribed strand compared to the non-transcribed strand of active cellular genes (Bohr *et al.*, 1985; Mellon *et al.*, 1987).

It has previously been reported that basal HCR (i.e. in non-irradiated cells) of the UVC-damaged Ad5HCMVsp1*lacZ* was significantly reduced in XP-C (GGR-deficient), CS (CS-A and CS-B; TCR-deficient) and XP-A (GGR/TCR-deficient) fibroblasts compared to that in normal human fibroblasts (Francis and Rainbow, 1999), suggesting that both TCR and GGR contribute to the repair, and subsequent expression, of the UVC-damaged *lacZ* gene in human cells. Using a quantitative PCR technique, a significant removal of UVC-induced photoproducts from the *lacZ* reporter gene was demonstrated in normal human fibroblasts, but CS-B and XP-C fibroblasts demonstrated reduced removal of these lesions (Boszko and Rainbow, 2002). These previous results suggest that differences in survival of β-gal activity in repair-proficient and deficient cells are likely due to differences in the removal of UVC-induced lesion by NER. Using the same experimental protocols for the HCR assay, we were interested in examining repair of the UV-damaged AdCA17*lacZ* construct (which contains a similar transgene as Ad5HCMVsp1*lacZ*) in non-irradiated normal and NER-deficient cells. In the present work, we show that HCR of the UV-damaged *lacZ* gene activity was reduced in CS-B, XP-C, XP-E and XP-A fibroblasts compared to that in the normal cell strain at 24 and 40

hr between AdCA17*lacZ* infection and scoring for β-gal activity (Figure 2-2; Tables 2-1 and 2-2). As indicated by the relative HCR values (Table 2-2), there was a significant reduction compared to normal for the XP-A, XP-C and CS-B strains, but not for the XP-E strain. The CS-B cell strain exhibited reduced levels of HCR in non-irradiated cells in comparison to normal cells at both 24 and 40 hr post-infection, while the XP-C cell strain exhibited significantly reduced levels of HCR in non-irradiated cells at 40 hr post-infection. However, the XP-A cell strain, which is deficient in both TCR and GGR pathways, showed the greatest deficiency in repair of the UV-damaged reporter gene. These results indicate that both TCR and GGR contribute to repair in the transcribed strand of the AdCA17*lacZ* encoded reporter gene, as previously reported for the Ad5HCMVSp1*lacZ* encoded reporter gene.

It has previously been reported that prior UVC irradiation of cells results in enhanced reactivation of the UVC-damaged *lacZ* reporter gene activity in normal human fibroblasts. This enhanced survival of β-gal activity for the UVC-damaged Ad5HCMVsp1*lacZ* construct was induced at low UVC fluences to normal and XP-C fibroblasts, but was delayed in CS-B fibroblasts and absent in other XP complementation groups examined (Francis and Rainbow, 1999; Pitsikas *et al.*, 2005; Rainbow *et al.*, in press). This suggested that the observed UV-enhanced HCR of the UV-damaged reporter gene activity in human cells results from an inducible DNA repair process that can involve both the TCR and the GGR pathway. We have shown in the current study that prior UV irradiation of both TCR-deficient XP-C and XP-E and GGR-deficient CS-B cell strains resulted in enhanced HCR of the UV-damaged reporter at 24 and 40 hr between

AdCA17*lacZ* infection and scoring for β-gal activity (Figures 2-3 and 2-4; Table 2-3). UV-enhanced HCR of the UV-damaged *lacZ* gene activity was observed for TCR-proficient XP-C and XP-E fibroblasts at both 24 and 40 hr post-infection, however this enhancement in reactivation was only significant at 40 hr post-infection (Table 2-3). These results give further support that both GGR and TCR are inducible upon UV-pretreatment of cells; increased repair of the transcribed strand of the reporter gene occurs in GGR-deficient (XP-C, XP-E) and TCR-deficient (CS-B) cells following UV exposure. UV-enhanced HCR of the UV-damaged reporter gene was not detected in the GGR- and TCR-deficient XP-A cell strain. The lack of UV-enhanced HCR of the UV-damaged *lacZ* gene activity in XP-A cell strain supports the requirement of NER for this process.

There are several lines of evidence that suggest that both GGR and TCR are inducible in mammalian cells. It has been reported that UV pre-treatment of normal, but not XP-A or XP-C, cells enhanced the recovery of semi-conservative DNA synthesis following subsequent UV treatment (Moustacchi *et al.*, 1979). The fact that XPC and XPA proteins were required for the enhanced recovery of DNA synthesis suggests that this phenomenon is dependent on GGR. In addition, pre-treatment of normal human fibroblasts with low doses of quinacrine mustard resulted in an enhanced rate of CPD removal by NER (Ye *et al.*, 1999). More recently, there has been evidence to suggest that TCR is inducible in rodent cells. Germanier and colleagues reported an increase in the rate of lesion removal from the actively transcribed dihydrofolate reductase (DHFR) gene following low dose UV-pre-treatment of Chinese hamster ovary (CHO) cells when

subsequently treated with a higher dose. The enhanced rate of lesion removal from the transcribed strand of the cellular DHFR gene suggests that TCR is inducible in CHO cells (Germanier *et al.*, 2000).

By an examination of D_{37} values for non-irradiated and UV-irradiated repair-deficient cell lines (Table 2-1) it can be seen that prior UV-irradiation of XP-C, XP-E or CS-B fibroblasts results in absolute D_{37} values that are reduced in comparison to those obtained for non-irradiated normal fibroblasts at either 24 or 40 hr post-infection. This indicates that UV-induction of either sub-pathway alone at this particular fluence (12 J/m^2) cannot compensate for the repair deficient sub-pathways of XP-C or CS-B cells.

Normal human fibroblasts are proficient in NER of UV-induced DNA damage for the transcribed strand of active genes, as well as the non-transcribed strand of active genes and non-coding regions of DNA. We have previously reported UV-enhancement in HCR of the *lacZ* reporter gene activity using UV-damaged Ad5HCMVsp1*lacZ* in normal cells (Pitsikas *et al.*, 2005; Francis and Rainbow, 1999; McKay *et al.*, 1997a). Enhanced HCR was readily detected in HCR assays of normal cells using the Ad5HCMVsp1*lacZ* virus and scoring for β-gal activity at 40 hr after infection. However, we were unable to detect enhanced HCR following UVC pre-treatment of normal cells when using the AdCA17*lacZ* virus under similar conditions (Table 2-3). Although significant UV-enhancement in HCR was not observed using the AdCA17*lacZ* virus in normal fibroblasts when scored for β-gal activity at 24 or 40 hr post-infection, our results indicate that UV-enhanced HCR is detectable when the basal HCR of the UV-damaged reporter gene in untreated cells is low. Upon plotting the relative D_{37} as a function of

basal HCR (D_{37} of non-irradiated cells) for each individual experiment, we observed a trend suggesting that relative D_{37} values decrease with increasing basal HCR values (Figure 2-5). This is consistent with the notion that UVC pre-treatment of cells increases the rate of repair in the transcribed strand of the reporter gene (Boszko and Rainbow, 2002), such that an enhancement in HCR is only detected at significant levels at early times after infection using the AdCA17*lacZ* virus when the HCR value in non-irradiated cells is low. Although UVC pre-treatment of cells enhances the rate of repair, at later times after infection (24 and 40 hr), the absolute levels of repair in the transcribed strand of the reporter gene are similar in UVC pre-treated compared to non-irradiated cells. Therefore, it was of no surprise that scoring for β-gal activity at 12 hr post-infection of the UVC-damaged AdCA17*lacZ* in normal cells resulted in significant UV-enhancement of HCR of the UV-damaged *lacZ* gene activity following immediate infection of cells.

In the current work, pre-UVC-treatment of cells immediately prior to infection with UVC-damaged AdCA17*lacZ* resulted in an enhanced HCR for CS-B cells when β-gal activity is scored at 40 hr after infection. However, UVC-enhanced HCR was only detected in CS-B cells when the time between UVC treatment of cells and Ad5HCMVsp1*lacZ* infection was delayed to between 40 and 60 hr (Pitsikas *et al.*, 2005). These results suggest that different conditions of the HCR assay, including the time after infection of scoring for β-gal activity and the time between UVC pre-treatment to cells and adenovirus infection, may be required in order for significant UV-enhancement in HCR to be detected using the UVC-damaged AdCA17*lacZ* construct compared to the Ad5HCMVsp1*lacZ* construct. The fact that UV-enhanced HCR is detected in CS-B, XP-

C and XP-E cell strains using AdCA17*lacZ* may suggest that the HCR levels in non-irradiated cells are reduced since constitutive levels are low, thus making enhanced repair more easily detected. However, basal HCR (D_{37}) values were significantly greater (~10-fold) in the cell lines using the Ad5HCMVsp1*lacZ* virus (at 10 MOI; see Francis and Rainbow, 1999) in comparison to assays involving AdCA17*lacZ* (MOI ~ 40 – 80). Both Ad5HCMVsp1*lacZ* and AdCA17*lacZ* contain a fragment of the HCMV-IE promoter from -299 to + 72, which has been shown to induce high levels of transgene expression in human cells. In addition, Graham and colleagues have demonstrated that AdCA17*lacZ*, which contains the *lacZ* reporter gene under the control of the HCMV-IE promoter inserted in the left to right orientation (see Figure 1-4), produced 7-fold higher levels of β-gal than another adenovirus construct carrying the same transgene except in the opposite orientation (Addison *et al.*, 1997). This orientation dependent difference may be due, in part, to the presence of the E1a enhancer, which remains in the adenovirus vector backbone upstream of the HCMV-IE promoter in AdCA17*lacZ*. The E1a enhancer can act to enhance E1A transcription, however deletion of this region results in a 4- to 5-fold decrease in E1A transcript levels (Hearing and Shenk, 1986). Even though the E1 region (E1A/E1B) of the adenovirus genome is removed and the transgene is inserted in its place, it is conceivable that in recombinant adenoviruses containing a rightwards-oriented transgene, transcription factors binding to the E1a enhancer and the HCMV-IE promoter enhancer sequences can act synergistically. Therefore, the presence of the E1a enhancer upstream of the HCMV-IE promoter may account for the increased β-gal protein levels (Addison *et al.*, 1997) and activity (this work) observed with the AdCA17*lacZ* construct.

Although the AdCA17*lacZ* and Ad5HCMVsp1*lacZ* constructs are not isogenic, the lack of an E1a enhancer upstream of the HCMV-IE promoter in Ad5HCMVsp1*lacZ* may result in differences in the timing of expression and repair of the reporter gene. Differences in the timing of expression for the reporter gene may in some way explain why, in contrast to previous reports using the Ad5HCMVsp1*lacZ* construct, UV-enhanced HCR of β-gal activity was observed in normal fibroblasts at 12 hr but not 24 and 40 hr post-infection; and in CS-B fibroblasts at both 24 and 40 hr post-infection using the AdCA17*lacZ* construct. Since inhibition of RNAPII results in the induction of p53 (Ljungman *et al.*, 1999) and subsequent p53-dependent transcriptional up-regulation of DDB2 and XPC proteins, it is possible that stalling of RNAPII transcription along the *lacZ* gene in AdCA17*lacZ* is increased compared to the less active *lacZ* gene in Ad5HCMVsp1*lacZ*, which may result in increased p53 up-regulation of DDB2 and XPC proteins in AdCA17*lacZ* infected, TCR-deficient CS-B cells. This may explain why UVC-enhanced HCR is observed in CS-B cells at 24 and 40 hr following AdCA17*lacZ* infection, but not until 40 - 60 hr following Ad5HCMVsp1*lacZ* infection. Future studies involving strand-specific repair may help determine whether orientation of the transgene in AdCA17*lacZ* compared to Ad5HCMVsp1*lacZ* can account for the different timing in the detection of UV-enhanced HCR observed in the various repair-deficient cells.

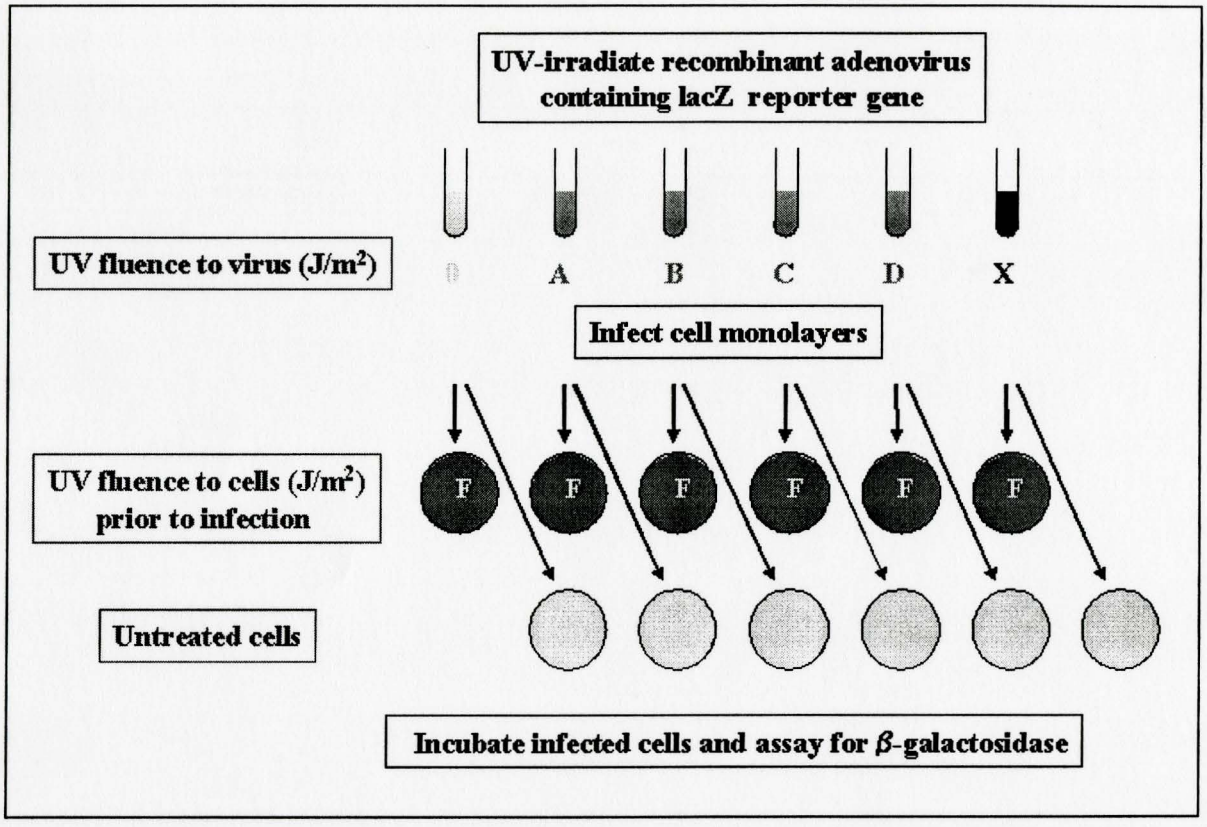

Figure 2-1. Schematic representation of host cell reactivation (HCR) and UV-enhanced HCR assays involving infection of a UV-damaged recombinant adenovirus-based reporter gene construct into human cells. Cells were seeded in 96-well microtitre plates at a density of 2×10^4 cells/well, 24 hours prior to UV treatment of cells. The growth medium was then aspirated, replaced with 40 ml of phosphate-buffered saline (PBS) and cell monolayers were either UV-irradiated with a fluence (F) of 20 J/m^2 (12 J/m^2 corrected fluence) or were mock-irradiated (no UVC treatment). Immediately after treatment (<1 hour), both UV-irradiated and non-irradiated cell monolayers were infected for 90 min at 37°C in a total volume of 40 µl containing non-irradiated AdCA17*lacZ* or AdCA17*lacZ* receiving a range of UVC fluences (A to D). Infected cells were incubated for 12, 24 or 40 hours before scoring for β-galactosidase activity. Cell monolayers infected with heavily irradiated AdCA17*lacZ* (8,000 J/m^2), X, served as a measure of background levels of β-galactosidase activity. Enhanced HCR in UV-treated compared to non-treated cells suggests inducible repair of the UV-damaged reporter gene activity. Adapted from Rainbow *et al.*, 2000.

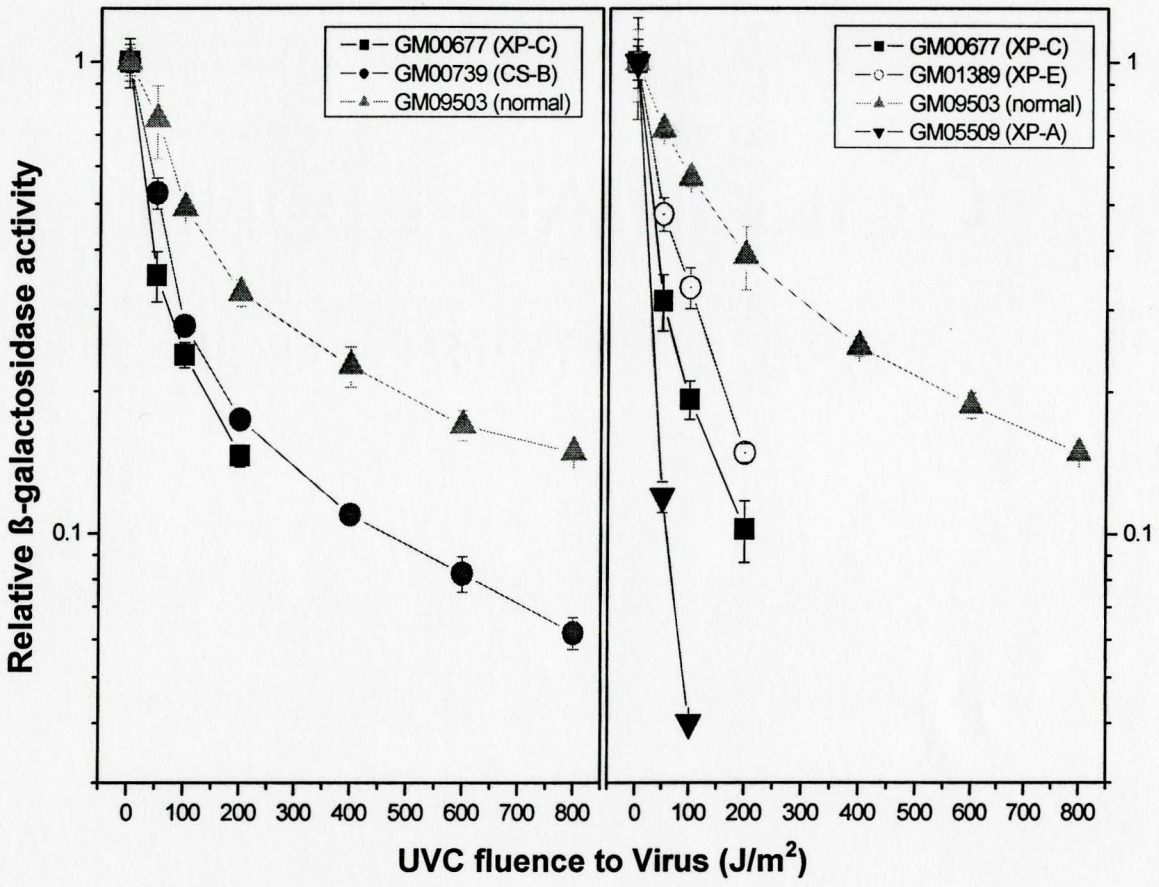

Figure 2-2. Host cell reactivation (HCR) of a UV-damaged *lacZ* gene activity reflects the nucleotide excision repair (NER) capacity of human cells. Human fibroblasts were infected with non-irradiated or UV-irradiated AdCA17*lacZ* and scored for β-gal activity 12, 24 or 40 hr later, as described previously. Representative results for GM09503 (normal; ▲), GM00677 (XP-C; ■), GM01389 (XP-E; ○), GM05509 (XP-A; ▼) and GM00739 (CS-B; ●) are depicted at 40 hours following infection of cells. Each point is the average of 3 replicates; error bars represent one standard error.

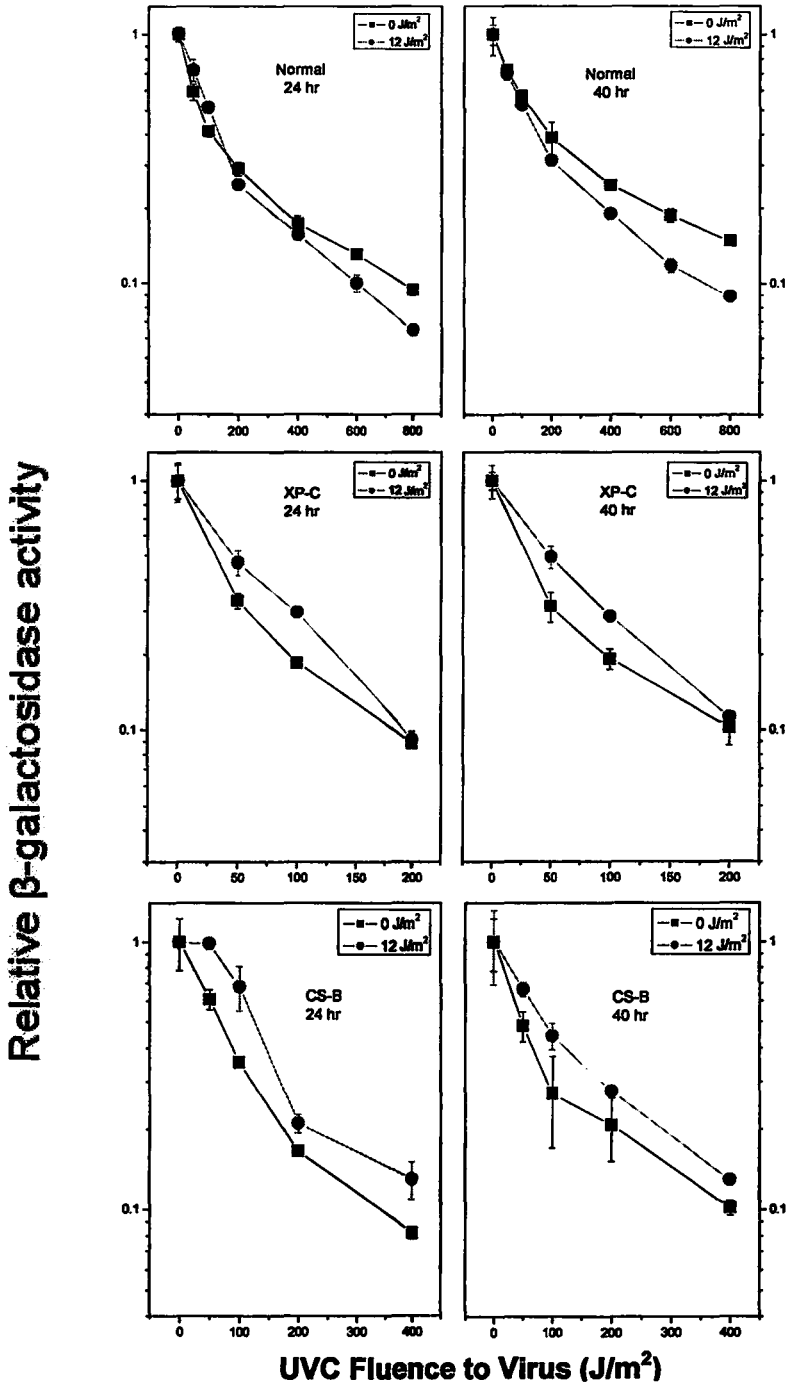

Figure 2-3. Effect of UV pre-treatment on HCR of a UV-damaged *lacZ* gene in normal, XP-C and CS-B fibroblasts. Results of typical experiments representing (■) non-irradiated and (●) UV-irradiated (12 J/m^2) primary human fibroblasts are shown. Immediately following UVC exposure, cells were infected with non-irradiated or UV-irradiated AdCA17*lacZ* and scored for β-gal activity at 24 or 40 hr post-infection. Cell strains presented are normal (GM09503), XP-C (GM00677) and CS-B (GM00739), respectively. Each point is the average of 3 replicates; error bars represent one standard error.

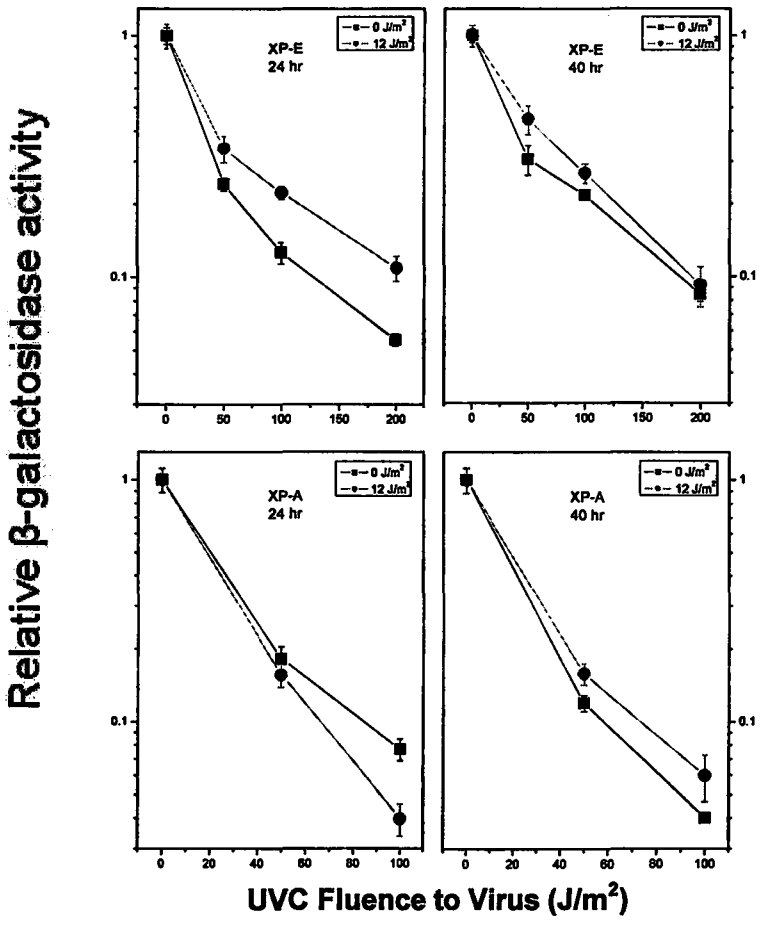

Figure 2-4. Effect of UV pre-treatment on HCR of a UV-damaged *lacZ* gene in XP-E and XP-A fibroblasts. Results of typical experiments representing (■) non-irradiated and (●) UV-irradiated (12 J/m^2) primary human fibroblasts are shown. Immediately following UVC exposure, cells were infected with non-irradiated or UV-irradiated AdCA17*lacZ* and scored for β-gal activity at 24 or 40 hr post-infection. Cell strains presented are XP-E (GM01389), and XP-A (GM05509), respectively. Each point is the average of 3 replicates; error bars represent one standard error.

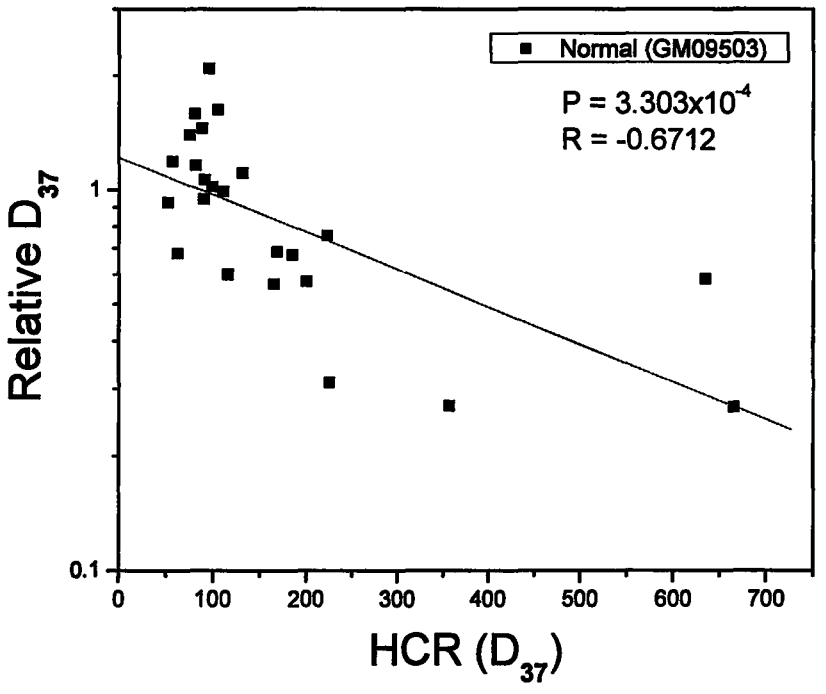

Figure 2-5. Enhanced HCR of β-gal activity (relative D_{37}) in normal cells plotted *versus* the basal HCR (D_{37}) values for each individual experiment. Each data point represents the relative D_{37} value for GM09503 (normal fibroblast) obtained from each individual experiment (at either 24 or 40 hr post-infection), plotted *versus* the HCR (D_{37}; GM09503 (non-irradiated)) value for the given experiment. D_{37} is the UVC fluence required to reduce the β-gal activity to 37%.Data points were fitted to a linear regression.

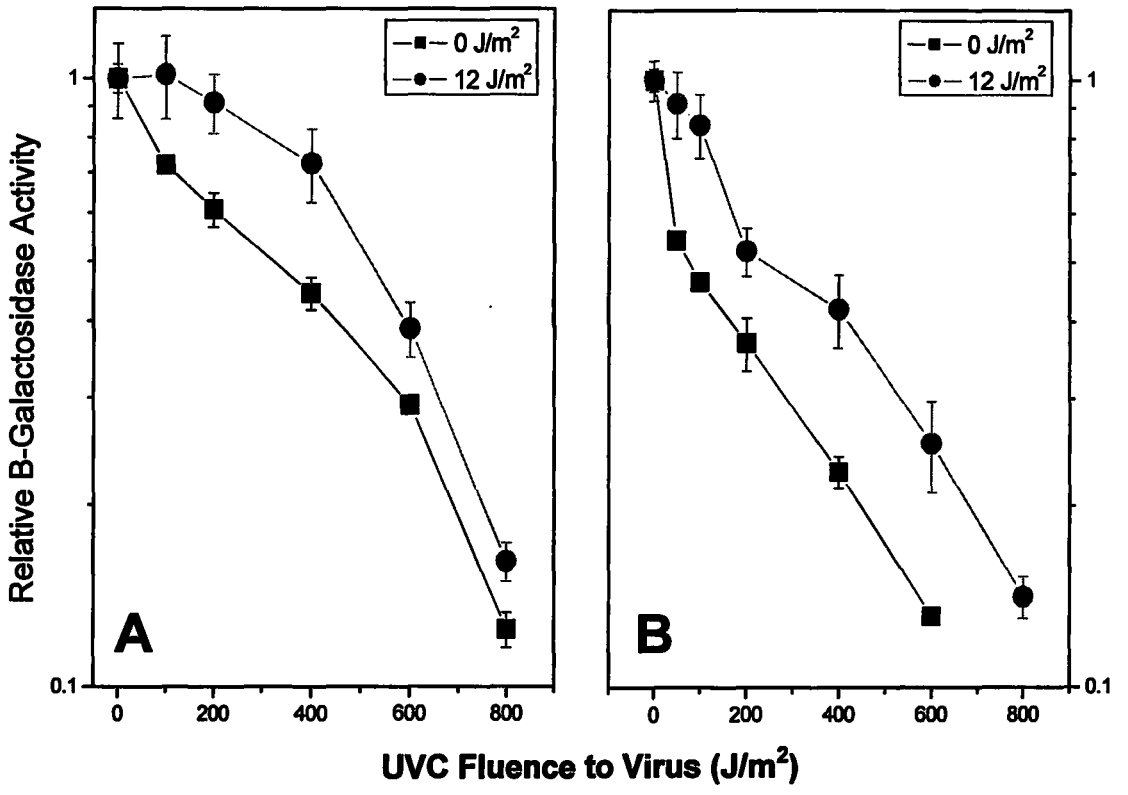

Figure 2-6. Effect of UV pre-treatment on HCR of a UV-damaged *lacZ* gene in normal fibroblasts, measured 12 hr following AdCA7*lacZ* infection. Representative results for (■) non-irradiated and (●) UVC pre-treated (12 J/m^2) GM09503 fibroblasts, in which the cells were infected with AdCA17*lacZ* either (A) immediately or (B) 24 hr following UVC pre-treatment and scored for β-gal activity at 12 hr post-infection. Each point is the average of 3 replicates; error bars represent one standard error.

Table 2-1. HCR of a UV-damaged *lacZ* gene activity in non-irradiated and UV-irradiated cells. Human fibroblasts were infected with non-irradiated or UV-irradiated AdCA17*lacZ* and scored for β-gal activity 24 or 40 hr later Results are shown as mean HCR (D_{37}) values ± SE for at least 3 independent experiments. D_{37} is the UVC fluence required to reduce the β-gal activity to 37%.

NIGMS Catelogue No.	Cell strain	Group	Non-irradiated Cells		UVC-irradiated Cells	
			D_{37} (J/m^2)	D_{37} (J/m^2)	D_{37} (J/m^2)	D_{37} (J/m^2)
			24 hrs	40 hrs	24 hrs	40 hrs
GM09503		Normal	152.8 ± 45.3	193.3 ± 49.8	132.6 ± 24.8	110.8 ± 11.4
GM00739	CS1AN	CS-B	73.1 ± 10.1	71.0 ± 8.5	105.4 ± 21.9	101.4 ± 19.7
GM00677	XP2BE	XP-C	73.5 ± 13.7	53.0 ± 9.7	107.5 ± 13.3	80.0 ± 12.3
GM01389	XP23PV	XP-E	80.7 ± 9.0	97.4 ± 24.0	119.4 ± 28.5	128.5 ± 23.7
GM05509	XP12BE	XP-A	27.3 ± 1.1	25.0 ± 1.1	28.2 ± 3.7	25.7 ± 0.8

Table 2-2. Relative HCR values for CS-B, XP-C, XP-E and XP-A fibroblast strains following infection with AdCA17*lacZ*. Relative HCR was calculated by basal HCR(D_{37} of non-irradiated cell strain) / basal HCR (D_{37} of non-irradiated normal cell) within individual experiments at 24 or 40 hr post-infection. D_{37} is the UVC fluence required to reduce the β-gal activity to 37%. * Relative HCR significantly less than 1 by an independent *t*-test (*P* value < 0.05).

NIGMS Catalogue No.	Complementation Group	Relative HCR 24 hr	Relative HCR 40 hr
GM09503	Normal	1.00	1.00
GM00739	CS-B	0.82 ± 0.08*	0.56 ± 0.11*
GM00677	XP-C	0.86 ± 0.21	0.34 ± 0.10*
GM01389	XP-E	0.95 ± 0.20	1.01 ± 0.44
GM05509	XP-A	0.22 ±0.06*	0.19 ± 0.11*

Table 2-3. Effect of UVC pre-treatment on HCR of a UV-damaged *lacZ* gene in repair-proficient and deficient cells. Pooled results are shown for cells infected at 24, 40 or 24-40 hr post-infection, and are represented as mean relative D_{37} (± SE). D_{37} is the UVC fluence required to reduce the β-gal activity to 37%. Enhanced HCR (relative D_{37}) of the UV-damaged *lacZ* gene activity is observed early in GGR-proficient fibroblasts (CS-B), and is delayed in TCR-proficient (XP-C and XP-E) fibroblasts pre-treated with 12 J/m^2. *Significantly > 1 by an independent *t*-test (*P* value < 0.05); (#) is the number of independent determinants, each performed in three replicates.

NIGMS Catalogue No.	Relative D_{37} 24 hr	*P* value	Relative D_{37} 40 hr	*P* value	Relative D_{37} 24 - 40 hr	*P* value
GM09503	1.05 ± 0.14 (12)	0.694	0.825 ± 0.13 (12)	0.182	0.939 ± 0.094 (24)	0.525
GM00739	1.38 ± 0.16* (6)	0.042	1.44 ± 0.23 (6)	0.085	1.41 ± 0.135* (12)	0.006
GM00677	1.56 ± 0.23 (4)	0.051	1.53 ± 0.11* (4)	0.002	1.55 ± 0.117* (8)	3.69×10^{-4}
GM01389	1.46 ± 0.25 (7)	0.088	1.45 ± 0.15* (7)	0.011	1.46 ± 0.139* (14)	0.003
GM05509	1.03 ± 0.13 (3)	0.815	1.03 ± 0.06 (3)	0.587	1.03 ± 0.06 (6)	0.607

Table 2-4. Effect of UV pre-treatment on HCR of a UV-damaged *lacZ* gene activity in normal fibroblasts when examined at 12 hr post-infection. Pooled results are shown for GM09503 fibroblasts infected either immediately or 24 hr following UVC irradiation (12J/m^2), and scored for β-gal activity at 12 hr post-infection. Results are represented as mean relative D_{37} (± SE). D_{37} is the UVC fluence required to reduce the β-gal activity to 37%. Enhanced HCR (relative D_{37}) of the UV-damaged *lacZ* gene was observed in normal cells that were UVC pre-treated (12 J/m^2) immediately prior to AdCA17*lacZ* infection. *Significantly > 1 by an independent *t*-test ($P < 0.05$); (#) is the number of independent determinants, each performed in three replicates.

	Immediate infection	**24 hr delay between UVC to cells and infection**
Relative D_{37} (J/m^2)	1.30 ± 0.19* (5)	1.42 ± 0.23 (5)
***P* value**	0.042	0.104

Chapter 3

Sensitivity of human keratinocytes, glioma and colon carcinoma cells following exposure to UVA, UVC and H_2O_2

Abstract

We have examined the colony survival of various human cells following exposure to UVC and UVA radiation. HaCAT, an immortalized human keratinocyte cell line, demonstrated increased resistance to UVC exposure compared to glioma (U373, T98G) and colon carcinoma (HT29) cell lines, while exhibiting increased sensitivity to UVA radiation and H_2O_2 treatment. This increased sensitivity towards both UVA and H_2O_2 treatment suggests that HaCAT cells have a reduced capacity to repair oxidative DNA damage. In addition, we show that p53-null keratinocytes (HPV-G) have reduced clonogenic survival compared to p53-mutant keratinocytes (HaCAT) following H_2O_2 treatment consistent with an involvement of p53 in the survival of keratinocytes following H_2O_2 treatment. We also examined whether the interaction of H_2O_2 with growth media resulted in chemical by-products that were toxic to cells. Even though the H_2O_2 levels in the media were reduced following 24 hr incubation, similar clonogenic survival curves of HaCAT cells were observed following treatment of cells with immediately-prepared or 24hr-incubated H_2O_2-containing media solutions. Thus, extended incubation (24 hr) of H_2O_2 with media did not alter its cytotoxicity towards HaCAT cells, indicating that no detectable levels of toxic chemical by-products were produced.

Introduction

Ultraviolet radiation (UV), a component of sunlight, is divided into three spectral regions: UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). UVC and the majority of UVB are absorbed by the stratospheric ozone layer, preventing such radiation from reaching the earth's surface. The remaining portion of solar UV radiation that can have deleterious effects on humans consists of UVA and UVB. UVB radiation accounts for approximately 5-10% of the terrestrial UV (IARC, 1992), yet it is considered to be more deleterious than UVA, since UVA fluences several orders of magnitude greater than UVB are required to produce similar cytotoxicity levels in vitro (Setlow, 1974).

The biological effects of different wavelengths vary across the UV spectrum. UVC and UVB radiation are maximally absorbed by DNA (Tornaletti and Pfeifer, 1996), capable of inducing mutagenic photolesions such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PP). UVA produces these lesions to a lesser extent as it is only weakly absorbed by DNA (Kvam and Tyrrell, 1997; Kielbassa *et al.*, 1997; Perdiz *et al.*, 2000). Instead, UVA is believed to be primarily responsible for the photo-oxidation of DNA. One of the most studied oxidative lesions results from the oxidation of guanine to produce 8-oxoguanine (8-oxoG). Both the photo-excitation and photo-oxidation of DNA can have cytotoxic and mutagenic consequences on cells.

Exposure of cells to various environmental sources, such as ultraviolet radiation, can also lead to DNA damage mediated by reactive oxygen species (ROS). UVA treatment of cell culture media results in significant hydrogen peroxide (H_2O_2)

production (Mahns *et al.*, 2003), and the conversion of H_2O_2 to hydroxyl radicals (OH·) has been suggested to be responsible for UVA-induced single-stranded break (SSB) formation (Petersen *et al.*, 2000). In addition, UVA radiation has been implicated in the oxidative modification of DNA bases, however, there are likely to be differences in the spectrum of oxidative lesions generated by UVA and H_2O_2. UVA radiation has a greater tendency to induce the formation of oxidative lesions by a type II photosensitization reaction, resulting in the production of singlet oxygen species ($^1O_2$), which react exclusively with guanine bases to produce 8-oxoG (Ravanat *et al*, 2001). While hydrogen peroxide is considered to be relatively unreactive with DNA (Bielski *et al.*, 1985), its decomposition into reactive OH· radicals allows for a wide range of oxidized lesions to be produced, including 8-oxoG and thymine glycol (Tg). In addition to DNA, cellular proteins and lipids have been shown to be damaged by H_2O_2 (Ramirez *et al.*, 2005; Zhu *et al*, 2005) and $^1O_2$ (Davies, 2003) in mammalian cells, rendering them non-functional and targets for degradation. Therefore, the formation of DNA, lipid and protein damage induced by oxidizing agents, such as UVA and H_2O_2, is mediated by ROS and/or $^1O_2$ produced within the cell upon exposure to these agents.

Although UVA is responsible for the formation of oxidative DNA damage, it is capable of causing DNA photolesions to some extent as previously mentioned. Therefore, in order to examine the deleterious effects of different UV wavelengths, we examined the cytotoxicity of UVC, UVA and H_2O_2 treatment on various human cells. By comparing the sensitivity of cell lines to these environmental and chemical agents, we

hoped to examine whether photo-excitation or photo-oxidation were predominantly responsible for UV-induced cell killing in UVA-irradiated cells.

Materials and Methods

Cell Lines

HaCAT, U373 and T98G cells were obtained as a gift from Dr. Michael Joiner (Wayne State University, Michigan). HaCAT cells are immortal, non-transformed human keratinocytes, exhibiting mutations in both alleles of the p53 gene (Boukamp *et al.*, 1990). Both T98G and U373 are human malignant glioma cell lines, each carrying a missense mutation in both alleles of the p53 gene (Ishii *et al.*, 1999). HT29 cells were obtained from Dr. Gurmit Singh (McMaster University, Canada). These cells are derived from a human colon carcinoma, which carry a point mutation in the p53 gene product, TP53, at $Arg^{273}His$ (Rodrigues *et al.*, 1990). HPV-G cells are keratinocytes obtained from a human neonatal foreskin that have been transfected with the human papillomavirus (HPV) to cause immortalization (Pirisi *et al.*, 1987). This cell line was obtained as a gift from Dr. Carmel Mothersill (McMaster University, Canada). All cell cultures were grown at 37°C in a humidified incubator in 5% CO_2 and cultured in RPMI medium supplemented with 10% fetal bovine serum, 2.05 mM L-glutamine and antimycotic/antibiotic (100 μg/ml penicillin. 100 μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL).

UV radiation sources

The UVC radiation source was a General Electric germicidal lamp (model G8T5), which emitted predominantly at 254 nm at a fluence rate of 1 W/m^2, as measured using a Black-Ray-J-255 short-wave UV meter. UVA irradiations were carried out using Sciencetech Model 200-1K light source housing a Sciencetech 100-1KMX, 1KW Hg-Xe lamp containing a UVA transmittance (335) filter, in order to prevent UVB radiation from being transmitted. The UVA radiation source emitted a fluence rate of ~70 W/m^2 as measured by a Black-Ray J-221 long-wave UV meter.

Clonogenic Survival Assays

Confluent 75 cm^2 flasks of each cell line were trypsinized, cells were counted with a haemocytometer and plated in 6-well tissue culture plates at a density of 500 cells/well. Cells were incubated in a humidified 37°C incubator at 5% CO_2 for 12 hours prior to UV exposure. Following incubation, the media from each well was aspirated, and 0.5 ml of warmed phosphate-buffered saline (PBS) (Hyclone; 9 g/L NaCl, 0.8 g/L Na_2HPO_4, 0.14 g/L KH_2PO_4 [pH 7 – 7.2]) was overlayed on the cells for UVC irradiation and 1.5 ml of PBS for UVA irradiation. Cells were treated (or mock irradiated) to increasing UVC fluences (J/m^2) at a fluence rate of 1 W/m^2. For UVA treatments, cells were treated to increasing UVA fluences (kJ/m^2) at a fluence rate of ~70 W/m^2. UVA irradiations at higher fluences resulted in an observable reduction (up to ~2/3) in the PBS volume, therefore, sterile ddH_2O was added to cells UVA-irradiated for long durations in order to restore the PBS concentration. After irradiation, RPMI supplemented media was

added to each well (1.5 ml for UVC and 3.5 ml for UVA irradiations). Cells were further incubated for 9 days in a humidified 37°C incubator in an atmosphere of 95% air/5% CO_2 to allow for surviving cells to form colonies. In order to stain the colonies after 9 days of incubation, the media was aspirated from each of the wells and the plates were stained with crystal violet (0.5% in 70% ethanol and 10% methanol) for 30 min. To de-stain the wells, the plates were briefly submerged in water and allowed to dry. Colonies containing at least 32 cells were counted as surviving colonies. Clonogenic survival is expressed as a ratio of the number of colonies in treated samples compared to their mock-treated counterparts.

Hydrogen Peroxide treatment of various cell lines

Known concentrations of H_2O_2 (BDH Inc.) were added to solutions of PBS/supplemented RPMI (1.5 ml: 3.5 ml mixed proportions) in order to produce H_2O_2 solutions of varying concentrations (μM). The H_2O_2 concentrations of each solution were verified using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes; A-22188) immediately prior to transferring the H_2O_2 solutions to cells. This assay provides a one-step fluorometric method for measuring the H_2O_2 concentration. The detection of H_2O_2 is possible with the Amplex red reagent, which in the presence of horseradish peroxidase and H_2O_2, produces the fluorescent resorufin product (Molecular Probes, product information, September 2003). Fluorescence was measured using a SAFIRE (Tecan) microplate reader (excitation λ 563 nm, emission λ 587 nm; Z-position 7257 μm). The H_2O_2 concentration was determined using linear regression analysis from

a standard curve generated from known concentrations of H_2O_2; the standard samples were also prepared in a PBS/supplemented RPMI (1.5 ml: 3.5 ml) solution lacking phenol red. In order to reduce fluorescence interference when determining the actual H_2O_2 concentrations present in media, supplemented RPMI medium lacking phenol red was used.

After determining the H_2O_2 concentration within the PBS/supplemented RPMI solutions, 2 ml of each of the H_2O_2 solutions were added to wells containing HaCAT, U373, T98G, HT29 and HPV-G cells, seeded 12 hr earlier according to the clonogenic survival assay protocol. A mock-treated solution was also included, and was administered to all the cell lines. The cells were incubated in a humidified 37°C incubator in an atmosphere of 95% air/5% CO_2, and were stained with crystal violet (and de-stained) according to the clonogenic survival assay protocol.

Effect of incubating H_2O_2 in PBS/RPMI on the survival of HaCAT colonies

Known concentrations of H_2O_2 (BDH Inc.) were added to solutions of PBS/supplemented RPMI (1.5 ml: 3.5 ml mixed proportions) in order to produce H_2O_2 solutions of varying concentrations (μM). The H_2O_2 concentrations of each solution were verified using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit, and 2ml of each solution was added to the aspirated wells containing HaCAT cells seeded 12 hr earlier according to the clonogenic survival assay protocol. The remainder of each solution was placed in individual wells of a 6-well plate and incubated in a humidified 37°C incubator for 24 hr. After 24 hr of incubation, the H_2O_2 concentration of the

solutions was measured again using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit, and 2ml of each solution was added to the aspirated wells containing HaCAT cells seeded 12 hr earlier. Mock-treated solutions were also included for both freshly prepared and incubated solutions. The HaCAT cells were incubated in a humidified 37°C incubator in an atmosphere of 95% air/5% CO_2, and were stained with crystal violet (and de-stained) according to the clonogenic survival assay protocol.

Results

Differences in clonogenic survival of human cells following UV-C, UV-A and H_2O_2 treatment

Typical colony survival curves for HaCAT, U373, T98G and HT29 cells following UVC and UVA treatments are presented in Figures 3-1 and 3-2, respectively. It can be seen that HaCAT cells are more resistant to UVC exposure than the other cell lines, whereas HaCAT cells are more sensitive to UVA treatment (Table 3-1). The reduction in clonogenic survival observed following treatment of HaCAT cells with increasing fluences of UVA were similar to the reduction in HaCAT cell viability reported following UVA treatment with similar UVA fluences (0-300 kJ/m^2) (Huang *et al.*, 2005).

A typical colony survival curve for HaCAT, U373, T98G and HPV-G cells following H_2O_2 treatment is presented in Figure 3-3. It can be seen that following exposure of cells to increasing H_2O_2 concentrations (μM), HaCAT cells demonstrate increased sensitivity to H_2O_2 treatment in comparison to U373, T98G and HT29 cells

(Figure 3-3; Table 3-1), while HPV-G cells exhibited increased sensitivity to H_2O_2 treatment compared to HaCAT cells. In addition, HaCAT cells demonstrated similar levels of sensitivity following H_2O_2 treatment, in a dose-dependent manner, as previously reported (Thorn *et al.*, 2001).

Prior incubation (24 hr) of H_2O_2 with the PBS/RPMI solution does not alter its cytotoxicity on HaCAT cells.

A comparison of the effect of prior incubation (24 hr) and immediate treatment of increasing concentrations (μM) of H_2O_2 (prepared using a PBS/RPMI solution) on the survival of HaCAT cells is demonstrated in Figure 3-4. The results of three independent experiments were pooled in order for survival curves to be generated using the quadratic formula, $y = e^{-(\alpha x+\beta x^{\wedge}2)}$. Prior incubation of increasing concentrations of H_2O_2 with the PBS/RPMI solution did not significantly alter its cytotoxicity on HaCAT cells.

Discussion

The ability of cells to survive exposure to radiation and avoid cell death is a frequently measured cellular response to radiation, commonly assayed by their ability to form colonies. Colony formation (or clonogenic survival) reflects a cell's ability to restore the integrity of its DNA following exposure to a cytotoxic and/or mutagenic agent, such that its ability (and the ability of its progeny) to undergo cell division is possible. The sensitivity of immortalized human keratinocytes (HaCAT), human glioma (U373 and T98G) and colon carcinoma (HT29) cell lines to radiation exposure of varying

UV wavelengths was examined by irradiating cells with increasing fluences of UVC or UVA radiation. Typical colony survival curves for these cell lines following UVC exposure is represented in Figure 3-1, which demonstrates that HaCAT and HT29 cells are less sensitive to UVC exposure compared to the glioma cell lines, T98G and U373. Since UVC-induced DNA photo-excitation produces predominantly CPD and 6-4PP adducts within the genome, one possible reason for the increased sensitivity of glioma cells to UVC radiation is that these cells are less able to repair UVC-induced DNA damage.

Cellular responses to UV radiation are dependent on the incident UV wavelength. While HaCAT cells were the most resistant to UVC radiation compared to the other tumor cell lines, they were the most sensitive to UVA exposure (Figure 3-2). This suggests that HaCAT cells are more sensitive to UVA-induced damage than the UVC-induced production of DNA photoadducts (CPDs and 6-4PPs). Although UVA produces CPDs and 6-4PPs to a lesser extent than photo-oxidative lesions such as 8-oxoG (Kvam and Tyrrell, 1997; Kielbassa *et al.*, 1997), the increased sensitivity of HaCAT cells to UVA radiation may result from the production of UV-induced photoadducts and oxidative DNA lesions. Previous studies have shown that UVA-treatment of NER-deficient (XP-C and XP-D) keratinocytes demonstrated increased sensitivity to UVA exposure compared to wild-type keratinocytes (Otto *et al.*, 1999). These experiments were carried out within a similar UVA fluence range (0 – 500 kJ/m^2) as our experiments, however, the D_{37} values obtained for wild-type and NER-deficient keratinocytes were greater than the D_{37} values we obtained for HaCAT cells (Table 3-1). These results are

consistent with an involvement of p53 in the survival of keratinocytes following UVA treatment.

To further examine cell sensitivity towards oxidative damage, various human cells were treated with increasing H_2O_2 concentrations (μM). Colony survival curves for human cells treated with media containing H_2O_2 showed that human keratinocytes (HaCAT, HPV-G) were more sensitive compared to the other cell lines. As the decomposition of H_2O_2 to OH· radicals has been suggested to be responsible for producing oxidized DNA lesions, these results suggest that the increased sensitivity of HaCAT cells to both UVA and H_2O_2 treatments is the result of a reduced ability to repair such lesions. In addition, preliminary results with HPV-transfected keratinocytes (HPV-G) showed that these cells demonstrate a reduced clonogenic survival compared to HaCAT keratinocytes, suggesting that the base excision repair (BER) pathway, the repair process responsible for removing oxidized lesions from the human genome, may be p53-dependent. HPV infection of cells results in the degradation of TP53, which may account for the reduced survival of HPV-G cells compared to HaCAT cells. While HaCAT cells exhibit mutations in both alleles of the p53 gene (Fusenig and Boukamp, 1998), these cells do not demonstrate similar levels of sensitivity towards H_2O_2 treatment as HPV-G cells. One possible explanation is that the mutations present in the p53 gene in HaCAT cells may not completely render the gene (and the resulting TP53 protein) non-functional, particularly in its involvement in BER of H_2O_2-mediated DNA damage. Other studies have reported that p53 is involved in the BER pathway by interacting and enhancing the DNA polymerase β (polβ) subunit (responsible for DNA re-synthesis in BER) in the

repair of abasic sites (Zhou *et al.*, 2001), formed as a result of the removal of oxidized and alkylated base lesions from the genome. Since the immortalized colon carcinoma (HT29) and glioma (U373, T98G) cell lines also carry p53 mutations, but demonstrate an increased resistance to UVA and H_2O_2 treatments, suggests that the involvement of p53 in BER may be more pronounced in different cell types and/or there are other factors contributing to BER which are altered in the tumor cell lines.

Hydrogen peroxide is a reactive oxygen species, capable of interacting with and modifying cellular macromolecules, such as DNA, proteins and lipids. Since various proteins, lipids, free amino acids and nucleic acid residues are present in the supplemented medium, we examined whether incubating growth media with H_2O_2 (for 24 hr) had any effect on the survival of HaCAT cells, in an attempt to determine whether H_2O_2 modifies the biomolecules present in media such as to increase its cytotoxicity towards cells. Figure 3-4 shows the pooled colony survival results of HaCAT cells treated with H_2O_2-containing medium (PBS/RPMI solution) immediately and 24 hr post-incubation. It can be seen that incubating H_2O_2 with media for 24 hr resulted in similar levels of clonogenic death as freshly-prepared H_2O_2-containing media. However, the H_2O_2 concentration levels differed in these two media preparations since incubation caused the H_2O_2 concentration to decrease with time, as measured within each individual experiment (data not shown). Since the survival curves were plotted as a function of the actual H_2O_2 concentration when added to the cells, these results show that the incubation of hydrogen peroxide with media for 24 hr did not produce alterations to biomolecules in the growth medium that resulted in additional toxicity towards HaCAT cells.

In summary, human keratinocytes, glioma and colon carcinoma cells demonstrated varying sensitivities to the environmental and chemical agents UVC, UVA and hydrogen peroxide. While HaCAT cells were more resistant to UVC exposure than the other human tumor cell lines, it demonstrated increased sensitivity towards UVA and H_2O_2 treatment. These differences are likely due to differences in the spectrum of DNA lesions generated by UVC, UVA and H_2O_2 treatment of cells, and the capacity of the cells to repair these lesions. In addition, our preliminary results may provide additional support for the involvement of p53 in BER of oxidative damage, as we have shown that p53-null keratinocytes demonstrated reduced survival towards H_2O_2 treatment compared to p53-mutated keratinocytes. We also show that although H_2O_2 levels were reduced following 24 hr incubation with growth medium, similar levels of clonogenic survival of HaCAT cells were observed following treatment with immediately-prepared or 24hr-incubated H_2O_2-containing media solutions. This indicated that incubating H_2O_2 with growth media for 24 hr did not produce additional alterations in biomolecules that are cytotoxic towards HaCAT cells.

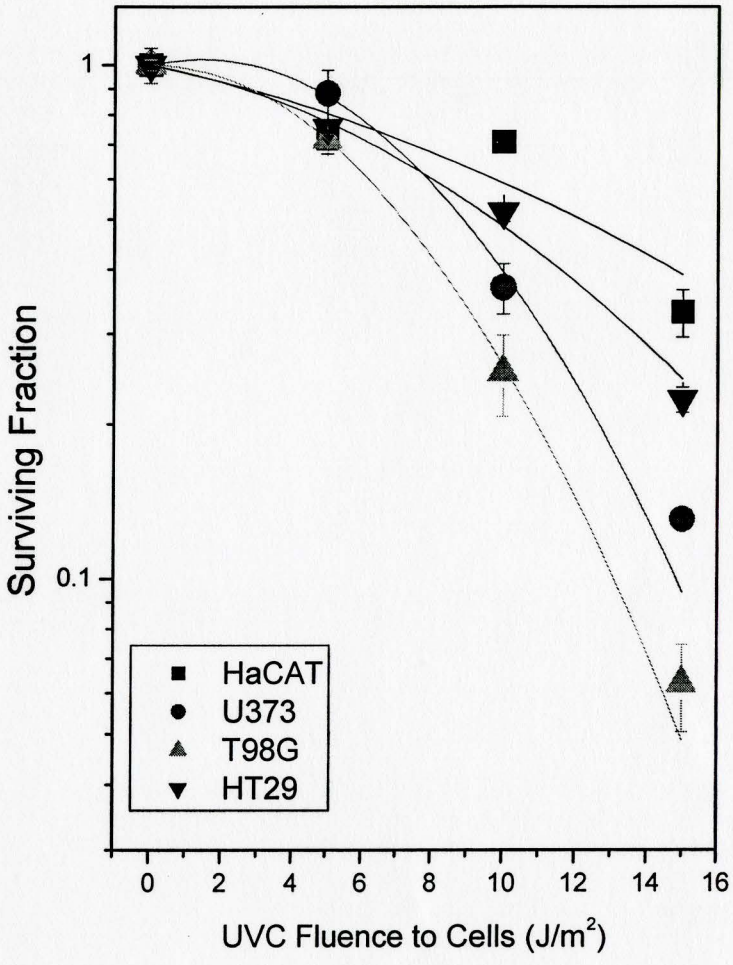

Figure 3-1. Colony survival of various human cell lines following UVC irradiation. Results show typical colony survivals of HaCAT, U373, T98G and HT29 cell lines exposed to increasing fluences (J/m^2) of UVC radiation. Various cell lines were seeded 500 cells/well in 6-well plates and incubated for 12 hours at 37°C in a humidified incubator at 5% CO_2. After incubation, the medium was aspirated and cells were exposed to UVC (254 nm) (or mock-irradiated) in 0.5 ml of PBS solution. Immediately following irradiation, 1.5 ml of supplemented RPMI media was added to each well and the cells were incubated for 9 days at 37°C. After incubation, each well was stained with crystal violet for 30 min. Results were graphed with respect to the quadratic formula, $y = e^{-(\alpha x+\beta x^{\wedge}2)}$. D_{37} values (fluence required to reduce the survival to 37%) were determined from the survival curve of each cell line within each individual experiment, and the average D_{37} values are shown in Table 3.1.

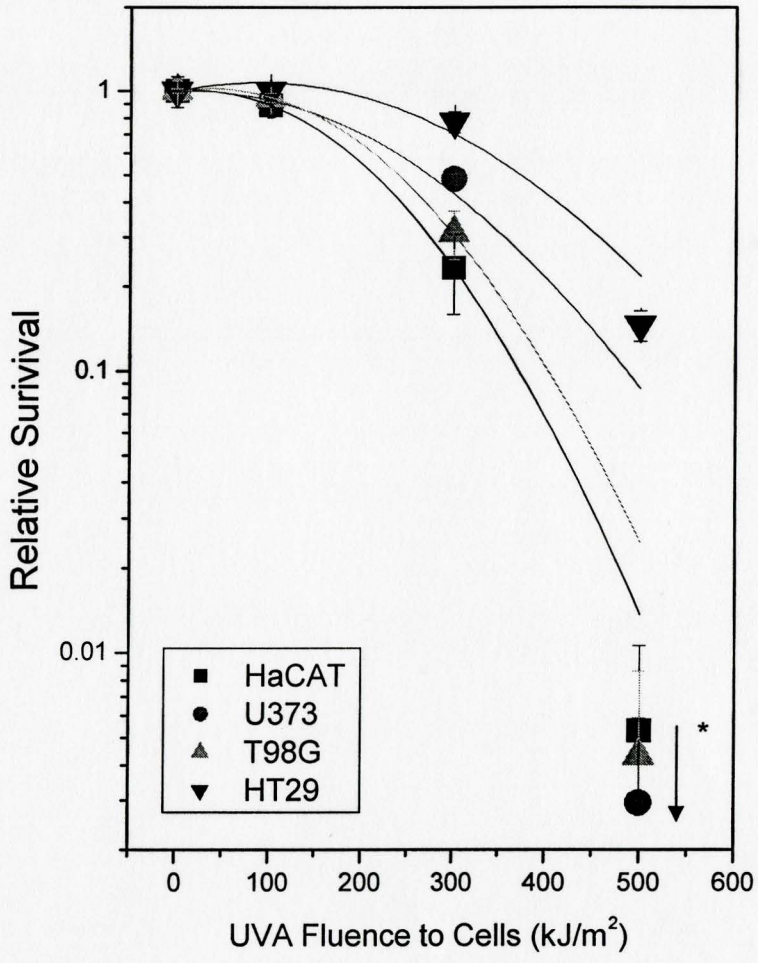

Figure 3-2. Colony survival of various human cell lines following UVA irradiation. Results show typical colony survivals of HaCAT, U373, T98G and HT29 cell lines exposed to increasing fluences (kJ/m^2) of UV-A radiation. Various cell lines were seeded 500 cells/well in 6-well plates and incubated for 12 hours at 37°C in a humidified incubator at 5% CO_2. After incubation, the medium was aspirated and cells were exposed to UVA (or mock-irradiated) in 1.5 ml of PBS solution. Immediately following irradiation, 3.5 ml of supplemented RPMI media was added to each well and the cells were incubated for 9 days at 37°C. After incubation, each well was stained with crystal violet for 30 min. Results were graphed with respect to the quadratic formula, $y = e^{-(\alpha x+\beta x^{\wedge}2)}$. *Represent "less than" points; points which possess a value that is less than if 1 colony survived UVA-treatment at the given fluence. D_{37} values (UVA fluence required to reduce the survival to 37%) were determined from the survival curve of each cell line within each individual experiment, and the average D_{37} values are shown in Table 3.1.

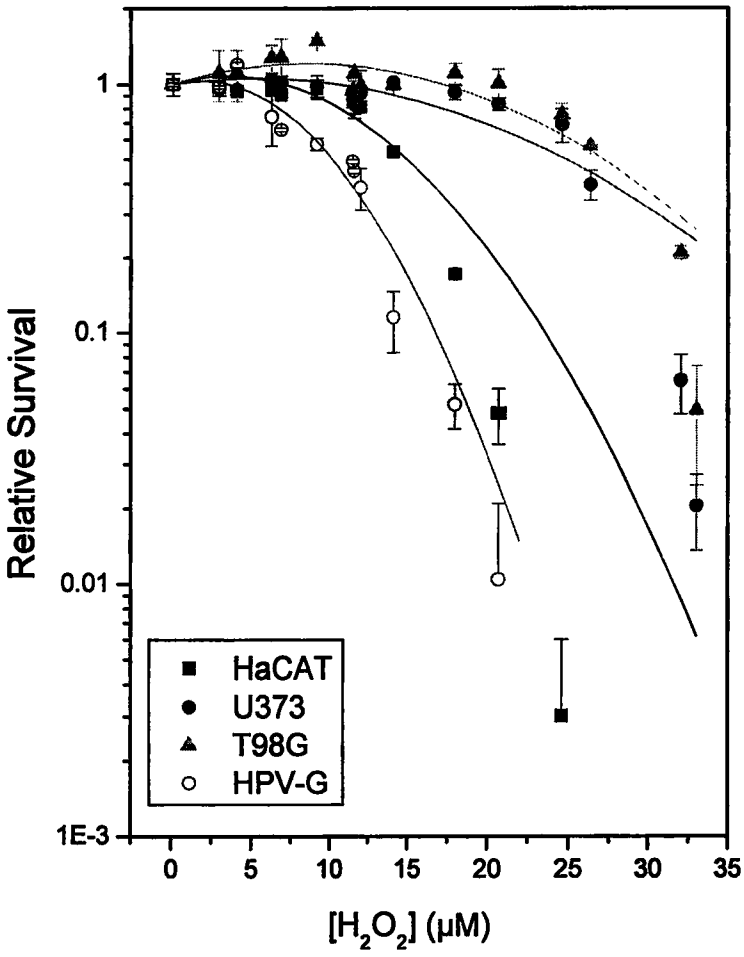

Figure 3-3. Colony survival of various human cell lines following hydrogen peroxide treatment. Results show typical colony survivals of HaCAT, U373, T98G and HT29 cell lines following H_2O_2 treatment. Various cell lines were seeded 500 cells/well in 6-well plates and incubated for 12 hours at 37°C in a humidified incubator at 5% CO_2. After incubation, the medium was aspirated and cells were treated with increasing concentrations of H_2O_2, prepared in a PBS/RPMI (1.5 ml: 3.5 ml mixed proportions) solution. Actual H_2O_2 concentration in the growth medium was measured immediately prior to treating cells using the Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probes). Results were graphed with respect to the quadratic formula, $y = e^{-(\alpha x+\beta x^{\wedge}2)}$. D_{37} values (dose required to reduce the survival to 37%) were determined from the survival curve of each cell line within each individual experiment, and the average D_{37} values are shown in Table 3.1.

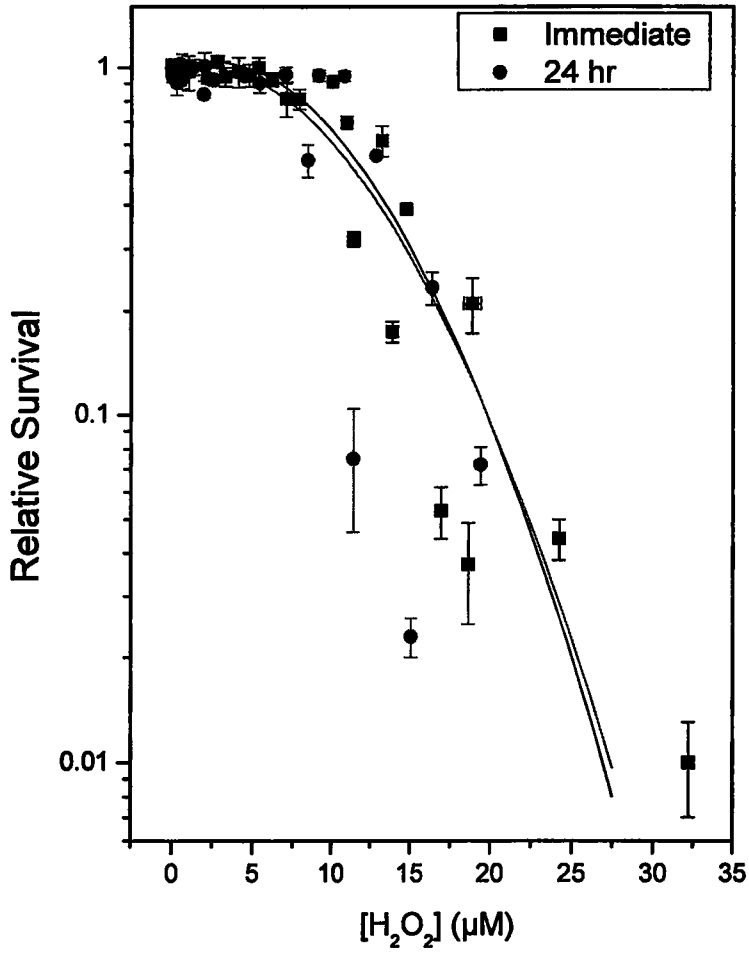

Figure 3-4. Prior incubation (24 hr) of H_2O_2 with RPMI/PBS solution does not alter its cytoxicity on HaCAT cells. Increasing concentrations of H_2O_2 were prepared in a PBS/RPMI solution (1.5 ml:3.5 ml mixed proportions), and were transferred onto HaCAT cells (500 cells/well) immediately or 24 hr later. Actual H_2O_2 concentration in the (freshly-prepared (immediate) or incubated (24 hr)) growth medium was measured immediately prior to treating cells using the Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probes). Results were pooled and graphed with respect to the quadratic formula, $y = e^{-(\alpha x+\beta x^2)}$.

Table 3-1. Clonogenic survival following UVC, UVA and H_2O_2 treatment of various human cell lines. D_{37} refers to the fluence (UVC, UVA) or dose (H_2O_2; μM) required to reduce the colony survival to 37%. D_{37} values were determined for each given cell line within each individual experiment. The resulting D_{37} values are given as mean ± SE of at least 2 independent experiments, except where indicated. (#) refers to the number of independent determinants, each performed in duplicate.

	UVC Treatment		UVA Treatment		H_2O_2 Treatment	
Cell Line	D_{37} (J/m^2)	SE	D_{37} (kJ/m^2)	SE	D_{37} (μM)	SE
HaCAT	15.35 (2)	0.24	274.6 (3)	47.5	20.5 (3)	5.1
U373	9.37 (2)	0.9	365.4 (3)	21	40.2 (3)	4.6
T98G	8.60 (2)	0.01	345.6 (3)	47.8	41.0 (3)	3.8
HT29	12.04 (2)	0.15	381.3 (3)	32.5	58.2 (3)	14.7
HPV-G	–	–	–	–	11.7 (1)	–

Chapter 4

Cytotoxicity and effects on cell growth of UVA-irradiated saline and growth medium from UV-irradiated human cell cultures: An examination of UV-induced Bystander Effects

Abstract

Ultraviolet irradiation of cells can have both cytotoxic and/or mutagenic consequences, resulting in increased levels of cell death or the induction of a state of genomic instability that persists for several cell generations after irradiation. However, the extent of the effects induced in non-irradiated cells by UV-irradiated cells has not been fully investigated. Using the medium transfer technique, we have tried to address whether UV irradiation of cells can induce biological effects in non-irradiated cells. Medium obtained from UVA, but not UVC, irradiation of various human cell lines in phosphate-buffered saline (PBS) solution was capable of reducing the relative clonogenic survival and colony size of non-irradiated HaCAT cells. In the absence of cells, UVA-irradiation of PBS reduced the clonogenic survival, but not the colony size, of various non-irradiated human cells. These results indicate a cytotoxicity of UVA-irradiated PBS towards non-irradiated cells. Hydrogen peroxide, a reactive oxygen species (ROS) generated following UVA-irradiation, was measured following UVA-treatment (or mock-treatment) of PBS (in the presence or absence of cells) either immediately or 24 hr post-irradiation. Hydrogen peroxide levels increased immediately following UVA irradiation, suggesting that it may contribute to the reduced survival of non-irradiated human cells. However, human glioma T98G and U373 cells produced elevated H_2O_2 levels in mock-irradiated conditions at 24 hr post-incubation, while demonstrating different sensitivities towards treatment with medium containing UVA-irradiated PBS. These results suggest that UVA-induced H_2O_2 is not responsible for the reduction in clonogenic survival of non-irradiated human cells. We also examined colony number and size at different times

following the addition of media containing UVA-irradiated PBS and media from UVA-irradiated cell cultures. Increasing the time before scoring for colonies resulted in an increased clonogenic survival and a decrease in relative colony size for HaCAT cells. These preliminary results suggest that the relative survival and relative colony size of non-irradiated cells are inter-related, and indicate that treating non-irradiated HaCAT cells with medium from UVA-irradiated cells or medium containing UVA-irradiated PBS reduced the growth rate of HaCAT colonies. Therefore, in order to properly evaluate clonogenic survival, a time-dependent examination of relative survival and relative colony size should be conducted in order to address whether the treatment results in an inhibition of cell growth and/or true cell killing.

Introduction

The mechanism of radiation-induced DNA damage formation within an irradiated cell is believed to occur as a result of direct ionization of DNA or by the indirect production of reactive oxygen species (ROS), which can react with and modify DNA. However, several studies have reported that irradiation of cells can induce biological effects in non-irradiated cells in close proximity or in direct contact with the irradiated cells, causing the cells to behave as though they had been irradiated by demonstrating signs of genetic instability or increased cell death. The term designated to describe the induction of a biological effect in non-irradiated cells by a factor(s) generated by irradiated cells is referred to as a radiation-induced bystander effect.

In vitro studies on radiation-induced bystander effects have primarily focused on ionizing radiation. Experimental evidence that supports bystander responses induced by sparsely ionizing (or low LET) radiation, such as X-rays and gamma rays, has been provided by two pioneers in low-LET *in vitro* bystander effect studies, Dr. Carmel Mothersill and Dr. Colin Seymour. Mothersill and Seymour demonstrated that medium from gamma-irradiated epithelial cells could reduce the clonogenic survival of non-irradiated cells. This effect was dependent on the number of cells present at the time of irradiation and the length of time the irradiated cells remained in contact with the medium, but the irradiated medium did not effect the survival of non-irradiated cells in the absence of cells during irradiation (Mothersill and Seymour, 1997). This suggested that the medium from irradiated cells contained a factor(s) that was produced as a result of gamma (γ) irradiation, and that was cytotoxic towards non-irradiated cells.

Radiation-induced bystander effects have also been implicated in inducing genetic instability in non-irradiated cells. Genetic instability is the result of non-clonal mutations in the progeny of apparently healthy cells that have survived radiation exposure. This concept was investigated by re-plating the surviving progeny of cells exposed to medium from irradiated cell cultures. The progeny of the cells that survived treatment to irradiated cell conditioned medium demonstrated reduced cloning efficiency in comparison to their mock-treatment controls (Seymour and Mothersill, 2000; Mothersill *et al.*, 2004b), suggesting that the bystander effect is transmissible to the progeny of cells exposed to the cytotoxic factor(s) in media generated by irradiating cells.

In addition to studies on bystander effects induced by ionizing radiation, other studies have focused on examining whether UV radiation can mediate bystander effects in mammalian cells. UVC irradiation of human fibroblast cells has been shown to stimulate the release of factors in the surrounding medium, capable of mimicking the UVC-induced synthesis of various gene products (Schorpp *et al.*, 1984). UVA and UVB radiation have also been the focus of UV-mediated bystander effect studies. In a study involving UVA treatment of Madin Darby canine kidney cells (MDCK II), the irradiated cultures exhibited an increased occurrence of isolated dead cell clusters rather than random cell death throughout the culture (Dahle *et al.*, 2001). Based on these observations, the authors suggested that these dead cell clusters are the result of the UVA-damaged cells enhancing the predisposition for damage in their neighbouring cells. In another study, Dahle and colleagues suggested that the bystander effect may modulate UVA and UVB-induced genomic instability in rodent cells through gap junction intercellular communication (GJIC), demonstrating an increased mutation frequency among cells allowed to be contact with their progeny (Dahle *et al.*, 2005). More recently, a study demonstrated reduced cell survival of non-irradiated human keratinocytes upon treatment with conditioned media from UV(A+B)-irradiated cells, and implicated apoptotic cell death due to the expression of certain apoptotic markers (Banarjee *et al.*, 2005). Therefore, although it is a relatively new focus in radiation-induced bystander effect studies, there is evidence to suggest that UV radiation can induce bystander effects.

In the present work, we report that the media obtained from UVA, but not UVC, irradiation of various human cell lines in phosphate-buffered saline (PBS) solution was

capable of reducing the clonogenic survival (~20-25%) and colony size of non-irradiated HaCAT cells. However, UVA irradiation resulted in an observable reduction (~ 2/3) in the PBS volume due to evaporation, suggesting that the increased salinity of the PBS solution may have contributed towards the reduced clonogenic survival of HaCAT cells. A clonogenic survival assay in which cells were treated with PBS/RPMI solutions of increased PBS concentration confirmed that increased salinity was responsible for the reduced clonogenic survival, and possibly, reduced colony size formation. Therefore, in subsequent experiments, sterile ddH_2O was added to UVA-irradiated PBS, in the presence or absence of cells, in order to restore the PBS concentration. After adjusting the PBS concentration following UVA irradiation, the observed reduction in HaCAT colony survival was less reduced (~10% reduction), however, relative colony size remained significantly reduced. In the absence of cells, UVA irradiation of PBS also reduced clonogenic survival (~10% reduction), but not colony size, of various non-irradiated human cells at 9 days post-incubation. Hydrogen peroxide has frequently been discussed as the major ROS generated following UVA irradiation (Mahns *et al.*, 2003) and has been shown to be responsible for mediating UVA-induced biological effects such as DNA damage (Peterson *et al.*, 2000) and reduced cell number (Wondrak *et al.*, 2003). Hydrogen peroxide levels were measured following UVA treatment (or mock-treatment) of PBS (in the presence or absence of cells) either immediately or 24 hr post-irradiation. Hydrogen peroxide levels increased immediately following UVA irradiation, suggesting that H_2O_2 may contribute to the reduced survival of non-irradiated human cells. However, human glioma (T98G, U373) cells produced elevated H_2O_2 levels (~2.2-2.3

μM) in mock-irradiated conditions at 24 hr post-incubation, suggesting that these cells release H_2O_2 into the medium under normal growth conditions. In addition, T98G cells were more sensitive (compared to U373 cells) to treatment with medium containing UVA-irradiated PBS. Taken together, these results indicate that H_2O_2 is not responsible for the reduction in clonogenic survival of non-irradiated human cells. We also examined colony number and size at different times following the addition of media containing UVA-irradiated PBS and media from UVA-irradiated cell cultures. Increasing the time before scoring for colonies resulted in an increased clonogenic survival and a decrease in relative colony size for HaCAT cells. These results suggest that the relative survival and relative colony size of non-irradiated cells are inter-related, and indicate that treating non-irradiated HaCAT cells with UVA-irradiated PBS and medium from UVA-irradiated cells reduces the growth rate of HaCAT colonies.

Materials and Methods

Cell Lines

HaCAT, U373 and T98G cells were obtained as a gift from Dr. Michael Joiner (Wayne State University, Michigan). HaCAT cells are immortal, non-transformed human keratinocytes, exhibiting mutations in both alleles of the p53 gene (Boukamp *et al.*, 1990). Both T98G and U373 are human malignant glioma cell lines, each carrying a missense mutation in both alleles of the p53 gene (Ishii *et al.*, 1999). HT29 cells were obtained from Dr. Gurmit Singh (McMaster University, Canada). These cells are derived

from a human colon carcinoma, which carry a point mutation in TP53 at $Arg^{273}His$ (Rodrigues *et al.*, 1990). All cell cultures were grown at 37°C in a humidified incubator in 5% CO_2 and cultured in RPMI medium supplemented with 10% fetal bovine serum, 2.05 mM L-glutamine and antimycotic/antibiotic (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B, Gibco BRL).

Clonogenic Survival Assay - Bystander Effect Studies

Flasks containing semi-confluent cultures were treated with 2X trypsin/EDTA (GibcoBRL) solution in order to remove the cells from the flask. When the cells had detached, they were re-suspended in supplemented RPMI medium and syringed gently to produce a single cell suspension. Cells destined to donate conditioned medium were counted with a haemocytometer and seeded in 6 well-plates (Falcon, Lincoln Park, NJ) at a density of 2×10^5 cells/well. Cultures were incubated in 2 ml of growth medium in a humidified 37°C incubator, with an atmosphere of 95% air/5% CO_2, for approximately 6 hours. Following incubation, the cells were UV-irradiated (or mock-irradiated) as described below, and were incubated for an additional 24 hr.

Gamma Irradiation of Cells

After seeding and incubating the donor cell cultures, the media was aspirated from each well and 5 ml of warmed RPMI supplemented media was placed on the cells. Cells were irradiated with the lid on at room temperature using a ^{137}Cesium (Cs) source which delivers gamma (γ) rays at a dose rate of 0.1 Gy/min. Controls for "RPMI alone"

effects were included in each experiment; 5 ml of RPMI supplemented medium was added to wells lacking cells and exposed to the ^{137}Cs source with the appropriate dose. The source to plate distance was 55.5 cm. Following irradiation, cultures were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 for 24 hr.

UVC Irradiation of Cells

After seeding and incubating the donor cell cultures, the media was aspirated from each well and 1 ml of warmed phosphate-buffered saline (PBS) (Hyclone; 9 g/L NaCl, 0.8 g/L Na_2HPO_4, 0.14 g/L KH_2PO_4 [pH 7 – 7.2]) was overlayed on the cells. Cells were UV-irradiated (or mock irradiated) with various fluences at a fluence rate of 2 W/m^2, as measured using a Black-Ray-J-255 short-wave UV meter. Cells were exposed to UVC using a General Electric germicidal bulb (emitting predominantly at 254 nm). Following UVC treatment (or mock treatment) of cells, 4 ml of supplemented RPMI medium (lacking phenol red) was added to each well. Controls for "PBS alone" effects were included in each experiment; 1 ml of PBS was added to wells lacking cells, exposed to UVC (or mock-irradiated) with the appropriate fluence(s) and 4 ml of supplemented RPMI medium (lacking phenol red) was added following irradiation. Cultures were incubated in a humidified 37°C incubator in an atmosphere of 95% air/5% CO_2 for 24 hr.

UVA Irradiation of Cells

After 6 hr of incubating the donor cell cultures, the media from donor cells was aspirated and 1.5 ml of warmed PBS was overlayed on the cells. Cells were UV-

irradiated (or mock irradiated) at a fluence rate of ~70 W/m^2, as measured by a Black-Ray J-221 long-wave UV meter. Cells were exposed with the lid off to UVA (325-475 nm) using a Sciencetech Model 200-1K light source housing a Sciencetech 100-1KMX IKW Hg-Xe lamp containing a UV-A transmittance (335) filter. UVA irradiations at higher fluences (particularly at 700 kJ/m^2) resulted in an observable reduction (up to ~ 2/3) in the PBS volume. Therefore, sterile distilled/deionized water (ddH_2O) was added to cells irradiated for long durations in order to restore the PBS concentration. The temperature of the PBS solution was monitored during the course of the irradiation, which remained below 37°C. Following UVA treatment (or mock-treatment) to cells, 3.5 ml of supplemented RPMI medium (lacking phenol red) was added to each well. Controls for "PBS alone" effects were included in each experiment; 1.5 ml of PBS was added to wells lacking cells, exposed to UVA (or mock-irradiated) with the appropriate fluence(s) and 3.5 ml of supplemented RPMI medium (lacking phenol red) was added following irradiation. Cultures were incubated in a humidified 37°C incubator in an atmosphere of 95% air/5% CO_2 for 24 hr.

UVA Irradiation of various Solutions

In experiments involving UVA irradiation of distilled/deionized water (ddH_2O), 1X Saline (9 g/L NaCl), 1X Phosphate buffer (PB; 0.8 g/L Na_2HPO_4, 0.14 g/L KH_2PO_4 [pH 7 – 7.2]) or 1X Phosphate-buffered Saline (PBS; 9 g/L NaCl, 0.8 g/L Na_2HPO_4, 0.14 g/L KH_2PO_4 [pH 7 – 7.2]), 1.5 ml of each solution was added to wells of a 6-well plate and UVA-irradiated with a fluence of 700 kJ/m^2, as described above. Following

irradiation, appropriate volumes of ddH_2O, 1X Saline or 1X PB solutions were added to the wells, such that each irradiated well contained 1.5 ml of a final (reconstituted) 1X PBS solution. To each well containing UVA-irradiated (or mock-irradiated) 1X PBS solutions, 3.5 ml of RPMI supplemented media (lacking phenol red) was added, and the final PBS/RPMI solutions were incubated for 24 hr at 37°C in an atmosphere of 95% air/5% CO_2.

Medium Transfer

The conditioned medium (PBS/RPMI supplemented solution) was removed from the irradiated (or mock-irradiated) cells 24 hr following UV exposure. In the case of UVA treatment of various solutions, the resulting PBS/RPMI supplemented (medium) solutions were removed from the irradiated (or mock-irradiated) wells after 24 hr of incubation. The medium was filtered through a 0.22-μm syringe (Acrodisc) filter used for sterilizing solutions in order to prevent bacterial contamination and to ensure that no cells were present in the transferred medium. Intact cells were not present (upon examination of aliquots of medium under the microscope) for any of the cell lines. Culture medium from various cell lines (seeded 12 hr earlier in 6-well plates at a density of 150-500 cells/well) was removed and 2 ml of the filtered medium was immediately added to these recipient wells (2 wells/cell line/condition). The non-irradiated recipient cells were left to incubate (at 37°C in 95% air/5% CO_2 atmosphere) in the presence of the filtered medium for 9 days to allow for colony growth before staining and counting. In

time-dependent studies, recipient cells were incubated in the filtered medium for 6, 9, 12 or 15 days prior to staining for colonies.

Colony Staining

In order to stain the colonies after incubation, the media was aspirated from each of the wells and the plates were stained with crystal violet solution (0.5% in 70% ethanol and 10% methanol) for 30 min. To de-stain the wells, the plates were briefly submerged in water and allowed to dry. Colonies containing at least 32 cells were counted as surviving colonies. Clonogenic survival is expressed as a ratio of the number of colonies in treated samples compared to their mock-treated counterparts. Colony area was examined using the Fluorchem™ (Alpha Innotech) imaging system and quantified using Fluorchem™ IS-8800 software (version 3.1). Relative colony size is expressed as a ratio of the average colony area (in pixels) in treated samples compared to their mock-treated counterparts.

Clonogenic Survival Assay – Effect of Increased Salinity on Human cells

Confluent 75 cm^2 flasks of each cell line were trypsinized, cells were counted with a haemocytometer and plated in 6-well tissue culture plates at a density of 300 cells/well. Cells were incubated in a humidified 37°C incubator at 5% CO_2 for 12 hours. Using a 10X PBS stock solution (Hyclone; 90 g/L NaCl, 8.0 g/L Na_2HPO_4, 1.4 g/L KH_2PO_4 [pH 7 – 7.2]), solutions of 1X through 5X PBS were prepared using ddH_2O and mixed with RPMI supplemented media (lacking phenol red) in order to make PBS/RPMI

supplemented solutions (mixed in 0.5 ml: 3.5 ml proportions of each solution, respectively). Following incubation of the cells, the media from each well was aspirated, and 2 ml of the warmed PBS/RPMI supplemented solutions of varying salinity were placed on the cells. Cells were further incubated for 9 days in a humidified 37°C incubator in an atmosphere of 95% air/5% CO_2 to allow for surviving cells to form colonies. In order to stain the colonies after 9 days of incubation, the media was aspirated from each of the wells and the plates were stained with crystal violet solution and de-stained with water, as previously discussed. Colonies containing at least 32 cells were counted as surviving colonies. Clonogenic survival is expressed as a ratio of the number of colonies in treated samples compared to their mock-treated counterparts.

Extracellular Hydrogen Peroxide Production Assay

Extracellular hydrogen peroxide (H_2O_2) production was determined using the Amplex Red Hydrogen Peroxide / Peroxidase Assay Kit (Molecular Probes, A-22188). The H_2O_2 concentration was measured immediately and 24 hr following UVA treatment (or mock treatment) of PBS/RPMI growth solution in the presence (or absence) of cells (2×10^5 cells/well). Fluorescence was measured using a SAFIRE (Tecan) microplate reader (excitation λ 563 nm, emission λ 587 nm; Z-position 7257 μm). The H_2O_2 concentration was determined using linear regression analysis from a standard curve generated from known concentrations of H_2O_2; the standard samples were prepared in a PBS/supplemented RPMI (1.5 ml: 3.5 ml) solution lacking phenol red. In order to reduce fluorescence interference when determining the actual H_2O_2 concentrations present in the

mock-irradiated and UV-irradiated media, supplemented RPMI medium lacking phenol red was used in UVA-related studies. In order to be consistent between experiments, supplemented RPMI medium lacking phenol red was also used in UVC-related studies, as well as experiments involving treatment of cells with medium of increasing PBS concentration.

Results

Toxicity of gamma-irradiated cells and medium.

HaCAT, T98G and HT29 cells were seeded in 6-well plates at a density of 2×10^5 cells/well. Six hours later, cells were exposed to a ^{137}Cs source (or mock-irradiated), which emitted gamma rays to a final dose of 5 Gray (Gy). This particular dose was chosen since previous work has shown that a reduction (~30%) in the clonogenic survival of HaCAT cells occurred following treatment with media from HaCAT cells exposed to a 5 Gy dose (Mothersill and Seymour, 1997). Twenty-four hours after irradiating the cells, the media was removed, filter-sterilized and transferred (2 ml/well) onto non-irradiated HaCAT cells (seeded 12 hr earlier at a density of 500 cells/well). Results show that transferring medium from γ-irradiated HaCAT or T98G cells did not effect the clonogenic survival of non-irradiated HaCAT cells compared to mock-irradiated medium (Figure 4-1; Table 4-1). In addition, irradiation of RPMI media in absence of cells (i.e. RPMI alone) did not alter the survival of non-irradiated HaCAT cells. In contrast, medium from γ-irradiated HT29 cells significantly reduced the clonogenic survival of HaCAT cells in 3 independent experiments.

Toxicity of UVC-irradiated cells and medium.

HaCAT, U373, T98G and HT29 cells were seeded in 6-well plates at a density of 2×10^5 cells/well. Six hours later, cells were UVC-irradiated (or mock-irradiated) with a fluence of 20 or 200 J/m^2 in 1 ml of PBS solution. Cells were exposed to UVC at this fluence (20 J/m^2) since similar levels of survival were observed in a UVC colony survival for these cell lines (see Chapter 3; Figure 3-1 and Table 3-1) compared to that observed in γ-induced bystander effects (Mothersill and Seymour, 1997). An additional 4 ml of RPMI supplemented media (lacking phenol red) was added to each well immediately following UVC (or mock) treatment. Twenty-four hours after irradiating the cells, the media was removed, filter-sterilized and transferred (2 ml/well) onto non-irradiated HaCAT cells (seeded 12 hr earlier at a density of 500 cells/well). Results show that transferring medium from various UVC-irradiated cell cell lines did not significantly effect the clonogenic survival of non-irradiated HaCAT cells compared to the mock-irradiated treatment (Figure 4-2; Table 4-2). In addition, no significant effect was also observed if medium containing UVC-irradiated PBS (cells absent during irradiation) was transferred onto HaCAT cells 24 hr post-irradiation.

Toxicity of UVA-irradiated cells and medium.

Donor cell lines and recipient HaCAT cells were seeded and prepared as described above. Six hours after seeding the donor HaCAT, T98G, U373 and HT29 cell lines, the cells were UVA-irradiated (or mock-irradiated) with a fluence of 350 or 700 kJ/m^2 in 1.5 ml of PBS solution. Cells were exposed to UVA at this fluence (350 kJ/m^2)

since similar levels of survival were observed in a UVA colony survival for these cell lines (see Chapter 3; Figure 3-2 and Table 3-1) compared to that observed in γ-induced bystander effects (Mothersill and Seymour, 1997). An additional 3.5 ml of RPMI supplemented media (lacking phenol red) was added to each well immediately following UVA (or mock) treatment. Twenty-four hours after irradiating the cells, the media was removed, filter-sterilized and transferred onto non-irradiated HaCAT cells. Results show that transferring medium from either UVA-irradiated HaCAT or U373 cell cultures (at increasing fluences) significantly reduced the clonogenic survival and colony size of non-irradiated HaCAT cells (Figures 4-3; Table 4-4). A similar level of reduction in clonogenic survival was observed when HaCAT cells were treated with medium, whereby PBS was irradiated in the absence of cells. However, UVA irradiation resulted in an observable reduction (up to ~ 2/3) in the PBS volume due to evaporation, suggesting that increased salinity of the PBS solution may have contributed towards the reduced clonogenic survival of HaCAT cells. In order to address this, a clonogenic survival assay was conducted in which cells were treated with PBS/RPMI supplemented medium solutions (mixed in 0.5 ml: 3.5 ml proportions, respectively) of increased PBS concentration (Figure 4-4). Increased salinity of the resulting PBS/RPMI solution (lacking phenol red) reduced the clonogenic survival of the human cell lines (Table 4-3), with T98G cells demonstrating the highest level of sensitivity. A PBS/RPMI solution that contains 3 times the PBS concentration (3×[PBS]), which would be equivalent to a solution experiencing a 2/3 reduction in PBS volume due to evaporation, resulted in a 44% reduction in the clonogenic survival of HaCAT cells after 9 days of incubation.

This suggests that UVA-induced evaporation of water from the PBS solution (with or without cells present) may have contributed to the reduced HaCAT clonogenic survival observed following treatment with UVA-irradiated medium (Figure 4-3; Table 4-4).

The medium from UVA-irradiated T98G and HT29 cells had undergone similar levels in PBS volume reduction due to evaporation, but did not reduce the survival of non-irradiated HaCAT cells to the same extent as UVA-irradiated PBS (irradiated in the presence or absence of HaCAT or U373 cells). According to Figure 4-4, it appears that HT29 and T98G cells were more sensitive to media of increased salinity compared to HaCAT and U373 cells. It is possible that the combination of UVA exposure and salinity may have resulted in more damage induced in HT29 and T98G cells compared to U373 and HaCAT cells, resulting in the induction and release of a factor(s) into the media that cause differences in survival of non-irradiated HaCAT cells.

In order to eliminate the effects of salinity in UVA-irradiated medium transfer experiments, appropriate volumes of distilled/deionized water (ddH_2O) was added to wells in order to restore the 1X PBS concentration immediately following UVA irradiation. Differences in HaCAT clonogenic survival following treatment with medium, where ddH_2O was added (or not added) immediately following UVA irradiation of PBS (with or without cells present), are shown in Table 4-4. The addition of ddH_2O immediately following UVA irradiation of PBS, in the presence or absence of cells, increased the relative survival of non-irradiated HaCAT cells treated with the UVA-irradiated PBS and medium (in the presence or absence of HaCAT or U373 cells). However, a significant decrease (~10%) in the relative clonogenic survival of HaCAT

cells was observed following treatment with medium from UVA-irradiated HT29 donor cells. In addition, medium containing UVA-irradiated PBS (no cells present) significantly reduced (~10%) the relative clonogenic survival of non-irradiated HaCAT cells. However, The relative clonogenic survival of non-irradiated HaCAT cells treated with medium from UVA-irradiated HaCAT, U373 or T98G cells was not significantly reduced.

Clonogenic Survival of various human cell lines following treatment with medium containing UVA-irradiated PBS.

To determine whether cell lines other than keratinocytes (HaCAT) are sensitive towards treatment with UVA-irradiated PBS and RPMI supplemented medium, non-irradiated glioma (U373, T98G) and colon carcinoma (HT29) cell lines were treated with medium containing UVA-irradiated PBS. Treatment of T98G and HaCAT cells with medium containing UVA-irradiated PBS (i.e. PBS alone) resulted in similar reductions in colony survival at 9 days post-treatment (Figure 4-5; Table 4-5). However, U373 and HT29 cells did not exhibit significant reductions in colony survival following treatment with UVA-irradiated PBS and growth medium compared to the mock-irradiated condition.

Reduction in the relative survival of HaCAT cells following medium treatment depends on the solution present during UVA irradiation.

To investigate the nature of the cytoxicity resulting from treating non-irradiated HaCAT cells with UVA-irradiated PBS and growth medium, the different solutions that

compose PBS (ddH_2O, 1X saline, 1X PB) were irradiated separately. Following irradiation, appropriate volumes of ddH_2O, 1X saline or 1X PB solutions were added to the wells, such that each irradiated well contained 1.5 ml of a reconstituted 1X PBS solution. UVA treatment of ddH_2O, 1X PB and 1X PBS solutions resulted in similar reductions in HaCAT colony survival when measured 9 days later (Figure 4-6; Table 4-6). However, UVA irradiation of a 1X saline solution did not significantly reduce HaCAT colony survival.

Increased extracellular hydrogen peroxide levels following UVA irradiation of PBS (in the presence or absence of cells) may be partially responsible for the reduction in HaCAT colony survival.

When examining the levels of hydrogen peroxide (H_2O_2) generated immediately following UVA irradiation, the results show a significant production of H_2O_2 in PBS, with lower levels (~2.5 to 4.5-fold) being generated when cells are present during irradiation (Table 4-7). Following the 24 hr incubation of the irradiated medium, these H_2O_2 concentration levels reduce to basal levels. However, the extracellular H_2O_2 concentration in the media from mock-irradiated U373 and T98G cells was approximately 4 to 10-fold greater after 24 hr post-incubation, suggesting that these cells, in the absence of UVA irradiation, release H_2O_2 into their extracellular environment.

Reduction in the Relative Colony size of HaCAT cells treated with UVA-irradiated medium requires the presence of human cells at the time of UVA treatment.

Treatment of HaCAT cells with medium from various UVA-irradiated human cell cultures also resulted in an observable reduction in HaCAT colony size, even after the

addition of ddH_2O immediately following irradiation as shown in Figure 4-7. From a qualitative perspective, it can be seen that treatment of non-irradiated HaCAT cells with medium from UVA-irradiated cells (HaCAT, T98G, HT29) resulted in an observable reduction in the size of HaCAT colonies, while medium containing PBS (irradiated in the absence of cells) did not demonstrate a reduction in the size of HaCAT colonies after 9 days of incubation. The area of HaCAT colonies were quantified (in pixels) for cells that received medium from various donor sources in order to determine the distribution of colony area following treatment with UVA-irradiated media compared to mock-irradiated media. Figure 4-8 demonstrates a representative distribution of HaCAT colony area following treatment of cells with medium containing UVA-irradiated PBS (irradiated in the presence or absence of HaCAT cells). The results demonstrate that there is a greater frequency of smaller HaCAT colonies when cells were treated with conditioned medium from UVA-irradiated HaCAT cells compared to conditioned medium from mock-irradiated cells. Medium containing UVA-irradiated or mock-irradiated PBS resulted in similar frequencies of HaCAT colonies of varying size.

Based on the size distributions of HaCAT colonies receiving medium from various donor sources, the mean colony area for cells receiving UVA-irradiated medium compared to mock-irradiated medium was determined, and subsequently used to determine the relative HaCAT colony size (Table 4-8). Medium from UVA-irradiated HaCAT, U373 and T98G cell cultures significantly reduced the colony size of non-irradiated HaCAT cells compared to mock-irradiated medium after 9 days of incubation. Medium from UVA-irradiated HT29 cell cultures, as well as medium containing UVA-

irradiated PBS, did not significantly reduce HaCAT colony size compared to the mock-irradiated treatment. Since medium containing UVA-irradiated PBS (no cells present) did not exhibit an observable reduction in HaCAT colony size compared to treatment with mock-irradiated medium, treatment of human glioma (U373, T98G) and colon carcinoma cells (HT29) with medium containing UVA-irradiated PBS was also examined (Table 4-5). In fact, treatment of various human cells, including HaCAT cells, with medium containing UVA-irradiated PBS resulted in an increase in their relative colony size after 9 days of incubation (Table 4-5).

Reduced colony survival and colony size of HaCAT cells following treatment with UVA-irradiated medium is dependent on the time of scoring for cell colonies.

The effect of increased length of incubation was examined for HaCAT cells treated with UVA-irradiated medium. HaCAT cells were incubated in the presence of UVA-irradiated (and mock-irradiated) medium for 6, 9, 12 and 15 days. Medium from UVA-irradiated HaCAT cells was transferred onto non-irradiated HaCAT cells, and colony survival and colony size were measured for both UVA-irradiated and mock-irradiated conditions. In addition, the effects of increased length of treatment of UVA-irradiation of PBS (no cells present during irradiation) and RPMI supplemented medium on the clonogenic survival and colony size of non-irradiated HaCAT cells was also examined. Preliminary results of a single experiment indicated that increased incubation with medium from UVA-irradiated donor HaCAT cells increased the relative clonogenic survival and reduced the relative colony size of non-irradiated HaCAT cells. Only after incubating HaCAT cells with UVA-irradiated PBS and medium for 15 days was a

reduction in the relative colony size observed (Table 4-9; Figure 4-9). When comparing the mean colony area of cells receiving mock-treated medium from HaCAT cells or PBS alone, the mean colony area was greater when cells received pre-conditioned medium (Figure 4-10, A). However, when the cells were treated with UVA-irradiated medium (in the presence or absence of cells), the mean colony areas were not significantly different from each other after incubating for various lengths of time (Figure 4-10, B).

Discussion

Bystander Effects following exposure to ^{137}Cs γ-rays

Using the medium transfer technique, we have tried to replicate the results of Mothersill and Seymour by exposing non-irradiated HaCAT cells to supernatants from HaCAT, human glioma (U373, T98G) and human colon carcinoma (HT29) cell lines exposed to ^{137}Cs γ-rays at a final dose of 5 Gray (Gy). Under these conditions, we could not demonstrate a reduction in HaCAT clonogenic survival following treatment with cell conditioned medium from irradiated HaCAT cells (see Figure 4-1), even though Mothersill and Seymour demonstrated ~30% reduction in the survival of HaCAT cells under similar conditions. Similar to their observations, irradiation of medium in the absence of cells did not have an effect on the clonogenic survival of non-irradiated HaCAT cells. The fact that sparsely-ionizing radiation from different sources produced different responses in HaCAT cells treated with irradiated cell conditioned media may be an indication that radiation-induced medium borne cytotoxic factor(s) are produced

depending on whether cells are exposed to radiation from ^{137}Cs or ^{60}Co sources, however this is unlikely since both sources produce γ-radiation, resulting in similar levels of ionization. Although medium from irradiated HaCAT cells did not reduce the survival of non-irradiated HaCAT cells, medium from irradiated HT29 cells was transferred onto non-irradiated HaCAT cells and a significant reduction in HaCAT clonogenic survival was observed (see Figure 4-1; Table 4-1). Previous studies have shown that HT29 cells do not demonstrate a reduction in survival following transfer of medium from γ-irradiated HT29 cells (Mothersill *et al.*, 2002 and 2004). However, transferring the same irradiated (5 Gy) medium onto SW48 cells, another colon carcinoma cell line, resulted in ~36% reduction in survival of these cells (Mothersill *et al.*, 2004b). Taken together, these results suggest that the induction of bystander effects may not only be specific to the cell line exposed to gamma radiation, but also specific to the cell line exposed to the irradiated cell conditioned medium.

Bystander Effects following UVC Exposure

Our desire to examine whether other forms of radiation, such as ultraviolet (UV) radiation, are capable of inducing bystander effects was in an attempt to investigate whether the clinical implications associated with sunlight exposure is only due to the direct exposure of cells to UV radiation. We began by investigating whether the medium from UVC-irradiated human cell lines would have any effect on the survival of non-irradiated HaCAT cells. Although UVC radiation does not account for any of the terrestrial UV due to absorption of UVC by the ozone layer, it is capable of inducing

DNA lesions, such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PP), which have been associated with human skin cancer risk (Sarasin and Giglia-Mari, 2002). Human immortalized keratinocytes (HaCAT), glioma (U373, T98G) and colon carcinoma (HT29) cell lines were exposed to UVC fluences of 20 and 200 J/m^2, and the cell conditioned medium was transferred onto non-irradiated HaCAT cells 24 hr post-incubation. The transfer of conditioned medium from cells exposed to increasing fluences of UVC radiation onto non-irradiated HaCAT cells did not significantly reduce their clonogenic survival (see Figure 4-2; Table 4-2). In addition, medium containing UVC-irradiated PBS (irradiated in the absence of cells) had no significant effect on the clonogenic survival of non-irradiated HaCAT cells. These results suggest that UVC irradiation of various human cells does not result in the production and secretion of a cytotoxic factor(s) that affects the survival of non-irradiated cells, particularly HaCAT cells. In the past, UVC irradiation of human fibroblast cells has been shown to stimulate the release of an extracellular factor(s) in the surrounding medium, which upon transfer to non-irradiated cells substitutes for UVC treatment by mimicking the UV-induced synthesis of various gene products (Schorpp *et al.*, 1984). In addition, the transfer of conditioned medium from UV-irradiated cells onto non-irradiated cells had no apparent effect in the growth properties of the recipient cells. Taken together with the results presented here (Figure 3-1; Table 3-1), it is unlikely that the extracellular factor(s) secreted by UVC-irradiated cells into the medium is cytotoxic towards non-irradiated cells, as we have not observed an apparent change in the number of surviving colonies.

Bystander Effects following UVA Exposure

Exposure of cells to different wavelengths of ultraviolet radiation can result in the production of different types of DNA lesions within the genome. While UVC radiation produces predominantly helix-distorting (bulky lesions) such as CPDs and 6-4PPs, UVA exposure can result in the oxidation of pyrimidine and purine bases, while producing CPDs and 6-4PPs to a lesser extent (Kvam and Tyrrell, 1997; Kielbassa *et al.*, 1997; Perdiz *et al.*, 2000). Since different wavelengths of ultraviolet radiation cause different biological effects, we examined whether transferring conditioned medium from various UVA-irradiated (compared to mock-irradiated) human cells onto non-irradiated HaCAT cells had any effect on colony survival after 9 days of incubation. However, in the initial experiments it was difficult to ascertain whether medium from UVA-irradiated cells was able to reduce the survival of non-irradiated cells since the increased salinity of the medium, caused as a result of evaporation occurring with increased UVA exposure, overshadowed the effect. Upon correcting for the reduction in the PBS volume observed immediately following UVA irradiation, treatment of HaCAT cells with medium containing UVA-irradiated PBS (irradiated in the absence of cells) or conditioned medium from UVA-irradiated HT29 cells resulted in a significant reduction (~10%) in the clonogenic survival of HaCAT cells. In contrast, the relative clonogenic survival of non-irradiated HaCAT cells treated with medium from UVA-irradiated HaCAT, U373 or T98G cells was not significantly reduced (Table 4-4), suggesting that the presence of these cells during UVA irradiation removes the cytotoxic factor(s) within the medium, either by preventing its production or by causing its degradation.

Treatment of non-irradiated T98G cells (but not U373 or HT29 cells) with medium containing UVA-irradiated PBS resulted in a similar, significant reduction in colony survival as non-irradiated HaCAT cells (see Figure 4-5; Table 4-5). This suggests that UVA irradiation of PBS may result in the production of a cytotoxic factor(s) in the medium, to which HaCAT and T98G cells exhibit sensitivity following treatment. In addition, HaCAT and T98G cells exhibited increased sensitivity to UVA exposure compared to U373 and HT29 cells (see Chapter 3; Figure 3-2, Table 3-1). The fact that HaCAT and T98G cells are more sensitive in response to UVA irradiation, as well as in response to treatment with medium containing UVA-irradiated PBS suggests that cells that are sensitive to direct UVA exposure are also likely to be sensitive to the by-products (indirect effects) of UVA irradiation. As mentioned previously, medium from UVA-irradiated HT29 cells reduced the survival of non-irradiated HaCAT cells, but medium contain UVA-irradiated PBS did not affect the survival on non-irradiated HT29 cells. Taken together, these results suggest that depending on the cell line exposed to UVA radiation and the cell line treated with the UVA-irradiated medium will determine whether the effect of reduced colony survival will be observed.

- Cytotoxicity of UVA-Irradiated PBS

To elucidate the identity of the UVA-induced cytotoxic factor in UVA-irradiated PBS, we examined whether a reduction in the survival of non-irradiated HaCAT cells was still possible if we irradiated the different solutions that compose 1X PBS separately. Solutions of ddH_2O, 1X saline, 1X phosphate buffer (PB) and 1X phosphate-buffered

saline (PBS) were UVA-irradiated, and appropriate volumes of ddH_2O, 1X saline or 1X PB solutions were added back immediately following UVA treatment in order to treat cells with a reconstituted 1X PBS and medium solution. Irradiation of ddH_2O or 1X PB solutions results in similar reductions in the survival of HaCAT cells as treating cells with UVA-irradiated 1X PBS solution (Figure 4-6; Table 4-6). However, irradiation of 1X saline solution does not cause a significant reduction in HaCAT colony survival, suggesting that phosphate ions are required in a salt solution in order for the UVA-induced cytotoxic factor to be produced. The fact that UVA irradiation of ddH_2O, in the absence of salt, was able to significantly reduce clonogenic survival when transferred onto non-irradiated HaCAT cells suggests that the cytotoxic factor is likely to be a reactive oxygen species (ROS), with H_2O_2 being the most likely candidate due to its long half-life (~1ms) compared to other ROS. Another possibility is that impurities in the water may have become reactive upon UVA exposure, causing the cytotoxic effect observed following transfer of the medium onto non-irradiated cells. The fact that UVA-irradiation of 1X saline solution did not have a similar effect upon treating HaCAT cells as irradiated ddH_2O, 1X PB or 1X PBS solutions was an interesting observation, one which requires further examination.

UVA irradiation of PBS has previously been shown to result in the production of low concentrations (μM) of hydrogen peroxide (H_2O_2) (Mahns *et al.*, 2003), and we have detected similar levels of H_2O_2 produced immediately following UVA-irradiation (700 kJ/m^2) of PBS (Table 4-7). The levels of hydrogen peroxide generated following UVA irradiation are consistent with the H_2O_2 levels that are capable of reducing the clonogenic

survival of various human cell lines to approximately 10% within statistical error (see Chapter 3, Figure 3-3). Hydrogen peroxide is considered to be a relatively stable reactive oxygen species that is capable of diffusing across biological membranes (Darr and Fridovich, 1994). In order to reduce the excessive extracellular H_2O_2 levels entering the cell, cells contain antioxidant enzymes such as catalase which degrades H_2O_2 into water and dioxygen. UVA irradiation of PBS in the presence of cells resulted in lower (~2.5 to 4.5-fold) levels of H_2O_2 being produced compared to UVA irradiation of PBS alone. These reduced levels of H_2O_2 observed following UVA irradiation of PBS is likely due to the enzymatic degradation of H_2O_2 due to the presence of cells. In the absence of UVA treatment, the levels of H_2O_2 within the medium (PBS/RPMI supplemented solution) from human glioma cell lines (U373 and T98G) were elevated (~ 4 to 10-fold) at 24 hr post-incubation, suggesting that T98G and U373 cells secrete H_2O_2 into their surrounding medium. This observation conflicts with the hypothesis that media containing UVA-induced H_2O_2 is responsible for reducing the clonogenic survival of T98G cells (Figure 4-5; Table 4-5), since H_2O_2 is normally released into the medium when culturing T98G cells. Therefore, it is also possible that UVA irradiation results in the production of a cytotoxic factor other than H_2O_2, a factor which has yet to be identified.

Effects of UVA-treated PBS and media from UVA-irradiated cells

Although treatment of cells with UVA-irradiated medium has been shown to cause a reduction in their clonogenic survival, a more prominent feature observed following treatment of non-irradiated HaCAT cells with medium from UVA-irradiated

cells is a reduction in the area (or size) of the resulting colonies. Treatment of HaCAT cells with medium from UVA-irradiated cells, particularly from HaCAT, U373 and T98G cultures, resulted in a significant reduction in the relative size of HaCAT colonies at 9 days post-incubation. These reductions in relative colony size may indicate that treatment of HaCAT cells with UVA-irradiated medium from various human cell cultures may reduce the growth rate of HaCAT colonies. In contrast, UVA-irradiated PBS (irradiated in the absence of cells) and medium administered to non-irradiated HaCAT cells did not significantly reduce the HaCAT colony size compared to the mock-irradiated treatment, nor did it reduce the relative size of colonies when administered to non-irradiated T98G, U373 or HT29 cells (see Table 4-5). Taken together, these results may suggest that UVA-irradiated cells secrete an extracellular factor into the medium which reduces the growth rate of HaCAT colonies, while irradiation of medium (PBS-irradiated) in the absence of cells does not have any effect on colony size after 9 days of incubation; evidence that may support a bystander effect in UVA-induced colony growth inhibition.

Previous reports have demonstrated that UV irradiation can inhibit proliferation of mammalian cells. UVA radiation was found to inhibit the proliferation of rodent fibroblasts in a dose-dependent manner, resulting in the accumulation of cells in the S-phase of the cell cycle (Banrud *et al.*, 1995). In addition, solar simulated light (SSL; consists of a combination of UVA and UVB) treated filtrates, containing proteins of the extracellular matrix (ECM), were shown to significantly reduce the proliferation (cell number) of human fibroblasts cells compared to cells treated with mock-irradiated filtrate

(Wondrak *et al.*, 2003). The anti-proliferative effect of SSL-treated ECM proteins could be reversed by pre-irradiating the filtrates in the presence of catalase, suggesting that H_2O_2, formed as a result of ECM protein-sensitization to SSL, is responsible for the effect on human fibroblasts. Although the medium transfer technique requires that the medium be filter-sterilized, it is possible that photosensitized cellular debris and by-products of photosensitized reactions can be present in the filtrate used to treat non-irradiated HaCAT cells in our study. More recently, a study by Banarjee and colleagues showed that transferring medium from UV(A+B)-irradiated HaCAT cells (irradiated in PBS) compared to mock-irradiated cells reduced the number of viable cells over a 72 hr period. However, these authors did not examine whether transferring UVA-irradiated PBS (in the absence of cells) and medium has any effect on HaCAT cell survival compared to the mock-treatment.

In order to examine whether the rate of growth of HaCAT cells treated with medium (irradiated in the presence or absence of cells) was altered due to UVA irradiation, the results of mean colony area of HaCAT cells incubated for several days with medium from UVA-irradiated (and mock-irradiated) HaCAT cells, or medium containing UVA (and mock) irradiated PBS, were fit to a linear regression. Analysis of the slopes indicated that the growth rate of HaCAT cells was significantly reduced following treatment with medium from UVA-irradiated HaCAT cells (0.1484±0.0081; R^2=0.9998) compared to mock-irradiated HaCAT cells (0.1719±0.0045; R^2=0.9998). Similarly, the growth rates of HaCAT cells were significantly reduced following treatment with medium containing UVA-irradiated PBS (0.1446±0.0014; R^2=0.9998)

compared to mock-irradiated PBS (0.1760±0.0021; R^2=0.9997). Since a similar decrease in the growth rate of HaCAT cells occurred following incubation with medium irradiated in the presence or absence of HaCAT cells (compared to the mock-irradiated treatment), these results suggest that the presence of cells during UVA irradiation of PBS may not have any bearing on the growth-inhibiting nature of the resulting medium on non-irradiated HaCAT cells. Although these results are preliminary, there is evidence to suggest that transferring medium, UVA-irradiated in the presence or absence of cells, onto non-irradiated HaCAT cells can reduce their proliferation, and therefore, reduce the growth rate of HaCAT colonies.

Although the results of relative clonogenic survival (number) and relative colony size (area) measure different phenomena, they are inter-related. By examining the relative survival of HaCAT cells treated with UVA-irradiated cell conditioned medium or UVA-irradiated medium (PBS-irradiated; no cells present) over various lengths of incubation, we observed that the surviving fraction was reduced (on average) after 6 days of medium incubation, but gradually increased with greater lengths of incubation (Figure 4-11). The fact that a reduction in the surviving fraction was observed earlier rather than later suggests that if the number of cells constituting a colony is less than our cut-off threshold (≥ 32 cells/colony), then the colony will be too small to be included as part of the surviving fraction. With increased time of incubation, a greater proportion of colonies will meet the cut-off threshold, and will be large enough to be counted and included as part of the surviving fraction, thus making the reduction in surviving fraction less impressive with time as shown in Table 4-9. These preliminary results suggest that

in order to properly evaluate clonogenic survival, a time-dependent examination of relative survival and relative colony size should be conducted in order to address whether the treatment results in an inhibition of cell growth and/or true cell killing.

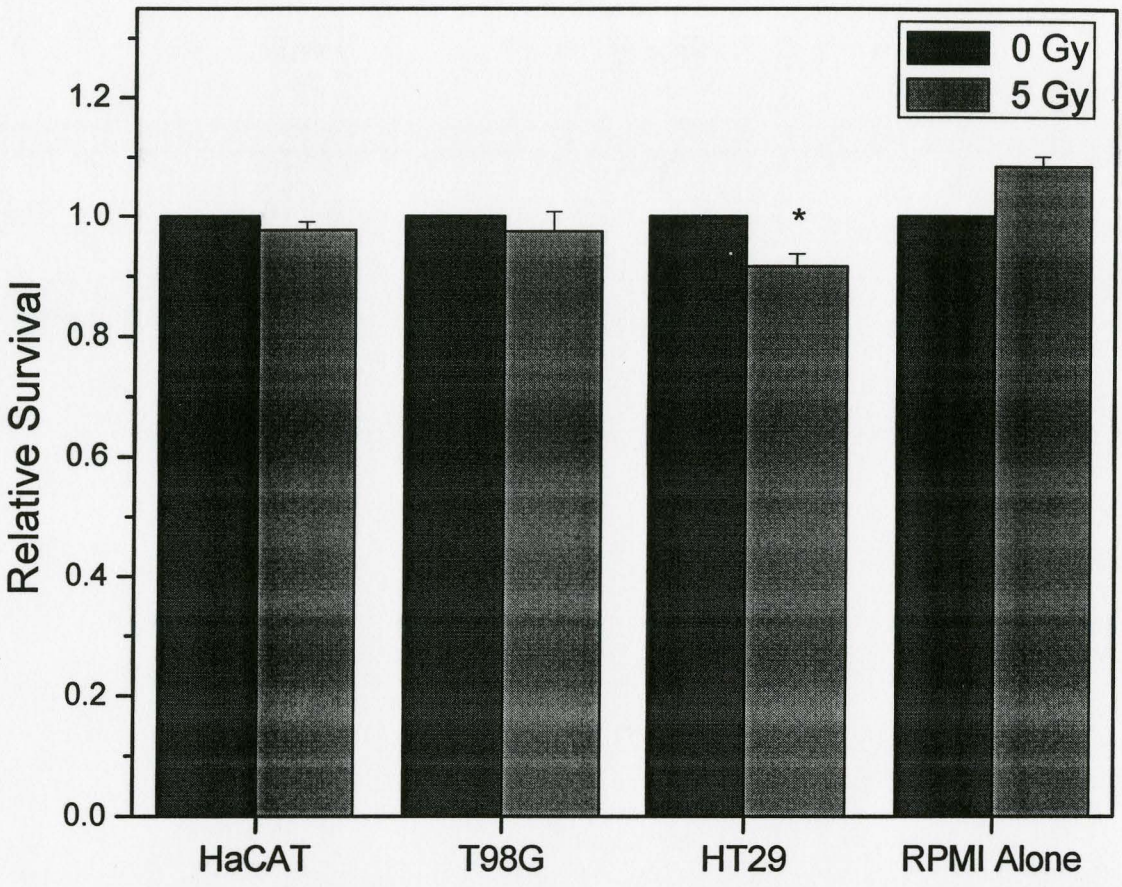

Figure 4-1. Relative survival of non-irradiated HaCAT cells upon transfer of gamma-irradiated conditioned media from various human cell lines at 24 hr post-irradiation. A dose of 5 Gray (Gy) was administered to various human cell lines (seeded 6 hr earlier at a density of 2×10^5 cells/well or RPMI supplemented media alone (5 ml). The medium was removed from the irradiated (or mock-irradiated) cells at 24 hr post-incubation, filter-sterilized and transferred (2 ml/well) onto non-irradiated HaCAT cells (seeded 12 hr earlier at a density of 500 cells/well). Relative survival refers to the survival of HaCAT colonies treated with gamma-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of 3 independent experiments, each performed in duplicate. Results show that conditioned media from gamma-irradiated HT29 cells significantly reduced ($P < 0.05$ in an independent *t*-test) the clonogenic survival of non-irradiated HaCAT cells.

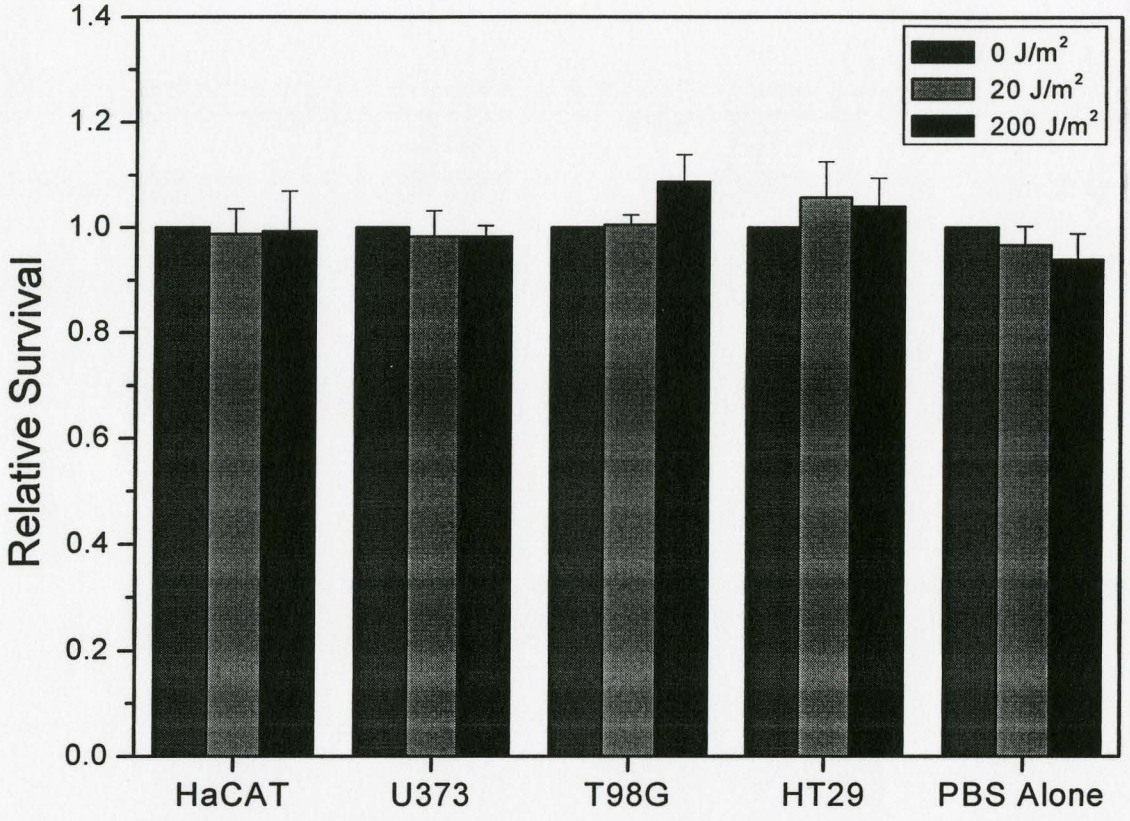

Figure 4-2. Relative survival of non-irradiated HaCAT cells upon transfer of UVC-irradiated PBS and conditioned media from various human cell lines at 24 hr post-irradiation. Increasing UVC fluences (J/m^2) were administered to various human cell lines (seeded 6 hr earlier at a density of 2×10^5 cells/well or phosphate-buffered saline (PBS) alone (1 ml), and 4 ml of supplemented RPMI media was added after irradiation. The medium was removed from the irradiated (or mock-irradiated) cells at 24 hr post-incubation, filter-sterilized and transferred (2 ml/well) onto non-irradiated HaCAT cells (seeded 12 hr earlier at a density of 500 cells/well). Relative survival refers to the survival of HaCAT colonies treated with UVC-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of at least 3 independent experiments, each performed in duplicate. Results show that conditioned media (UVC-irradiated PBS) from UVC-treated cells did not significantly reduce ($P > 0.05$ in an independent *t*-test) the clonogenic survival of non-irradiated HaCAT cells.

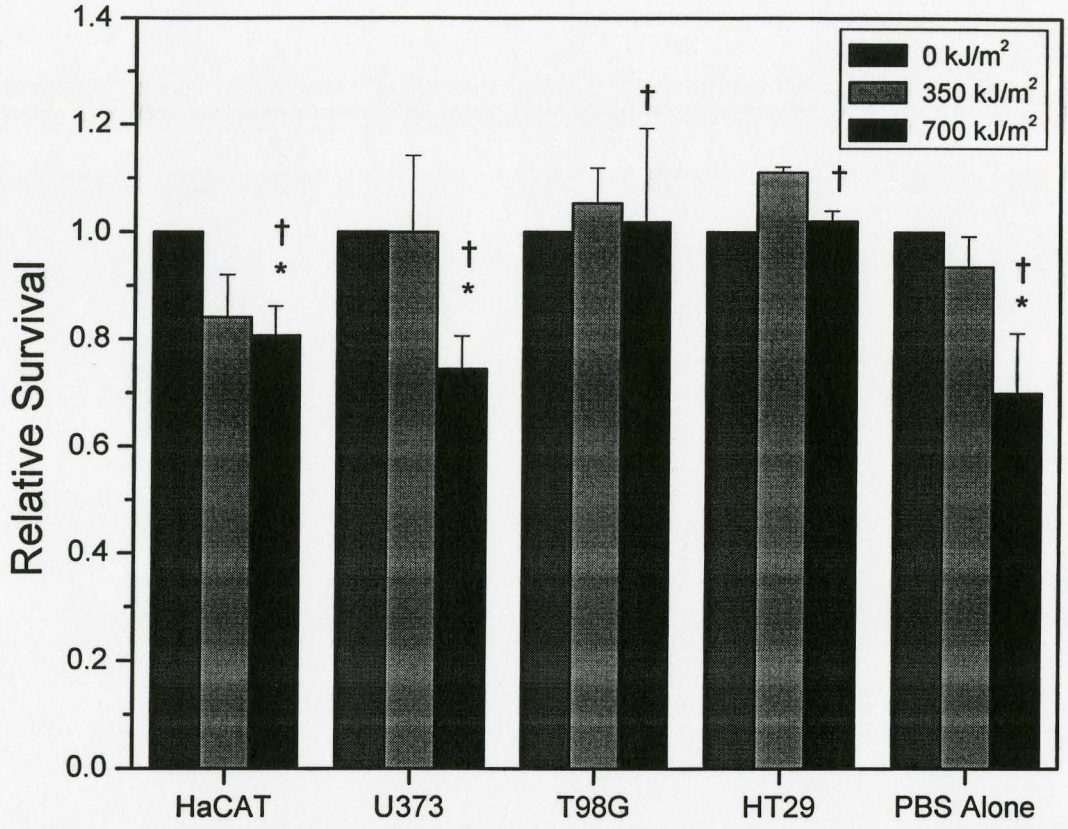

Figure 4-3. Relative survival of non-irradiated HaCAT cells upon transfer on UVA-irradiated PBS and conditioned media from various human cell lines at 24 hr post-irradiation. A UVA fluence of 350 or 700 kJ/m^2 was administered to various cell lines (seeded 6 hr earlier at a density of 2×10^5 cells/well or phosphate-buffered saline (PBS) alone (1.5 ml), and 3.5 ml of supplemented RPMI media was added after irradiation. The medium was removed from the irradiated (or mock-irradiated) cells at 24 hr post-incubation, filter-sterilized and transferred (2 ml/well) onto non-irradiated HaCAT cells (seeded 12 hr earlier at a density of 300 cells/well). Results show that UVA exposure increased the toxicity of conditioned media obtained from HaCAT and U373 cultures (irradiated in 1.5 ml of PBS), such as to significantly reduce the clonogenic survival (*) and/or colony size (†) of non-irradiated HaCAT cells. Relative survival refers to the survival of HaCAT colonies treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of at least 3 independent experiments, each performed in duplicate. *Significantly less than 1 in an independent *t*-test ($P < 0.05$); †Reduced colony sizes (relative to mock-irradiated condition).

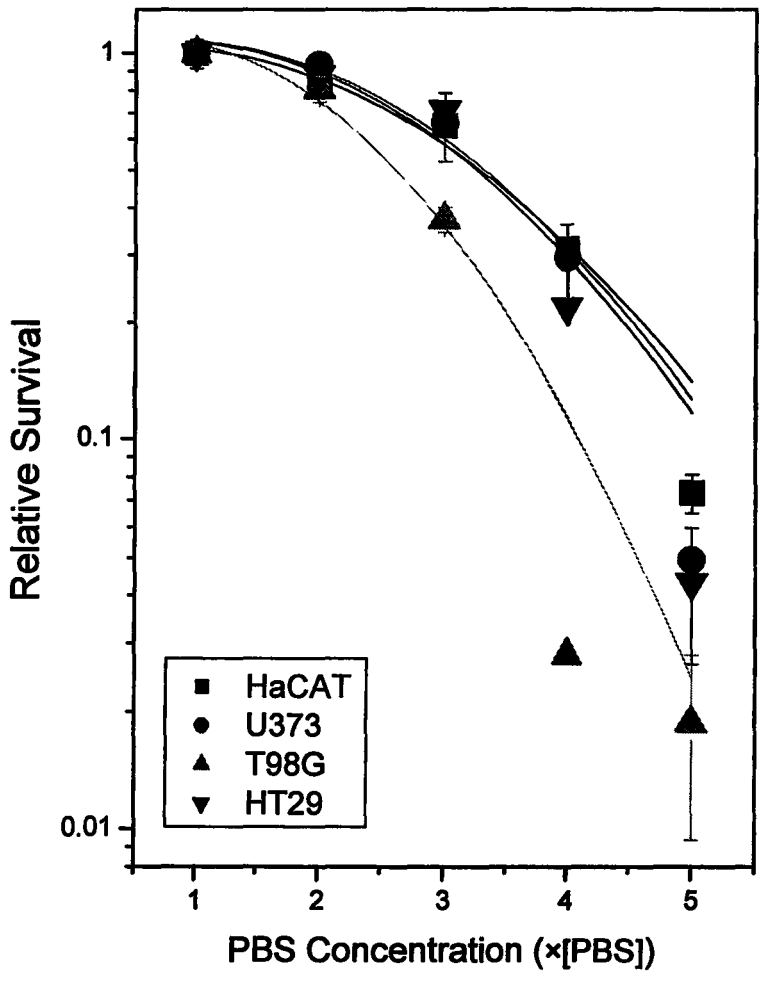

Figure 4-4. Effects of increased concentration of phosphate-buffered saline (PBS) on the survival of various human cell lines. Increasing concentrations of PBS solution were prepared and added to RPMI supplemented medium (mixed in 0.5 ml: 3.5 ml proportions, respectively). The resulting PBS/RPMI solutions were added to various human cell lines. Solutions of increasing salinity reduced the clonogenic survival of the human cells. Each individual data point is the mean ± SE of 3 independent experiments, each performed in triplicate.

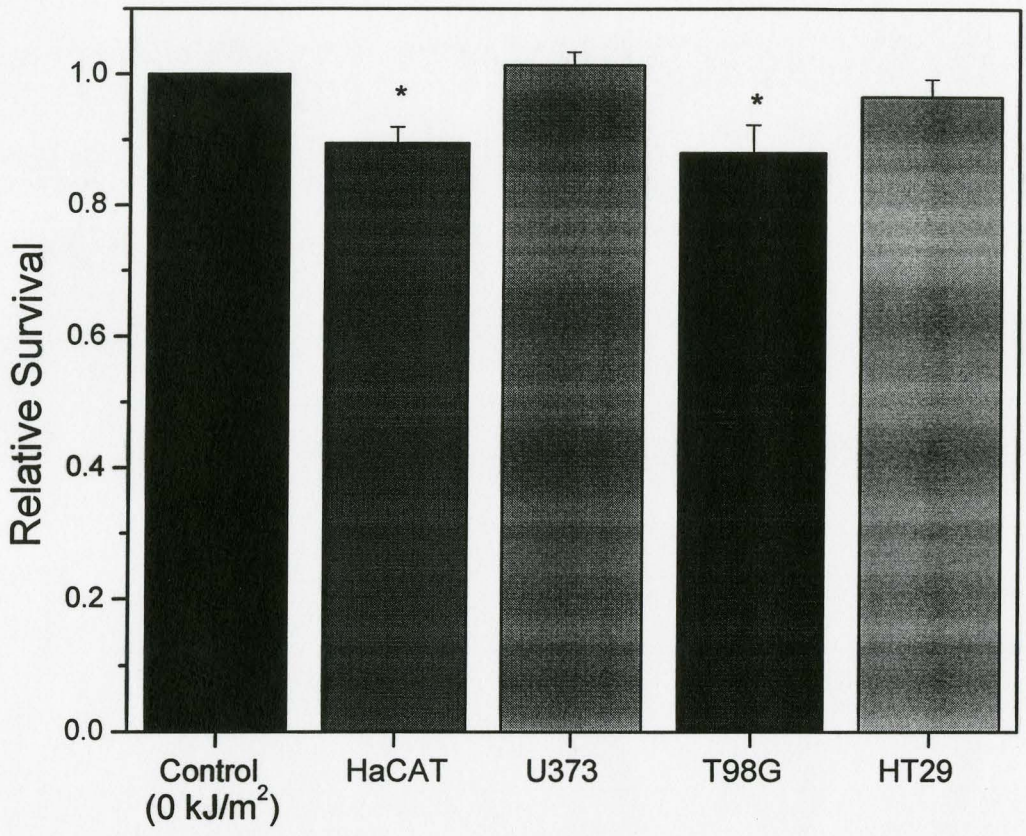

Figure 4-5. Treatment of various human cells lines with UVA-irradiated PBS and RPMI supplemented medium reduces their clonogenic survival. 1X Phosphate-buffered saline (1.5 ml) was UVA-irradiated with a 700 kJ/m^2 fluence. Since UVA irradiation (700 kJ/m^2) resulted in a reduction in the PBS volume by ~2/3 due to evaporation, distilled/deionized water (ddH_2O) was added in order to restore the PBS concentration immediately following irradiation. To each well containing UVA-irradiated (or mock-irradiated) 1X PBS solutions, 3.5 ml of RPMI supplemented media (lacking phenol red) was added, and the final PBS/RPMI solutions were incubated for 24 hr. After incubation, the resulting media solutions were removed from the wells, filter-sterilized and transferred (2 ml/well) onto various non-irradiated human cells. Colonies were stained 9 days later with crystal violet solution. Results show that UVA irradiation of 1X PBS resulted in significant reductions in the clonogenic survival of HaCAT and T98G cells, but not for U373 or HT29 cells. Relative survival refers to the survival of human cells treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of at least 3 independent experiments, each performed in duplicate. [a]Significance assessed using an independent *t*-test ($P < 0.05$).

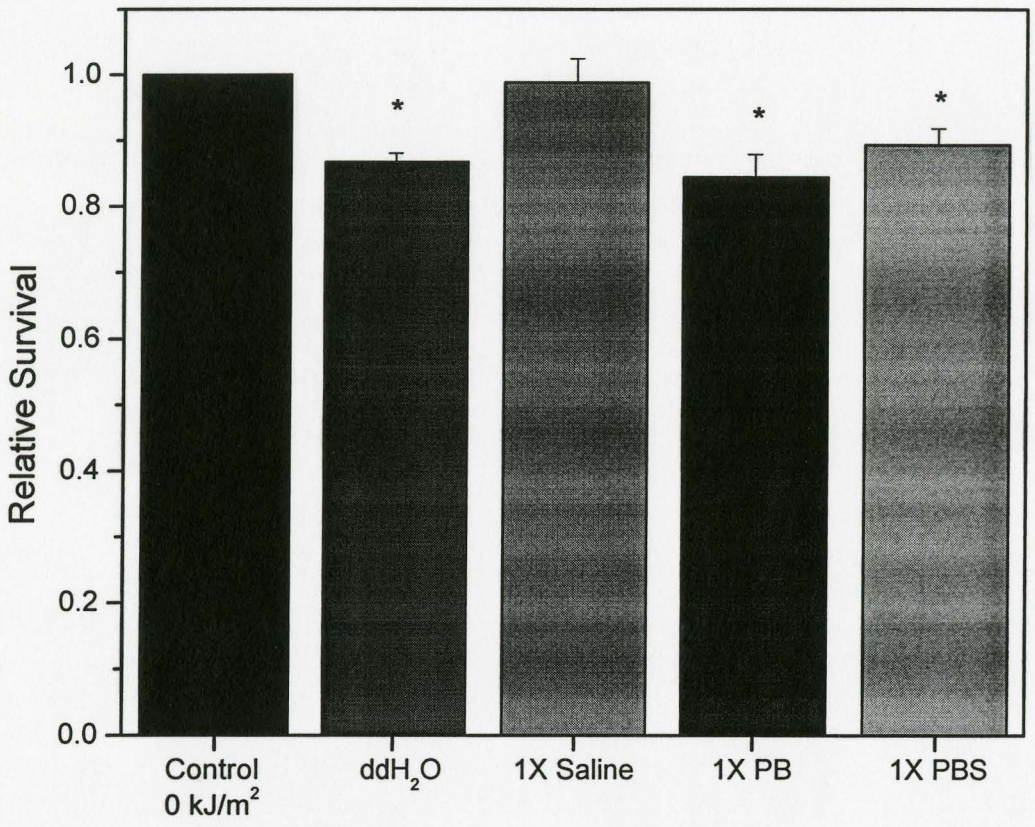

Figure 4-6. UVA treatment of various solutions results in a reduction of HaCAT colony survival. The individual solutions that compose 1X phosphate-buffered saline (PBS) were UVA-irradiated at fluence of 700 kJ/m^2. Immediately following UVA treatment, appropriate volumes of distilled/deionized water (ddH_2O), 1X saline or 1X phosphate buffer (PB) solutions were added to the wells, such that each irradiated well contained 1.5 ml of a final 1X PBS solution. To each well containing UVA-irradiated (or mock-irradiated) 1X PBS solutions, 3.5 ml of RPMI supplemented media (lacking phenol red) was added, and the final PBS/RPMI solutions were incubated for 24 hr. After incubation, the resulting media solutions were removed from the wells, filter-sterilized and transferred onto non-irradiated HaCAT cells (2 ml/well). HaCAT colonies were stained 9 days later with crystal violet solution. Results show that UVA irradiation of ddH_2O, 1X PB and 1X PBS solutions resulted in similar reductions in the clonogenic survival of HaCAT cells, while UVA treatment of 1X saline solution did not have an effect. Relative survival refers to the survival of HaCAT colonies treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of at least 3 independent experiments, each performed in duplicate. *Significantly less than 1 in an independent *t*-test ($P < 0.05$).

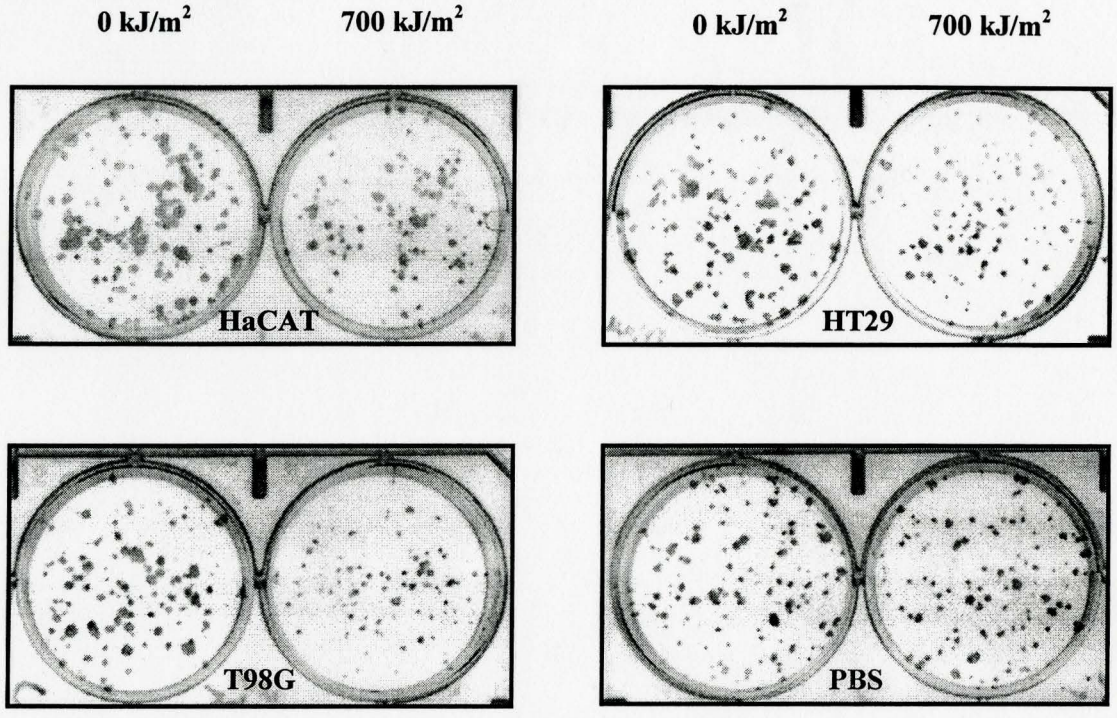

Figure 4-7. Size differences of HaCAT colonies following transfer of UVA-treated (or mock-treated) conditioned media from various human donor cell lines. UVA-treated (700 kJ/m^2) PBS and supplemented conditioned media from various human cell lines (HaCAT, T98G, HT29) reduced the size of non-irradiated HaCAT colonies. In the absence of cells (PBS alone), colony size was not reduced after treating HaCAT cells with a UVA-irradiated (700 kJ/m^2) medium. Following UVA irradiations, the volume of the irradiated 1X phosphate-buffered saline (PBS) solution was reduced (<1.5 ml), and therefore, ddH_2O was added in order to restore the PBS concentration in each well.

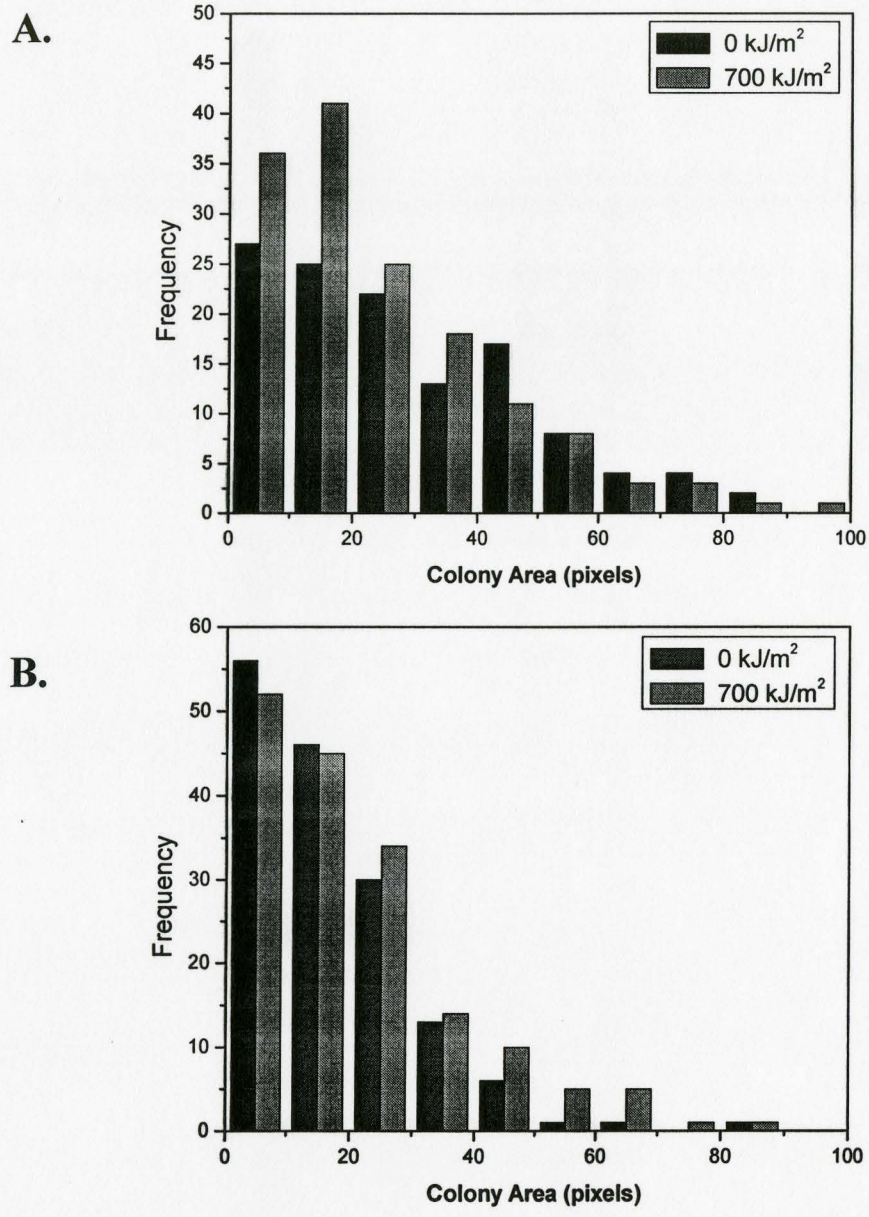

Figure 4-8. Representative distribution of HaCAT colony area following treatment with UVA-irradiated media compared to mock-irradiated media. Recipient HaCAT cells were treated with UVA-irradiated (or mock-irradiated) medium. This medium consists of RPMI supplemented media (3.5 ml) and UVA irradiated (or mock-irradiated) PBS (1.5 ml), in the **A)** presence or **B)** absence of HaCAT cells. Distilled/deionized water (ddH_2O) was added (in appropriate volumes) immediately after irradiation in order to replace the evaporated water and restore the PBS concentration. Twenty-four hours after incubation, the medium was removed, filter-sterilized and transferred onto the recipient HaCAT cells (seeded 12 hr earlier at a density of 300 cells/well). Cells were incubated for 9 days prior to being stained with crystal violet solution.

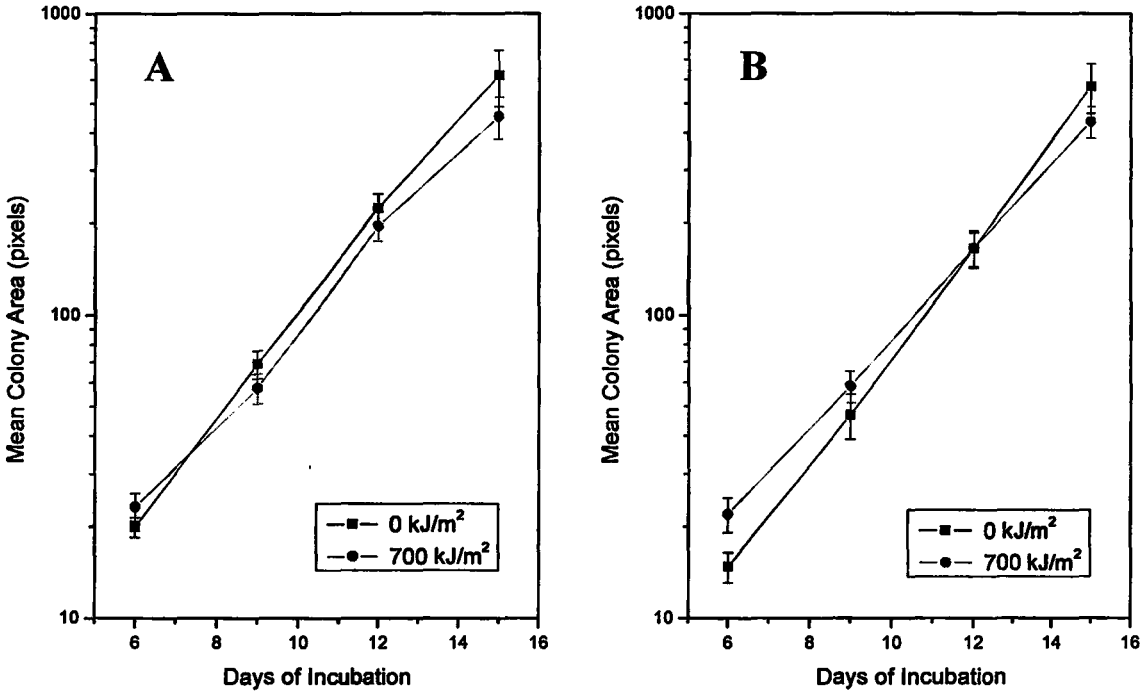

Figure 4-9. Mean colony area decreases following increased incubation with UVA-irradiated medium. Recipient HaCAT cells were treated with UVA-irradiated (or mock-irradiated) medium. This medium consists of RPMI supplemented media (3.5 ml) and UVA-irradiated (or mock-irradiated) PBS (1.5 ml irradiated in the **(A)** presence or **(B)** absence of HaCAT cells). Distilled/deionized water (ddH_2O) was added (in appropriate volumes) to the wells immediately after irradiation in order to replace the evaporated water and restore the PBS concentration. Twenty-four hours after incubation, the medium was removed, filter-sterilized and transferred (2 ml/well) onto the recipient HaCAT cells (seeded 12 hr earlier at a density of 150 cells/well). Cells were incubated for 6, 9, 12 or 15 days prior to being stained with crystal violet solution. The mean colony area (± SE) was quantified and plotted *versus* the length (days) of medium-incubation within the single experiment.

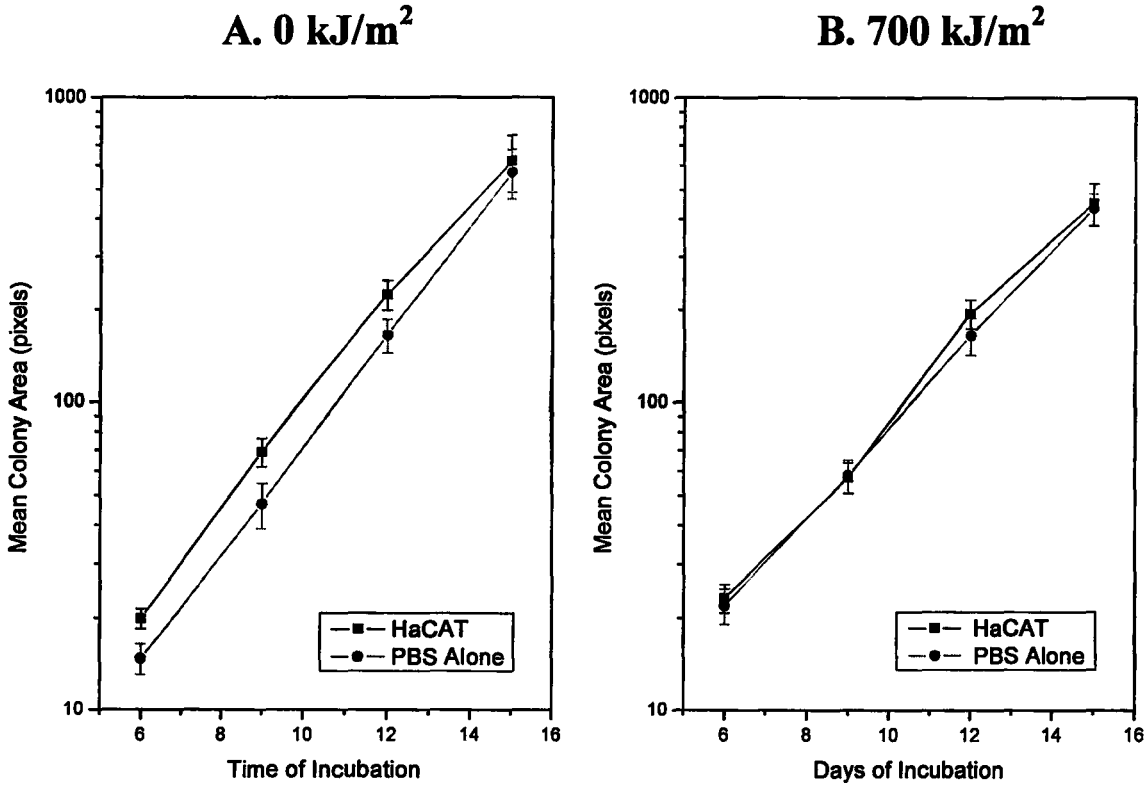

Figure 4-10. Differences in mean colony area of HaCAT cells treated with conditioned compared to unconditioned medium are not observed following UVA treatment. Recipient HaCAT cells were treated with UVA-irradiated (or mock-irradiated) medium. This medium consists of RPMI supplemented media (3.5 ml) and UVA-irradiated (or mock-irradiated) PBS (1.5 ml irradiated in the presence or absence of HaCAT cells). Distilled/deionized water (ddH_2O) was added (in appropriate volumes) to the wells immediately after irradiation in order to replace the evaporated water and restore the PBS concentration. Twenty-four hours after incubation, the medium was removed, filter-sterilized and transferred (2 ml/well) onto the recipient HaCAT cells (seeded 12 hr earlier at a density of 150 cells/well). Cells were incubated for 6, 9, 12 or 15 days prior to being stained with crystal violet solution. The mean colony area (± SE) was quantified and plotted *versus* the length (days) of medium-incubation within the single experiment. Results were plotted with respect to cells receiving medium that was **A)** mock-irradiated or **B)** UVA-irradiated (700 kJ/m^2) in the presence or absence of HaCAT cells.

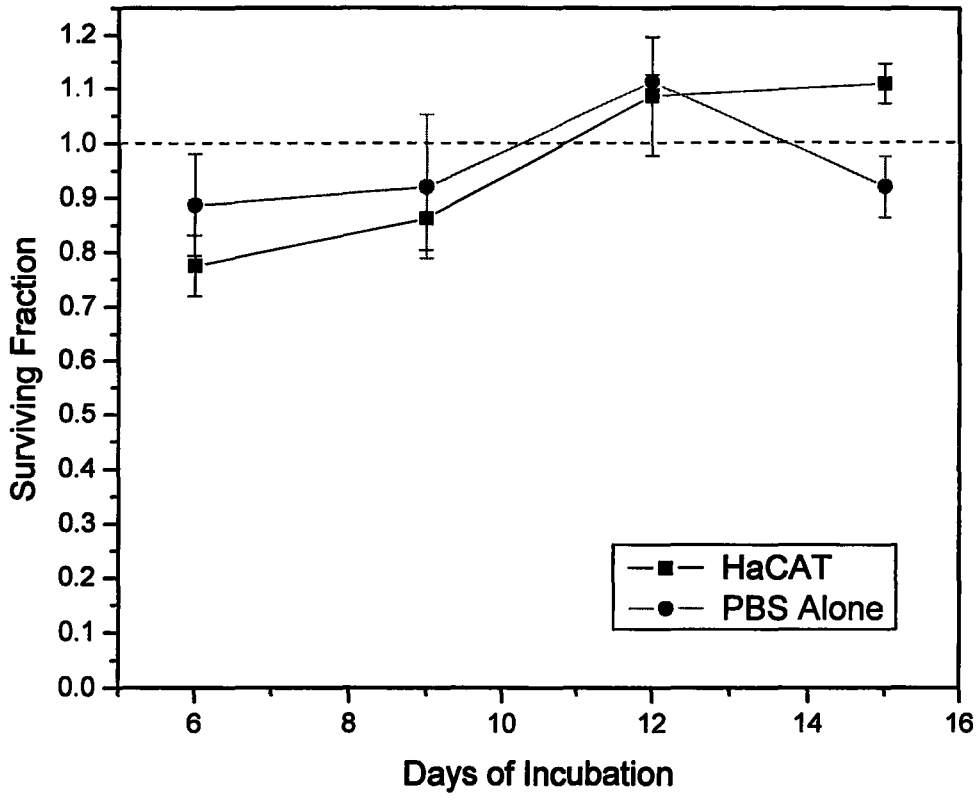

Figure 4-11. Relative clonogenic survival increases following increased incubation with UVA-irradiated medium. Recipient HaCAT cells were treated with UVA-irradiated (or mock-irradiated) medium. This medium consists of RPMI supplemented media (3.5 ml) and UVA-irradiated (or mock-irradiated) PBS (1.5 ml irradiated in the presence or absence of HaCAT cells). Distilled/deionized water (ddH_2O) was added (in appropriate volumes) to the wells immediately after irradiation in order to replace the evaporated water and restore the PBS concentration. Twenty-four hours after incubation, the medium was removed, filter-sterilized and transferred (2 ml/well) onto the recipient HaCAT cells (seeded 12 hr earlier at a density of 150 cells/well). Cells were incubated for 6, 9, 12 or 15 days prior to being stained with crystal violet solution. Surviving fraction (SF) refers to the survival of HaCAT colonies treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of a single independent experiment.

Table 4-1. Relative survival of non-irradiated HaCAT cells upon transfer of gamma-irradiated conditioned media from various human cell lines at 24 hr post-irradiation. Results show that conditioned media from gamma-irradiated HT29 cells significantly reduced ($P < 0.05$ in an independent *t*-test) the clonogenic survival of non-irradiated HaCAT cells. Surviving fraction (SF) refers to the survival of HaCAT colonies treated with gamma-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of 3 independent experiments, each performed in duplicate.

	Relative Survival		
	SF	**%SE**	***P* value**
Control (0 Gy)	1	–	–
HaCAT	0.977	0.014	0.182
T98G	0.975	0.033	0.489
HT29	0.917	0.021	0.017
RPMI Alone	1.085	0.017	0.007

Table 4-2. Relative survival of non-irradiated HaCAT cells upon transfer of UVC-ICCM from various human cell lines at 24 hr post-irradiation. Increasing UVC fluences (J/m^2) were administered to various human cell lines (seeded 6 hr earlier at a density of 2×10^5 cells/well or phosphate-buffered saline (PBS) alone (1 ml), and 4 ml of supplemented RPMI media was added after irradiation. The medium was removed from the irradiated (or mock-irradiated) cells at 24 hr post-incubation, filter-sterilized and transferred (2 ml/well) onto non-irradiated HaCAT cells (seeded 12 hr earlier at a density of 500 cells/well). SF is the surviving fraction of HaCAT cells receiving UVC-irradiated medium relative to mock-irradiated medium, and is given as mean ± SE of 3 independent experiments, each performed in duplicate. [a]Significance assessed using an independent *t*-test ($P < 0.05$); (#) is the number of independent determinants.

	UVC Fluence to Cells (J/m^2)								
	0			20			200		
	SF	%SE	*P* value[a]	SF	%SE	*P* value[a]	SF	%SE	*P* value[a]
HaCAT	1	–	–	0.987	0.049	0.809 (3)	0.993	0.076	0.932 (3)
U373	1	–	–	0.982	0.05	0.757 (3)	0.982	0.022	0.509 (3)
T98G	1	–	–	1.005	0.019	0.806 (3)	1.087	0.052	0.236 (3)
HT29	1	–	–	1.057	0.068	0.490 (3)	1.04	0.054	0.541 (3)
PBS Alone	1	–	–	0.966	0.036	0.398 (3)	0.939	0.05	0.289 (3)

Table 4-3. Treatment of human cells with PBS solutions of increasing concentration reduces their clonogenic survival. Increasing concentrations of PBS solution were prepared and added to RPMI supplemented medium (mixed in 0.5 ml: 3.5 ml proportions, respectively). The resulting PBS/RPMI solutions were added to various human cell lines. Solutions of increasing salinity reduced the clonogenic survival of the human cells. Results are given as mean ± SE of 3 independent experiments, each performed in triplicate. D_{37} refers to the dose (PBS concentration) required to reduce the survival of cells to 37%.

	PBS Concentration		Relative Survival	
Cell Line	D_{37} (×[PBS])	SE	3×[PBS]	SE
HaCAT	3.69	0.15	0.561	0.035
U373	3.9	0.22	0.646	0.063
T98G	2.92	0.03	0.343	0.009
HT29	3.83	0.2	0.628	0.062

Table 4-4. Addition of distilled/deionized water following UVA irradiation of human cells in order to restore the PBS concentration improves the clonogenic survival of non-irradiated HaCAT cells treated with the UVA-ICCM. UVA irradiation (700 kJ/m^2) resulted in a reduction in the PBS volume by 2/3 due to evaporation. In order to restore the PBS concentration following UVA irradiation of cells (or PBS alone), distilled/deionized water (ddH_2O) was added. Restoration of the PBS concentration improved the survival of non-irradiated HaCAT cells treated with the UVA-ICCM. However, treatment of HaCAT cells with UVA-irradiated medium (PBS-irradiated; no cells present) or conditioned medium from HT29 cells resulted in a significant reduction in the clonogenic survival of the HaCAT cells even after the addition of ddH_2O. Surviving fraction (SF) refers to the survival of HaCAT colonies treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of at least 3 independent experiments, each performed in duplicate. [a]Significance assessed using an independent *t*-test ($P < 0.05$); (#) is the number of independent determinants.

	- ddH_2O			+ ddH_2O		
	SF	**%SE**	***P* value[a]**	**SF**	**%SE**	***P* value[a]**
Control (0 kJ/m^2)	1	–	–	1	–	–
HaCAT	0.807	0.055	0.005 (7)	0.951	0.036	0.222 (4)
U373	0.744	0.062	0.006 (4)	0.977	0.047	0.654 (3)
T98G	1.018	0.176	0.924 (3)	0.932	0.036	0.132 (3)
HT29	1.02	0.02	0.359 (3)	0.912	0.024	0.021 (3)
PBS Alone	0.700	0.112	0.018 (8)	0.894	0.025	4.59×10^{-4} (11)

Table 4-5. Treatment of various human cells lines with UVA-irradiated PBS and RPMI supplemented medium reduces their clonogenic survival but not their colony size. 1X Phosphate-buffered saline (1.5 ml) was UVA-irradiated with a 700 kJ/m^2 fluence. Since UVA irradiation (700 kJ/m^2) resulted in a reduction (~2/3) in the PBS volume due to evaporation, distilled/deionized water (ddH_2O) was added in order to restore the PBS concentration immediately following irradiation. To each well containing UVA-irradiated (or mock-irradiated) 1X PBS solutions, 3.5 ml of RPMI supplemented media (lacking phenol red) was added, and the final PBS/RPMI solutions were incubated for 24 hr. After incubation, the resulting media solutions were removed from the wells, filter-sterilized and transferred onto various non-irradiated human cells (2 ml/well). Colonies were stained 9 days later with crystal violet solution. Results show that UV-A irradiation of 1X PBS resulted in significant reductions in the clonogenic survival of HaCAT and T98G cells, but not for U373 or HT29 cells. Surviving fraction (SF) refers to the survival of human cells treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of at least 3 independent experiments, each performed in duplicate. [a]Significance assessed using an independent *t*-test ($P < 0.05$).

Cell Line Recipient	Relative Survival			Relative Colony Area (Size)		
	SF	SE	*P* value[a]	Relative Size	SE	*P* value[a]
Control (0 kJ/m^2)	1	–	–	1	–	–
HaCAT	0.896	0.023	1.94×10^{-4} (12)	1.070	0.067	0.3136 (7)
U373	1.014	0.020	0.516 (3)	1.122	0.115	0.3467 (3)
T98G	0.880	0.043	0.047 (3)	1.236	0.030	0.0015 (3)
HT29	0.966	0.027	0.281 (3)	1.202	0.016	2.01×10^{-4} (3)

Table 4-6. UVA treatment of various solutions results in a reduction of HaCAT colony survival. The individual solutions that compose 1X phosphate-buffered saline (PBS) were UVA-irradiated at fluence of 700 kJ/m^2. Immediately following UVA treatment, appropriate volumes of distilled/deionized water (ddH_2O), 1X saline or 1X phosphate buffer (PB) solutions were added to the wells, such that each irradiated well contained 1.5 ml of a final 1X PBS solution. To each well containing UVA-irradiated (or mock-irradiated) 1X PBS solutions, 3.5 ml of RPMI supplemented media (lacking phenol red) was added, and the final PBS/RPMI solutions were incubated for 24 hr. After incubation, the resulting media solutions were removed from the wells, filter-sterilized and transferred onto non-irradiated HaCAT cells (2 ml/well). HaCAT colonies were stained 9 days later with crystal violet solution. Results show that UVA irradiation of ddH_2O, 1X PB and 1X PBS solutions resulted in significant reductions in the clonogenic survival of HaCAT cells, while UV-A treatment of 1X saline solution did not have an effect. Surviving fraction (SF) refers to the survival of HaCAT colonies treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of at least 3 independent experiments, each performed in duplicate. [a]Significance assessed using an independent *t*-test ($P < 0.05$).

	UVA Irradiation (700 kJ/m^2) of Various Solutions			
Irradiated Medium	**SF**	**SE**	***P* value[a]**	**No. of Determinants**
ddH_2O	0.868	0.013	0.001	3
1X PB	0.845	0.035	0.011	3
1X Saline	0.988	0.037	0.765	3
1X PBS	0.896	0.023	1.94×10^{-4}	12

Table 4-7. Hydrogen peroxide concentration of the PBS / RPMI media solution obtained from UVA-irradiated (and mock-irradiated) cells, as measured immediately and 24hr following irradiation. Results show that UVA treatment to PBS (with or without cells present) results in an increased production of hydrogen peroxide immediately following irradiation, as determined using the Amplex Red Hydrogen Peroxide / Peroxidase Assay Kit (Molecular Probes). In the absence of UV-A treatment, the hydrogen peroxide levels increased approximately 4 to 10-fold in the human glioma cell lines (T98G and U373) when measured 24 hr post-incubation. $[H_2O_2]$ is given as mean ± SE of at least 2 independent experiments, each performed in triplicate. [a]Refers to the number of determinants (Det.), each performed in triplicate.

		0 kJ/m^2				700 kJ/m^2			
		Immediate		24 hr		Immediate		24 hr	
Donor Source	[a]Det.	$[H_2O_2]$ (µM)	SE	$[H_2O_2]$ (µM)	SE	$[H_2O_2]$ (µM)	SE	$[H_2O_2]$ (µM)	SE
HaCAT	4	0.366	0.202	0.126	0.071	1.219	0.234	0.115	0.067
T98G	3	0.556	0.301	2.304	0.35	1.154	0.207	0.829	0.5
U373	3	0.218	0.137	2.217	0.76	0.691	0.23	0.374	0.185
HT29	2	0.501	0.42	0.755	0.134	0.964	0.513	0.368	0.057
PBS Alone	4	0.421	0.223	0.218	0.155	3.1	0.375	0.411	0.22

Table 4-8. Relative HaCAT colony size following transfer of irradiated cell conditioned medium from various human donor cell lines. UVA-treated (700 kJ/m^2) PBS and supplemented conditioned media from various human cell lines significantly reduced the size of non-irradiated HaCAT colonies. In the absence of cells (PBS alone), a reduction in colony size was not significantly reduced after treating HaCAT cells with a UVA-irradiated (700 kJ/m^2) medium. Following UV-A irradiations, the volume of the irradiated 1X phosphate-buffered saline (PBS) solution was reduced (<1.5 ml), and therefore, ddH_2O was added in order to restore the PBS concentration in each well. Relative size refers to the size of HaCAT colonies treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of at least 3 independent experiments, each performed in duplicate. [a]Significance assessed using an independent *t*-test ($P < 0.05$).

	Relative Colony Size of HaCAT recipients			
Donor Source	**Relative Size**	**SE**	***P* value[a]**	**No. of Determinants**
HaCAT	0.807	0.064	0.017	5
U373	0.714	0.101	0.048	3
T98G	0.598	0.024	7.79×10^{-5}	3
HT29	0.789	0.095	0.091	3
PBS Alone	1.070	0.067	0.3136	7

Table 4-9. HaCAT clonogenic survival increases and relative colony size decreases following increased incubation with UVA-irradiated medium from donor HaCAT cells. Recipient HaCAT cells were treated with UVA-irradiated (or mock-irradiated) medium. This medium consists of RPMI supplemented media (3.5 ml) and UV-A irradiated (or mock-irradiated) PBS (1.5 ml irradiated in the presence or absence of HaCAT cells). Distilled/deionized water (ddH_2O) was added (in appropriate volumes) to the wells immediately after irradiation in order to replace the evaporated water and restore the PBS concentration. Twenty-four hours after incubation, the medium was removed, filter-sterilized and transferred onto the recipient HaCAT cells (seeded 12 hr earlier at a density of 150 cells/well). Cells were incubated for 6, 9, 12 or 15 days prior to being stained with crystal violet solution. Surviving fraction (SF) refers to the survival of HaCAT colonies treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of a single independent experiment. Relative size refers to the size of HaCAT colonies treated with UVA-irradiated medium compared to mock-irradiated medium, and is given as mean ± SE of a single independent experiment, performed in duplicate. [a]Significance of mean colony area (size) of HaCAT cells receiving non-irradiated media compared to UVA-irradiated media.

		Relative Survival		Relative Colony Area (Size)		
	Time (day)	SF	%SE	Relatve Size	%SE	*P* value[a]
Control (0 kJ/m^2)		1	–	1	–	
HaCAT	6	0.775	0.056	1.168	0.123	0.237
	9	0.863	0.059	0.832	0.095	0.242
	12	1.087	0.109	0.872	0.096	0.389
	15	1.110	0.037	0.730	0.116	0.252
PBS Alone	6	0.887	0.094	1.5	0.2	0.027
	9	0.921	0.132	1.243	0.150	0.284
	12	1.113	0.013	1.004	0.141	0.983
	15	0.922	0.056	0.763	0.091	0.233

Chapter 5

Summary and Future Directions

Recombinant non-replicating adenovirus constructs have been used to introduce UV-damaged reporter genes into human and rodent cells in order to assess the cell's capacity to repair damaged DNA using host cell reactivation (HCR) of the reporter gene activity as a measured endpoint. Previous studies have shown that HCR of a UV-damaged adenovirus reporter gene construct (Ad5HCMVsp1*lacZ*), which contains the *lacZ* reporter gene under the control of the constitutive human cytomegalovirus immediately early (HCMV-IE) promoter inserted into the deleted E1 region of the adenovirus genome, was significantly reduced in xeroderma pigmentosum (XP) and Cockayne syndrome (CS) fibroblast strains relative to normal (McKay and Rainbow, 1996; McKay *et al.*, 1997a; Francis and Rainbow, 1999; Pitsikas *et al.*, 2005). The XP and CS disorders are each characterized by deficiencies in one or both of the sub-pathways that constitute the nucleotide excision repair (NER) pathway, the repair pathway involved in the removal of UV-induced helix-distorting lesions from the genome. Each of the two sub-pathways of NER, transcription-coupled repair (TCR) and global genome repair (GGR), have distinct roles: the TCR pathway specifically targets transcription-blocking lesions, resulting in the preferential removal of lesions from the transcribed strand of active genes, whereas the GGR pathway acts to remove lesions non-preferentially from coding and non-coding regions of the genome.

Using this *in vitro* approach, we were interested in investigating whether repair of the UV-damaged AdCA17*lacZ* construct (which contains a similar transgene as Ad5HCMVsp1*lacZ*) in non-irradiated normal and NER-deficient cells was dependent on the orientation of the transgene in these recombinant viruses. Cells were infected with

UVC-irradiated or mock-irradiated AdCA17*lacZ* and subsequently scored for β-gal expression at various times after infection. Similar to the results obtained for Ad5HCMVsp1*lacZ*, we were able to demonstrate that repair of the UV-damaged AdCA17*lacZ* and subsequent expression and *lacZ* reporter gene (β-galactosidase; β-gal) activity was reduced in TCR-proficient (XP-C, XP-E), GGR-proficient (CS-B) and NER (GGR/TCR)-deficient (XP-A) cell strains at both 24 and 40 hr post-infection, in comparison to normal fibroblasts. It has previously been reported that pre-treatment of normal human fibroblasts with low UVC fluences immediately prior to infection with the UVC-irradiated viral construct, Ad5HCMVspl*lacZ*, resulted in enhanced HCR of the UV-damaged reporter gene in normal and XP-C cell strains, but delayed in CS-B. The observed UV-enhanced HCR of the UV-damaged reporter gene activity in human cells has been suggested to result from an inducible DNA repair process that involves both the TCR and the GGR pathways (McKay *et al.*, 1997a; Francis and Rainbow, 1999; Pitsikas *et al.*, 2005; Rainbow *et al.*, in press). Using the AdCA17*lacZ* construct, we have shown that prior UVC irradiation of TCR-deficient (XP-C and XP-E) and GGR-deficient (CS-B) cell strains resulted in enhanced HCR of the UV-damaged *lacZ* gene activity at 24 and 40 hr post-infection, thus providing further support that both GGR and TCR are inducible upon UVC-pretreatment of cells. However, significant enhancement in HCR of the UV-damaged *lacZ* gene activity could only be observed in normal cells at 12 hr post-infection, suggesting that proficiency in both TCR and GGR results in the faster rate of repair of the transcribed strand of the reporter gene than in either a TCR or GGR-compromised cell line.

While differences in the rate at which enhancement in HCR of the UV-damaged reporter gene activity is observed when infecting with either of the two adenovirus constructs into NER-deficient and proficient cell lines, we believe that orientation of the transgene within the adenovirus genome may be responsible for demonstrating these repair rate differences. This orientation-dependent difference may be due, in part, to the presence of the E1a enhancer, which remains in the adenovirus vector backbone upstream of the HCMV-IE promoter in AdCA17*lacZ*. It is conceivable that transcription factors binding to both the E1a enhancer and the HCMV-IE promoter enhancer sequences can act synergistically, resulting in the increased repair and subsequent expression of β-galactosidase observed following AdCA17*lacZ* infection. The lack of an E1a enhancer upstream of the HCMV-IE promoter in Ad5HCMVsp1*lacZ* may result in differences in the timing of repair and expression of the reporter gene, particularly in normal and CS-B fibroblasts.

A limitation of the HCR assay is the fact that by measuring the reporter gene activity, repair of the transcribed strand of the reporter gene can only be examined in various repair-deficient and proficient cells. Using experimental assays that focus on strand-specific repair (Bohr assay, ligation-mediated polymerase chain reaction (LM-PCR) technique), the repair rates of the transcribed and non-transcribed strands can be detected and compared. In addition, future studies focusing on strand-specific repair may help to determine whether orientation of the transgene in the adenovirus constructs, Ad5HCMVsp1*lacZ* and AdCA17*lacZ*, can account for the differences in the ability to detect UV-enhanced HCR of the UV-damaged reporter gene activity in various human

cell lines. Since significant enhancement in HCR of the UV-damaged reporter gene activity was observed at 12 hr following AdCA17*lacZ* infection and scoring for β-gal activity, it would be interesting to examine whether UVC pre-treatment of TCR- and GGR-deficient cells can also result in significant enhancement in HCR at earlier times post-infection. While UVC pre-treatment of cells has been suggested to induce both the TCR and GGR sub-pathways, whether this induction in repair is the result of introducing cellular DNA damage, or the induction of a UV-induced stress signal, in the host cell has not been investigated. By introducing damaged DNA into non-irradiated cells prior to infecting with the UV-damaged AdCA17*lacZ* construct, we can examine whether exogenously introduced damaged DNA can induce the repair and subsequent reactivation of the *lacZ* reporter gene activity.

Much effort has gone into investigating and understanding the nature of the phenomenon known as radiation-induced bystander effects. Radiation-induced bystander effects refer to the induction of biological effects in non-irradiated cells that have occurred as a result of exposure of other cells to radiation. Literature regarding radiation-induced bystander effects has been present for many decades which have shown that exposure to ionizing radiation results in the production of a plasma-borne factor capable of causing chromosomal damage in non-irradiated cells. For instance, Emerit and colleagues reported the presence of radiation-induced clastogenic (chromosome-breaking) factors in the plasma of children exposed at Chernobyl (Emerit *et al.*, 1997).

In more recent years, *in vitro* studies have been used to study radiation-induced bystander effects. One approach to studying the effects communicated by irradiated cells

onto non-irradiated cells involves the "medium transfer" technique. This approach involves harvesting culture medium from irradiated and mock-irradiated cells and transferring the medium onto non-irradiated cells in order to determine the effect of any radiation-induced medium-borne factors. Experiments involving the transfer of medium from γ-irradiated cells onto non-irradiated cells have been investigated by Mothersill and Seymour, who demonstrated a reduction (~30%) in the clonogenic survival (or colony-forming ability) of immortalized human keratinocytes (HaCAT) treated with supernatants from HaCAT cells exposed to a 5 Gray (Gy) dose from a ^{60}Co gamma source (Mothersill and Seymour, 1997). In contrast, medium irradiated in the absence of cells had no effect on the non-irradiated HaCAT cells, suggesting that irradiation of cells results in the generation of extracellular factors that communicate the cytotoxic response to non-irradiated cells. We have attempted to reproduce the results of Mothersill and Seymour under similar conditions with little success. Non-irradiated HaCAT cells were treated with supernatants from HaCAT, human glioma (U373, T98G) and human colon carcinoma (HT29) cell lines exposed to a 5 Gy dose from a ^{137}Cs gamma source. Under these conditions, we could not demonstrate the ~30% reduction in HaCAT clonogenic survival following treatment with cell conditioned medium from γ-irradiated HaCAT cells. However, we demonstrated that irradiation of medium alone does not reduce the survival on non-irradiated HaCAT cells.

In addition to ionizing radiation, studies have focused on examining whether UV radiation can mediate bystander effects in mammalian cells. The transfer of irradiated cell conditioned medium from cells exposed to increasing fluences of UVC radiation onto

non-irradiated HaCAT cells did not significantly reduce their clonogenic survival. In addition, UVC-irradiated PBS and RPMI supplemented medium (irradiated in the absence of cells) had no effect on the clonogenic survival of non-irradiated HaCAT cells. Although UVC irradiation of cells has previously been shown to result in the secretion of extracellular factors into the medium, the transfer of conditioned medium from UV-irradiated cells onto non-irradiated cells had no apparent effect in the growth properties of the recipient cells, similar to our results. Therefore, it is unlikely that the extracellular factor(s) secreted by UVC-irradiated cells into the medium is cytotoxic towards non-irradiated cells, as we did not observe an apparent change in the number of surviving colonies.

In contrast to the effects of UVC radiation, medium from UVA-irradiated cells did affect the growth of non-irradiated colonies. Transferring medium from UVA-irradiated cells onto non-irradiated HaCAT cells resulted in an observable reduction in the area (size) of non-irradiated HaCAT colonies, while PBS irradiated in the absence of cells (medium alone) did not demonstrate a reduction in the size of HaCAT colonies after 9 days of incubation. However, medium irradiated in the presence or absence of cells was shown to reduce (~10%) the survival of non-irradiated colonies after 9 days of incubation. In order to examine whether growth inhibition of HaCAT colonies is occurring over time and whether the effects of reduced relative clonogenic survival and relative colony size are related, we examined the relative survival and relative size of HaCAT colonies incubated with the irradiated cell conditioned medium at various days following incubation. Preliminary results showed that the relative survival of HaCAT

colonies increased, while the relative colony size decreased (as early as 9 days), with increased incubation with cell conditioned medium from UVA-irradiated HaCAT cells. Medium irradiated (PBS-irradiated) in the absence of cells reduced the size of HaCAT colonies at 15 days post-incubation. In order to examine whether the rate of growth of HaCAT cells treated with medium (irradiated in the presence or absence of cells) was altered due to UVA irradiation, the results of mean colony area of HaCAT cells incubated for several days with medium from UVA-irradiated (and mock-irradiated) HaCAT cells, or medium containing UVA (and mock) irradiated PBS, were fit to a linear regression. Analysis of the slopes indicated that the growth rate of HaCAT cells was significantly reduced following treatment with medium from UVA-irradiated HaCAT cells or medium containing PBS-irradiated alone. Since a similar decrease in the growth rate of HaCAT cells occurred following incubation with medium irradiated in the presence or absence of HaCAT cells (compared to the mock-irradiated treatment), these results suggest that the presence of cells during UVA irradiation of PBS may not have any bearing on the growth-inhibiting nature of the resulting medium on non-irradiated HaCAT cells. Although these results are preliminary, there is evidence to suggest that transferring medium, UVA-irradiated in the presence or absence of cells, onto non-irradiated HaCAT cells can reduce their proliferation, and therefore, reduce the growth rate of HaCAT colonies.

The fact that the relative clonogenic survival of HaCAT cells treated with UVA-irradiated medium was reduced at earlier times suggests that fewer colonies met the cut-off threshold ($\geq$ 32 cells/colony) at earlier times after medium treatment. With increased

time of incubation, a greater proportion of colonies met the cut-off threshold and were counted and included as part of the surviving fraction. These preliminary results suggest that in order to properly evaluate clonogenic survival, a time-dependent examination of relative survival and relative colony size should be conducted in order to determine whether the treatment results in an inhibition of cell growth and/or true cell killing.

There are several experiments that can be conducted in order to gain insight into the effects induced upon UVA irradiation of cells or PBS. For instance, to address whether certain cell types are more susceptible to the cytotoxic factors produced by UVA irradiation, various repair-deficient and comparable repair-proficient cells can be treated with medium from a UVA-irradiated reporter cell culture or medium containing UVA-irradiated PBS alone. This would also address whether damage is induced upon incubating various repair-deficient and proficient cells treated with irradiated medium (i.e. cellular response to UVA-induced signal) and whether reduced growth of non-irradiated cells/colonies is the result of ongoing repair taking place. In addition, various repair-deficient and comparable repair-proficient cells can be UVA-irradiated and the resulting medium transferred onto a non-irradiated reporter cell line. This experiment may not identify the UVA-induced cytotoxic factor(s) generated following UVA-irradiation, but it may provide insight on the nature of the cytotoxic factor(s) (i.e. cytotoxic nature of the UVA-induced signal). A previous study by Mothersill and colleagues reported that DNA repair-deficient cell lines with a wide variety of different repair defects produced an irradiated cell conditioned medium that was more cytotoxic than their comparable repair-proficient cells when transferred onto non-irradiated HPV-G

reporter cells (Mothersill *et al.*, 2004b). Therefore, these experiments would address whether signal production or cellular response to the signal determines the type (or pattern) of response observed.

It should be noted that attempts were made to examine whether treating the medium (containing UVA-irradiated PBS) with bovine liver catalase could reduce the H_2O_2 levels generated as a result of UVA irradiation, in order to determine whether the UVA-induced effect (i.e. reduced clonogenic survival and reduced colony size) on HaCAT cells was due to H_2O_2. However, these experiments were conducted without the addition of ddH_2O immediately following UVA treatment, and therefore did not provide reliable evidence since the salinity of the medium was not controlled. In order to properly repeat these experiments, ddH_2O should be added immediately following UVA irradiation. In addition, we examined if the cytotoxic signal induced by UVA irradiation was generated during or after irradiation of cells or PBS alone. This experiment was conducted by replacing the PBS on UVA-irradiated cells/wells with fresh PBS immediately after UVA treatment, and subsequently transferring the medium on non-irradiated HaCAT cells 24 hr post-incubation. However, these results are not presented in this thesis since ddH_2O was not added to restore the PBS concentration of the irradiated control(s).

Finally, we examined the colony survival of various human cells following exposure to UVC, UVA and H_2O_2 treatment. Immortalized human keratinocytes (HaCAT) demonstrated increased resistance to UVC exposure compared to glioma (U373, T98G) and colon carcinoma (HT29) cell lines, while exhibiting increased

sensitivity to UVA radiation and H_2O_2 treatment. This increased sensitivity towards both UVA and H_2O_2 treatment suggested that HaCAT cells have a reduced capacity to repair oxidative DNA damage. In addition, we showed that p53-null keratinocytes (HPV-G) have reduced clonogenic survival compared to p53-mutant keratinocytes (HaCAT) following H_2O_2 treatment, which is consistent with an involvement of p53 in the survival of keratinocytes following H_2O_2 treatment. Additional experiments that examine the capacity of these cell lines to repair oxidative DNA damage may provide further insight into the involvement of p53.

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