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Invited Review

Detection of the Excised, Damage-containing Oligonucleotide Products of Nucleotide Excision Repair in Human Cells†

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ABSTRACT

The human nucleotide excision repair system targets a wide variety of DNA adducts for removal from DNA, including photoproducts induced by UV wavelengths of sunlight. A key feature of nucleotide excision repair is its dual incision mechanism, which results in generation of a small, damage-containing oligonucleotide approximately 24 to 32 nt in length. Detection of these excised oligonucleotides using cell-free extracts and purified proteins with defined DNA substrates has provided a robust biochemical assay for excision repair activity in vitro. However, the relevance of a number of in vitro findings to excision repair in living cells in vivo has remained unresolved. Over the past few years, novel methods for detecting and isolating the excised oligonucleotide products of repair in vivo have therefore been developed. Here we provide a basic outline of a sensitive and versatile in vivo excision assay and discuss how the assay both confirms previous in vitro findings and offers a number of advantages over existing cell-based DNA repair assays. Thus, the in vivo excision assay offers a powerful tool for readily monitoring the repair of DNA lesions induced by a large number of environmental carcinogens and anticancer compounds.

INTRODUCTION

DNA is constantly under assault by endogenous and exogenous agents, and therefore the maintenance of genomic integrity is essential for the survival and function of living cells. A major environmental source of DNA damage is ultraviolet (UV) light from the sun, which induces the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts ((6-4)PPs) between adjacent pyrimidine bases in DNA. The formation of these UV photoproducts causes structural distortions of the DNA helix that can interfere with normal cellular processes such as DNA replication and transcription. In humans, the only known repair mechanism for removing UV-induced photoproducts from DNA is nucleotide excision repair (1–3). Defects in excision repair cause the photosensitivity syndrome xeroderma pigmentosum (XP), which is a rare autosomal recessive genetic disorder characterized by a very high incidence of sunlight-induced skin cancer (4–6). Because of the development of assays for monitoring nucleotide excision repair, researchers have made remarkable progress in understanding the molecular mechanism and biological role of the nucleotide excision repair system in humans.

MECHANISM OF HUMAN NUCLEOTIDE EXCISION REPAIR

The basic mechanism of excision repair involves three basic steps: (1) damage recognition and the assembly of repair factors at the damage site; (2) a dual incision event at sites bracketing the lesion to release the damage in the form of a small DNA oligonucleotide; and (3) repair synthesis and ligation within the resulting gap (Fig. 1) (1,2,4). In humans, six repair factors (TFIIH, RPA, XPA, XPC, XPG and XPF-ERCC1) composed of 15 polypeptides are sufficient for the damage recognition and subsequent dual incision events of the general excision pathway that operates throughout the genome (Table 1) (7–9). However, in the absence of XPC, damage can still be removed from the genome through a subpathway of excision repair known as transcription-coupled repair. Unlike in the general or global genome repair pathway that utilizes the damage recognition factors RPA, XPA and XPC-TFIIH to assemble at the damage site in a cooperative manner, the transcription-coupled repair pathway involves RNA polymerase stalling at the lesion and the subsequent recruitment of the CSA and CSB (Cockayne syndrome A and B) proteins. Because of the lack of in vitro systems for reconstituting transcription-coupled repair, less is known about its molecular mechanism in comparison with the general repair system. Nonetheless, following damage recognition, the remaining steps of excision repair are thought to be identical in both repair pathways. The XPB and XPD helicase subunits of TFIIH unwind the DNA to open the helix locally at the damage site and form a stable intermediate termed preincision complex 1 (PIC1). XPG is then recruited into the complex and XPC leaves

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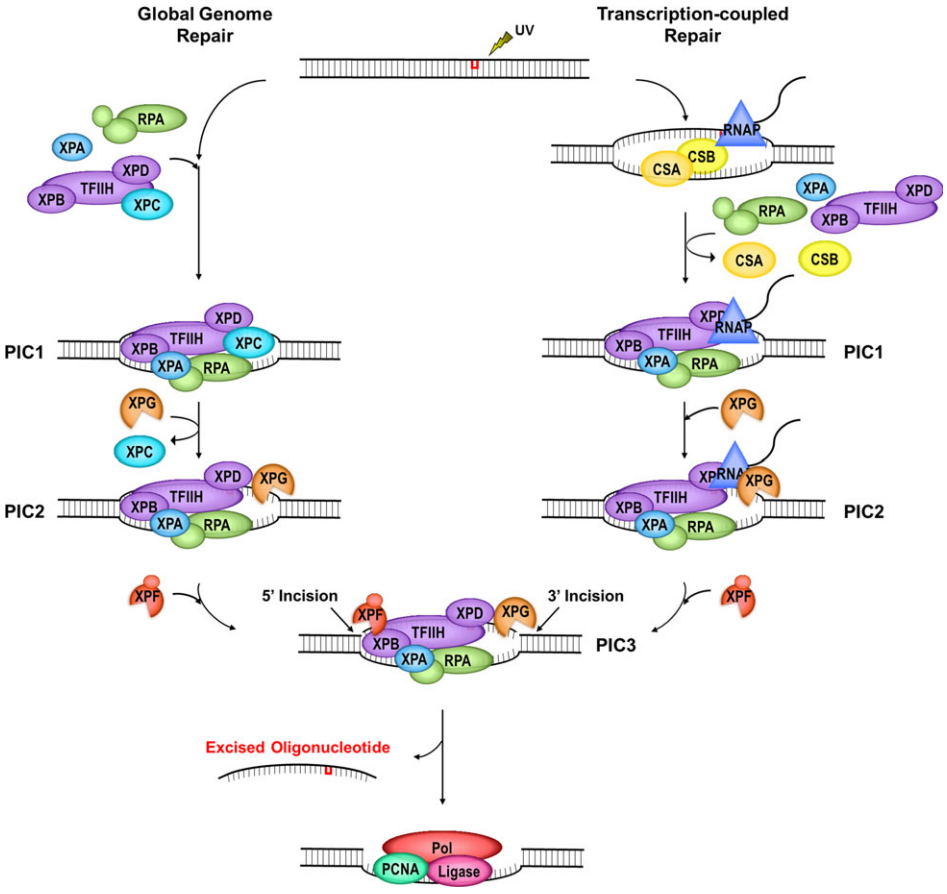

Figure 1. Model for the nucleotide excision repair pathway in human cells. The DNA lesion is recognized by the cooperative action of the damage recognition factors RPA, XPA and XPC-TFIIH, which leads to the formation of preincision complex 1 (PIC1) at the damage site. In the transcription-coupled repair pathway, RNA polymerase stalls upon encountering DNA damage and recruits the CSA and CSB proteins to the lesion site. Then, other repair factors (RPA, XPA and TFIIH) assemble to form the preincision complex. The XPB and XPD subunits in TFIIH act as helicases that unwind the damaged DNA. XPG then replaces XPC and stabilizes the other factors in PIC2. The subsequent recruitment of XPF-ERCC1 forms PIC3 and initiates the dual incision events on the damaged strand of DNA, which occur 5′ and 3′ to the lesion by XPF-ERCC1 and XPG, respectively. The damage-containing oligonucleotide then dissociates from the gap, which is subsequently filled in by the replication machinery using the nondamaged strand as a template and then ligated. Only recently, methods have been developed to isolate and detect the excised oligonucleotide products of excision repair in UV-irradiated cells.

the damaged DNA, resulting in the formation of preincision complex 2 (PIC2). Finally, XPF-ERCC1 is recruited to the complex, which leads to the formation of preincision complex 3 (PIC3). XPG makes an incision 3′ to the lesion and XPF-ERCC1 mediates the 5′ incision, which results in release of the lesion from DNA in the form of a small oligonucleotide. Nearly 25 years ago, in vitro biochemical studies using human cell-free extract and a UV-damaged DNA substrate first demonstrated that the excision process involves incisions at the 20th (±5) phosphodiester bond 5′ and the sixth (±3) phosphodiester bond 3′ to the DNA lesion, which release an oligonucleotide 24–32 nucleotides in length that contains the DNA lesion (10–12). The resulting gap is filled during repair synthesis by polymerase δ/ε and replication proteins (RPA, RFC and PCNA), and the remaining nicks are sealed by DNA ligase (13,14). Comprehensive, detailed overviews of the molecular mechanism of nucleotide excision repair have been presented elsewhere (1–4). In this review, we therefore focus on assays for detecting the excised oligonucleotide products of repair, which provide a convenient way to monitor the biochemical activity of the nucleotide excision repair machinery.

IN VITRO SYSTEMS FOR DETECTING EXCISED OLIGONUCLEOTIDE REPAIR PRODUCTS

Numerous studies both in vitro and in vivo have provided us with our current understanding of the mechanism of nucleotide excision repair. Indeed, the entire concept of excision repair was based on seminal studies by Howard-Flanders and Setlow in which radiolabeled thymidine incorporated into genomic DNA of *E. coli* cells was found to be released (excised) from DNA into the acid-soluble fraction of cells following UV irradiation (15,16). However, it took nearly 20 years to identify the core genes and purify the proteins (UvrA, UvrB and UvrC) responsible for this phenomenon and to ultimately reconstitute the bacterial nucleotide excision repair system in vitro (17). This accomplishment demonstrated that UV photoproducts and other related bulky adducts are removed from DNA by a dual incision mechanism in the form of a 12 to 13 nucleotide-long oligonucleotide.

It took an even longer period of time for the human nucleotide excision repair system to be characterized, and this process

Table 1. The six core factors of human nucleotide excision repair and their functions.

Factor	Subunit	Function
XPA	XPA/p31	Damage recognition, binding to a damaged strand, facilitates repair complex assembly
RPA	p70	Damage recognition, binding to single-stranded DNA
	p32	Binding to single-stranded DNA
	p11	Binding to single-stranded DNA
XPC	XPC/p106	Damage recognition, DNA binding
	HR23B/p58	Molecular matchmaker binds to XPC and stimulates activity
TFIIH	XPB/ ERCC3/p89	3′-5′ DNA helicase, unwinding the duplex
	XPD/ ERCC2/p80	5′-3′ DNA helicase, kinetic proofreading
	p62	TFIIH subunit stimulates XPB
	p52	TFIIH subunit
	p44	TFIIH subunit stimulates XPD
	p34	DNA binding
XPG	XPG/ ERCC5/ p135	DNA endonuclease for 3′-incision
XPF-ERCC1	XPF/ERCC4/ p112	DNA endonuclease for 5′-incision
	ERCC1/p33	Structure-specific endonuclease

involved the development of a variety of methods over the past few decades. In particular, the identification of excision repair genes and the establishment of in vitro excision repair systems using cell-free extracts and later purified repair factors led to characterization of the individual repair proteins and ultimately to a detailed elucidation of the molecular mechanism of nucleotide excision repair (7,8,18). The notion that UV photoproducts are removed from DNA in humans in the form of a small DNA oligonucleotide as in bacteria was first demonstrated using in vitro assays with human cell-free extracts and a plasmid substrate carrying thymine dimers near a radioisotope label. The results revealed that a series of DNA fragments consisting of 24 to 32 nucleotide oligomers were released from the DNA substrate (10). Human excision repair was then reconstituted in a highly defined system with the six purified repair factors (RPA, XPA, TFIIH, XPC, XPF-ERCC1 and XPG) that were shown to be sufficient for the excision reaction in vitro (7,8). These in vitro excision repair systems with defined DNA substrates and cell-free extracts or purified proteins have subsequently been applied for measuring DNA repair activity toward a large variety of DNA adducts, including CPDs, (6-4)PPs, benzo[a]pyrene-guanine adducts, acetylaminofluorene-guanine lesions and cisplatin-(GpG) diadducts (19–21). Moreover, the establishment of such a reliable in vitro repair assay has provided considerable insight into the biochemical steps of human excision repair. However, the relevance of these findings with in vitro systems to the mechanism of excision repair in vivo remained unclear. Similarly, the ultimate fate of the excised oligonucleotide products of excision repair had not been widely considered (22).

Recently, this latter issue was examined in greater detail through the use of the classical in vitro excision repair assay that employs an internally radiolabeled substrate containing a UV photoproduct and mammalian cell-free extracts and purified human excision repair factors (23). Interestingly, this work demonstrated that the excised oligonucleotide products of repair were found to be released from DNA in a tight complex with the repair factor TFIIH. The excised oligonucleotides were found to ultimately be slowly released from TFIIH and to associate with RPA, which is the major single-stranded DNA binding protein in eukaryotic cells, or become degraded in cell-free extract. However, the extent to which these in vitro findings regarding the excised oligonucleotide recapitulate what takes place in vivo was not clear owing to a lack of assays for detecting the excised oligonucleotide products of repair in living cells.

Furthermore, in vitro systems using purified repair proteins or cell-free extracts have primarily utilized naked DNA substrates and may therefore not accurately represent the repair of DNA damage that is embedded in nucleosomes and higher-order chromatin in cells in vivo. Previous studies had shown that nucleosomes can have inhibitory effects on various DNA transactions (24–26), and the Sancar laboratory indeed found that a DNA lesion in the nucleosome core is refractory to human excision repair by both the purified human excision proteins and mammalian cell extracts (27). Considering the lack of methodologies for monitoring excision repair and specifically the excised oligonucleotide products of the repair reaction in vivo, many questions remained regarding the mechanism of excision repair within chromatin and the status and fate of excised oligonucleotides in living cells.

DETECTION OF EXCISED OLIGONUCLEOTIDE REPAIR PRODUCTS IN VIVO

To address these issues, methods for detecting and isolating the UV photoproduct-containing oligonucleotide products of excision repair in human cells were recently developed (28,29). The approach is relatively straightforward and overwhelmingly confirms two decades of findings with in vitro approaches, including important observations regarding the length of the excised oligonucleotide (24–32 nt), the locations of the dual incision events relative to the damaged bases (17–22 phosphodiester bonds 5′ and 5–8 phosphodiester bonds 3′ to the lesion) and the release of excised oligonucleotides in a tight complex with TFIIH. To detect the generation of excision repair products containing UV photoproducts in vivo, low molecular weight DNA is extracted from UV-irradiated cells and then purified. The DNA is then subjected to immunoprecipitation with antibodies specific for (6-4)PPs or CPDs. The oligonucleotides containing UV photoproducts are subsequently radiolabeled at the 3′-terminus with terminal transferase and a radionucleotide, electrophoresed on a urea–polyacrylamide sequencing gel and detected via phosphorimaging. Using this approach, small DNA oligomers in the range of ~18–32 nucleotides in length are observed following UV irradiation, and these DNAs comprise both the primary excision products approximately 30 nt in length and the secondary products that have undergone partial nucleolytic degradation.

The in vivo excision assay has also been applied to better understand the postexcision processing of the excised oligonucleotide products of repair, including both their localization in the nucleus and the role that gap filling DNA repair synthesis and ligation play in excision repair (30). Moreover, using oligonucleotide standards, the in vivo excision assay can be utilized in a quantitative manner to study excision repair activity

and kinetics (29). The assay has also provided a mechanism for characterizing the process of nucleotide excision repair in previously unstudied organisms (31) and on novel potential substrates in genomic DNA in vivo (32). Furthermore, taking advantage of the association of excised oligonucleotides with TFIIH and next-generation sequencing, the development of the XR-Seq method has provided detailed maps of nucleotide excision repair events across the genome in UV-irradiated cells (33,34).

DEVELOPMENT OF A NONRADIOISOTOPIC IN VIVO EXCISION ASSAY

Although DNA lesion and TFIIH immunoprecipitation along with radioisotopic DNA labeling allow for the first detection of the excised oligonucleotide repair products of excision repair in UV-irradiated cells in vivo, the method involves several time-consuming steps and additional safety matters associated with the use of radioisotopes. We have therefore established a novel, nonradioisotopic approach that improves the applicability of the in vivo excision assay for studying UV photoproduct repair in living cells (29). The experimental procedure, which is outlined in Fig. 2, is similar to the radioisotopic approach in many regards, but instead utilizes biotin labeling of the excised oligonucleotides and detection with horseradish peroxide-conjugated streptavidin and chemiluminescence. Furthermore, this novel methodology allows for the detection of the excised oligonucleotide products of repair in human cells without the need for immunoprecipitation with antibodies specific for repair

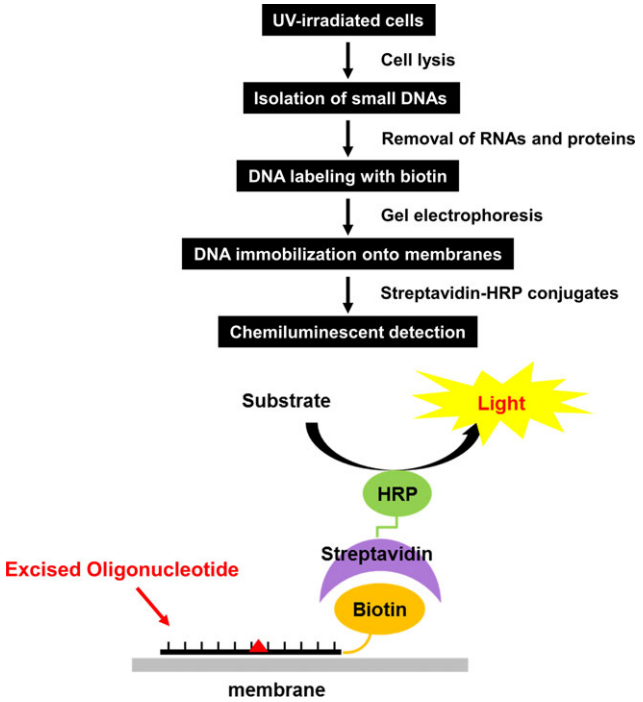

Figure 2. Schematic of the nonradioisotopic, chemiluminescent method for detecting the excised oligonucleotide products of nucleotide excision repair in human cells. Following lysis of UV-irradiated cells, small DNAs are purified, labeled at the 3′ end with a biotinylated nucleotide, electrophoresed on a sequencing gel, immobilized on a nylon membrane and then visualized with streptavidin-conjugated HRP and a chemiluminescent substrate.

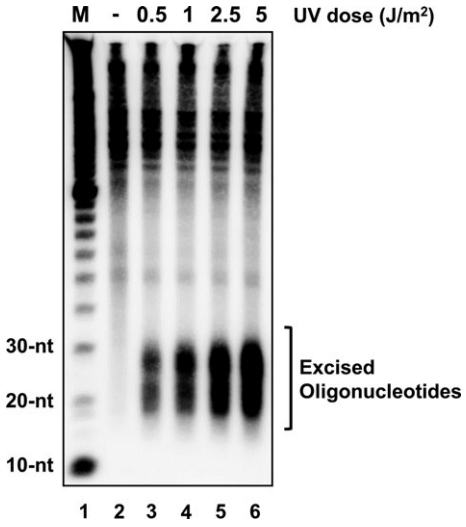

Figure 3. Detection of excised DNA oligomer products of nucleotide excision repair in human cells in vivo. HeLa cells were exposed to the indicated fluences of UVC and then harvested 30 min later. Following cell lysis, the excised DNA oligonucleotide products of excision repair were visualized as outlined in Fig. 2.

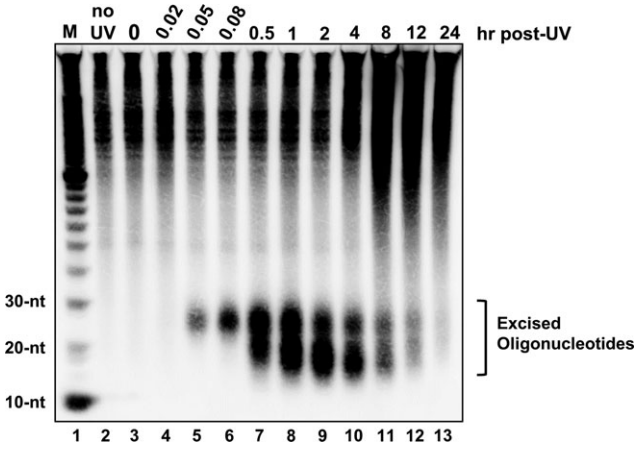

Figure 4. Time-course analysis of excised DNA oligonucleotide generation in UV-irradiated cells of excision repair. HeLa cells were exposed to 20 J m^{-2} of UVC and harvested at the indicated time points. Excised DNA oligomers containing UV photoproducts were extracted and processed as described in Fig. 2.

factors or photoproducts that were used in previous studies in vitro and in vivo described above.

Following UV irradiation, cells can be lysed using the Hirt method (35) that is widely used for extraction of low molecular weight DNAs from mammalian cells. However, we found that other cell lysis buffers containing nonionic or ionic detergents can also be used for this approach, indicating that the excised oligomer products of excision repair are highly soluble and readily extractable from cells. For the nonradioisotopic DNA labeling, we use terminal transferase and the nucleotide analog biotin-11-dUTP to label the 3′ end of the excised oligonucleotides (36). The labeled DNAs are then subjected to denaturing urea–polyacrylamide gel electrophoresis and transfer to a nylon membrane. Finally, the DNAs are visualized by HRP-conjugated streptavidin. This methodology readily visualizes DNA oligomers in the range of ~20–30 nt in length. Furthermore, the signals are

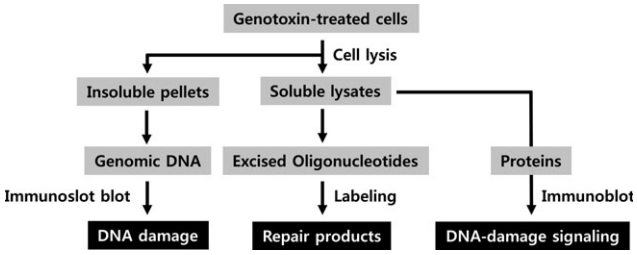

Figure 5. An integrated approach for monitoring the repair of genomic DNA damage, the generation of excised oligonucleotide repair products and the activation of DNA damage signaling pathways in a single culture of cells. Following the exposure of cells to DNA-damaging agents, cells are lysed with a nonionic detergent and centrifuged to generate soluble and insoluble fractions. Genomic DNA is purified from the insoluble pellet to measure the presence (and loss) of lesions using an immunoslot blot assay. The soluble cell lysate is processed for extraction and visualization of the oligonucleotide products of excision repair or used in immunoblot assays for monitoring DNA damage response signaling and apoptosis. Adapted from Choi *et al.* (37).

completely dependent on UV irradiation of cells and on an intact nucleotide excision repair system (29). Importantly, our methodology is very sensitive and is thus capable of detecting excised DNA oligomers at very low fluences of UVC. As shown in Fig. 3, excised oligonucleotides can be readily observed even at a low fluence of 0.5 J m^{-2}. These results therefore demonstrate the extremely high sensitivity of the methodology for detecting DNA excision repair events that are induced by nonlethal fluences of UV. Time-course experiments further show that the excised oligomers are readily detectable within minutes after UV irradiation (Fig. 4), which is not possible with conventional assays such as immunoslot blot and fluorescence microscopy. Thus, to our knowledge, there is no existing repair assay that is able to detect excision repair events so soon after damage formation in human cells in vivo. A limitation of the in vivo excision assay is that the excised oligomers are ultimately degraded in living cells, and thus, a significant reduction in signal is inevitable as repair is completed following UV irradiation. Therefore, additional quantitative measurements of nucleotide excision repair, such as immunoslot blotting with damage-specific antibodies, may continue to be useful in certain experimental settings for detecting a low rate of repair over long periods of time. Compared to the conventional radiolabeling method, the new biotinylation approach requires additional procedures to obtain signals, including DNA transfer onto a membrane and subsequent steps for chemiluminescent detection. Nevertheless, the novel nonradioisotopic method is still a powerful approach for detecting UV photoproduct-containing oligomers in UV-irradiated human cells.

Although this methodology was first applied for use with UV-irradiated cells, we have recently shown that the in vivo excision repair assay is capable of detecting nucleotide excision repair of a broad range of DNA lesions that are substrates for the nucleotide excision machinery (37). We have successfully used this assay to monitor the generation of excised oligomers in cells treated with a wide variety of environmental and occupational carcinogens and anticancer drugs, including benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), *N*-acetoxy-2-acetylaminofluorene (AAF), formaldehyde, mitomycin C (MMC) and cisplatin. These results demonstrate that our in vivo excision repair assay is capable of detecting nucleotide excision repair of a wide spectrum of DNA lesions and thus may be useful to a variety of researchers in many fields of cancer biology.

AN INTEGRATED APPROACH FOR MEASURING REPAIR, CHECKPOINT AND APOPTOSIS

In addition to DNA repair, DNA damage also induces other cellular responses in human cells, including DNA damage checkpoints and apoptosis (4). Because these DNA damage responses are closely coordinated with one another, there are advantages to be able to analyze all three cellular responses simultaneously in the same population of cells. In this regard, we recently reported a simplified, integrated approach that is capable of monitoring the full set of biochemical responses to DNA damage, including nucleotide excision repair, DNA damage checkpoints and apoptosis (37). Using a mild cell lysis method, we have simultaneously analyzed the repair of genomic DNA damage, the generation of excision repair products and the activation of cell cycle checkpoints and apoptotic signaling in a single culture of cells. A schematic of the integrated approach is presented in Fig. 5. Following UV irradiation, cells are lysed and centrifuged to separate soluble fractions and insoluble pellets. The insoluble pellet is used for extraction of genomic DNA that is analyzed for the presence of DNA lesions by immunoslot blot assay with antibodies specific for photoproducts. The soluble cell lysates are used for extraction of excised oligomers that are generated by nucleotide excision repair. The remaining lysates are used in immunoblot assay to monitor DNA damage checkpoints and apoptosis. This approach therefore allows simultaneous evaluation of three major cellular responses to DNA damage that include DNA repair, checkpoint and apoptosis using a single preparation of cells.

CONCLUSIONS AND PERSPECTIVES

The study of DNA damage and associated repair requires highly sensitive methods for detecting DNA repair events. The high sensitivity and versatility of the in vivo excision assay therefore offer researchers interested in a wide variety of DNA-damaging compounds a novel way to quantitatively measure repair with low, nontoxic doses of compounds and within short time frames of damage formation. Thus, we believe that the in vivo excision assay has the potential to become a routine approach in studies examining cellular responses to DNA damage that is targeted for repair by the nucleotide excision repair system in human cells.

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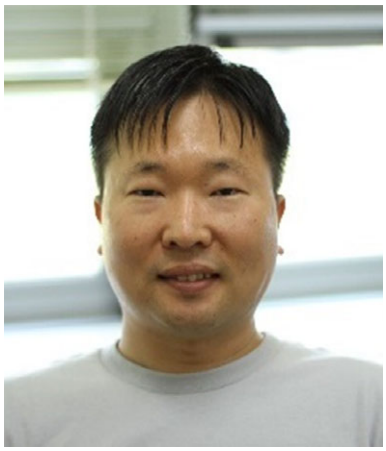

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