Cell cycle arrest and apoptosis provoked by UV radiation-induced DNA damage are transcriptionally highly divergent responses

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ABSTRACT

DNA damage caused by UV radiation initiates cellular recovery mechanisms, which involve activation of DNA damage response pathways, cell cycle arrest and apoptosis. To assess cellular transcriptional responses to UVC-induced DNA damage we compared time course responses of human skin fibroblasts to low and high doses of UVC radiation known to induce a transient cellular replicative arrest or apoptosis, respectively. UVC radiation elicited >3-fold changes in 460 out of 12,000 transcripts and 89% of these represented downregulated transcripts. Only 5% of the regulated genes were common to both low and high doses of radiation. Cells inflicted with a low dose of UVC exhibited transcription profiles demonstrating transient regulation followed by recovery, whereas the responses were persistent after the high dose. A detailed clustering analysis and functional classification of the targets implied regulation of biologically divergent responses and suggested involvement of transcriptional and translational machinery, inflammatory, anti-proliferative and anti-angiogenic responses. The data support the notion that UVC radiation induces prominent, dose-dependent downregulation of transcription. However, the data strongly suggest that transcriptional repression is also target gene selective. Furthermore, the results demonstrate that dose-dependent induction of cell cycle arrest and apoptosis by UVC radiation are transcriptionally highly distinct responses.

INTRODUCTION

Ultraviolet (UV) radiation of sunlight is the most prominent physical carcinogen in our natural environment and has an established role in skin carcinogenesis (1,2). Whereas longer UV wavelengths (UVB, UVA) also induce oxidative stress and protein denaturation, short wavelength UV radiation (UVC) causes predominantly DNA damage to cells in the form of pyrimidine dimers and 6-4 photoproducts (3,4). These bulky DNA lesions halt RNA polymerase II elongation and are repaired enzymatically by two types of nucleotide excision repair (NER): transcriptionally active genes are repaired by transcription coupled repair, whereas global genomic repair restores the rest of the genome (5). Due to incomplete repair, damaged bases may be misinterpreted during replication, resulting in accumulation of cancer predisposing mutations (3). Several DNA damage-induced signaling cascades, including ATR/Chk1, jun N-terminal kinase and p38 kinase pathways are activated following UVC radiation, leading to activation of NER and recovery mechanisms via transcription factors such as p53, NF-kB and AP-1 (6,7). These events lead to a complex transcriptional response of the cells resulting in regulation of DNA damage repair, cell cycle progression and apoptosis. However, the molecular mechanisms underlying the cellular UV responses remain to be elucidated in detail.

We have earlier reported the dose-dependent, highly distinct cellular responses induced by UVB and UVC radiation in diploid human skin fibroblasts (8). Following a low dose of UV radiation, DNA replication is inhibited and the cells undergo a transient arrest re-entering the cell cycle by 24 h. Instead, a high dose of UV damage leads to an initial replicative arrest followed by death of the cells by apoptosis. In this study we set out to determine differences in gene expression between responses to low and high doses of DNA damage induced by UV radiation, and thus chose to use UVC as a source to avoid possible changes due to, for example, protein denaturation and lipid peroxidation caused by the longer wavelengths. By applying oligonucleotide microarray technology we quantitatively screened the responses of over 12,000 transcripts detailing the biological pathways affected by UVC. We show here that transient cell cycle arrest and apoptosis elicited by UVC-induced DNA damage are transcriptionally highly distinct responses.

MATERIALS AND METHODS

Cell culture and irradiation

WS1 human skin fibroblasts (CRL-1502, ATCC) were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and...
non-essential amino acids at >37°C in a humidified atmosphere containing 5% CO₂. All cell culture reagents were provided by Gibco-BRL (Rockville, MD). UVC-treatment of cells was carried out essentially as described previously (9). Exponentially growing cultures were exposed to UVC (254 nm) at a dose of 10 (low) or 50 (high) J/m² with a Stratalinker 2400 UV crosslinker (Stratagene, La Jolla, CA) or were mock-treated, and incubated for the indicated periods of time. Two independent series of UVC-treated fibroblasts were entered for further analysis.

**RNA extraction and cRNA preparation**

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol and assessed for quality with the Bioanalyzer 2100 instrument and RNA 6000 Lab chip kit (Agilent Technologies, Palo Alto, CA). Double-stranded cDNA synthesis was carried out using the SuperScript Choice system (Life Technologies Inc., Rockville, MD), essentially following the manufacturer’s recommendations. Reactions were scaled to accommodate for a starting amount of 8 μg of total RNA. HPLC-purified T7-(dT)24 primer was purchased from Sigma Genosys Ltd (Pampisford, UK). Immediately after completion of the synthesis cDNA was purified by standard phenol/chloroform/isoamyl alcohol (25:24:1) extraction and precipitated in 1 M NH₄OAc and 60% EtOH in the presence of 0.05 g/l glycogen (AMBION, Austin, TX). cDNA product quality was assessed using a Bioanalyzer 2100 instrument as above.

Biotin-labeled cRNA was prepared using the BioArray HighYield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY) which takes advantage of T7 RNA polymerase and biotinylated nucleotides to produce single stranded labeled cRNA. The procedure was set up essentially according to the manufacturer’s suggestion. The reaction was scaled up to a total of 50 μl and the starting amount of cDNA was doubled. In order to further increase the yield the incubation time was extended to 6 h. To avoid background hybridization of unincorporated nucleotides, samples were subjected to a cleanup procedure using RNeasy spin columns (Qiagen).

In order to minimise the occurrence of secondary structures in the RNA that could impair the specificity and overall performance of the hybridization to the probe arrays, cRNA samples were fragmented to obtain a transcript size distribution of 50 to 200 bases by incubating 20 μg of cRNA in 40 mM Tris–OAc (pH 8.1), 125 mM KOAc and 30 mM MgOAc at 94°C for 35 min. Size distribution and overall quality of transcribed products and their fragmented counterparts were assessed using the Bioanalyzer 2100 instrument as above.

**Array hybridization and scanning**

Prior to running the full scale probe arrays cRNA preparation as well as hybridizing conditions and reagents were validated by hybridizing 5 μg of cRNA from selected samples, together with spiked non-eukaryotic controls, to Test3 control arrays (Affymetrix, Santa Clara, CA) following the protocol outlined in the Gene Expression Analysis Technical Manual (Affymetrix). More details on the controls present on the Test3 control array as well as other details regarding array design of Affymetrix GeneChip probe arrays are available at http://www.Affymetrix.com. Quality assessment involved analysis of the noise and background levels, 3' to 5' signal ratios for housekeeping controls, presence and relative magnitude of signals from the spiked controls and absence of signal for the cross-hybridization controls. In the full-scale hybridizations, 15 μg of fragmented cRNA and a selection of non-eukaryotic spiked controls were hybridized to Affymetrix HGU95Av2 GeneChip probe arrays. For both test and full scale probe arrays the hybridizations were performed in an Affymetrix GeneChip Hybridization Oven 640 under constant rotation at 60 r.p.m. for 16 h at 45°C. Washing and staining procedures were carried with Affymetrix GeneChip Fluidics Station 400 and the protocol for antibody amplification was selected. After completion, the probe arrays were immediately scanned using a GeneArray G2500A scanner (Agilent Technologies). Pixel size was set to 3 μm, filter wavelength to 570 nm and scan number was set to 2. Other parameters were set up according to the defaults specified by Affymetrix for the HGU95Av2 probe arrays.

**Data analysis**

MIAME standards (10) were followed in the data analysis and storage. The raw data from all analyses performed are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) by accession numbers GSE449 and GSE448 for low dose and high dose data series, respectively. Scanned images were analyzed with Affymetrix Microarray Suite 5 software employing the Statistical Expression Algorithm implemented in the Absolute Expression Analysis. All analysis parameters were set to the default values recommended by Affymetrix. Global scaling to a target intensity of 100 was applied to all arrays but no normalizations were performed at this point. Probe arrays were verified for absence of anomalies in the data registered for the spiked controls probes, the cross-species hybridization control probes and the housekeeping probe controls. Output files of result metrics were further processed using GeneSpring 4.2.1 data analysis software (Silicon Genetics, Redwood City, CA). To increase stringency, only genes for which the signal intensity was reliably detected, i.e. scored as ‘present’ by the Statistical Expression Algorithm, in at least two of the samples within a UV dose series, were included for further analysis.

UVC-treated samples were normalized by calculating ratios between the sample signal intensity and the corresponding non-irradiated control. In order to avoid loss of data for genes with low measurement levels in the controls, but significantly upregulated levels in the irradiated samples, or vice versa, a cut-off value was introduced. For each probe array signal intensities were log-transformed and the average value for the ‘absent’ calls was determined. An average of all the thus obtained averages was then calculated as well as the corresponding standard deviation. The cut-off value was defined as the total average plus two standard deviations, resulting in a value of 15.8. Signal intensities below this were thus adjusted to 15.8 thereby preventing genes from being artificially interpreted as up- or downregulated. Ratios were calculated as follows: signal intensities from replicate samples and controls were first averaged, then the averages of samples were divided by the average of the corresponding control sample (6 h control for 6 and 12 h UVC-treated samples; 24 h control for 18 and 24 h UVC-treated samples). Genes for which the calculated ratio exceeded 3:1 or was below or equal
to 1:3 in at least one time point were considered to be significantly up- or downregulated, respectively. These cut-off ratios were established by analyzing the experimental variation in samples and controls (Results) and were set as to attain a low enough proportion of false positives while keeping a reasonable level of false negatives.

As the HG95Av2 probe array includes multiple occurrences of probe sets recognizing a certain transcript, occasional multiple entries were recorded in the result tables. To avoid confusion, however, only one entry per transcript was included in the final list. When selecting the most representative entry several criteria were taken into consideration including signal reliability, i.e. the proportion of samples with ‘present’ calls, and the degree of reproducibility, i.e. the ratio between the standard deviation and the average of the signal intensities from the duplicate experiments.

Temporal profiles of expression for genes identified as significantly up- or downregulated, were classified into groups exhibiting distinct patterns of profiles using the K-means clustering algorithm implemented in the GeneSpring software. To focus pattern identification on the ‘shape’ of the expression profiles rather than the ‘amplitude’ at single time points, Pearson’s correlation was selected as distance measure. This correlation method assumes data centering around the value of one so there is no bias towards upregulated transcripts, as with other types of correlations. The algorithm was set to terminate after a maximum of 200 iterations. Clustering was performed for numbers of clusters ranging from 3 to 25 and for each number selected clustering was repeated a total of 50 times, each starting from a different random classification. The ‘optimal’ clustering result was determined by identifying the classification that maximized intra-cluster similarity but at the same time maximized the inter-cluster diversity. In addition, the best clustering also had to provide a good representation of the overall temporal profiles as demonstrated by a high value of explained variability. Clustering results were further validated by principal component analysis.

Functional classification of the genes was based on gene ontology categories established by the Gene Ontology Consortium (http://www.geneontology.org) and/or according to information obtained from the OMIM (http://www.ncbi.nlm.nih.gov) and SwissProt (http://www.expasy.org/sprot) databases. Functional groups were put together to represent main cell biological processes. Because of the broadness of possible biological functions some simplifications were made, e.g. signaling group includes both intracellular and intercellular signaling, and transcription includes both transcriptional and transrepression. For genes qualifying for several classes the most descriptive ones were selected.

**Northern analysis**

Either total RNA was extracted as above or mRNA was extracted with GenElute Direct mRNA Miniprep Kit (Sigma). RNA was separated in 1% agarose gels containing formaldehyde and transferred to nylon membrane (Hybond-N+, Amersham) using 20× standard sodium citrate (SSC). mRNA was detected by probing with the indicated cDNA insert labeled with [α-32P]dCTP by random priming (Ready- To-Go, Pharmacia) using ExpressHyb solution (Clontech, Palo Alto, CA). Sequences of the probes used are available upon request. Autoradiograms were quantitated by Fujiﬁlm BAS-2500 Image Analyzer and MacBAS 2.5 program. RNA levels were normalized to the level of GAPDH, and fold inductions were calculated by comparing signals of treated samples to the untreated control cells.

**RESULTS**

We have earlier shown that a low dose of UV radiation induces a transient p53 response and replicative cell cycle arrest in human skin fibroblasts (8). A high dose of UV radiation, on the other hand, initiates apoptosis and results in death of the cells within 72 h (8). Here we examined in genomic scale the differences in the transcriptional responses of UVC radiation-induced transient and fatal cellular events. For this, exponentially growing diploid human skin fibroblast cultures were irradiated with low (10 J/m²) or high (50 J/m²) doses of UVC and were incubated for 6, 12, 18 or 24 h. Control samples were mock-treated and were incubated for either 6 or 24 h. Total RNA was extracted from the cells and was further processed and used in microarray hybridization as described in Materials and Methods.

**Analysis of the experimental variability**

To assess the extent of variation accumulated during sample preparation, hybridization and scanning procedures, raw intensity values for replicates of the same sample were plotted against each other on a log scatter plot (Fig. 1A). No transcripts exhibited signal intensities differing by >4-fold, and only a few by >3-fold between the replicates. A considerably higher number of transcripts exhibited >2-fold differences. To illustrate the added contribution of biological noise to overall variation, similar plots were created for mock-treated controls from which RNA was isolated at 6 and 24 h timepoints (Fig. 1B). A very slight overall increase in variation was observed but this increase was, however, mainly limited to differences <2-fold, thus not significantly affecting the proportion of transcripts with higher folds of variation. Log scatter plots representing variation due to UVC treatment were compared (Fig. 1C). The highly augmented spreading of the data, dramatically increasing the proportion of differences exceeding 3-fold, clearly demonstrates the validity of the experimental set-up. Based on these observations, using a cut-off of 3-fold resulted in a false positive proportion of <5%.

**Analysis of transcriptional responses by UVC radiation induced cell cycle arrest and apoptosis**

To identify gene transcripts regulated by UVC radiation, ratios were calculated between the averaged intensity values of the replicates of the irradiated samples and the averaged values of the control samples. Each dose series (low, high) was examined for genes up- or downregulated by >3-fold at any time point. Only genes reliably detected in the array, i.e. scored as ‘present’ in at least two of the samples within a dose series (5618 genes for low and 5109 genes for high dose series), were included in further analyses. Identification of false positives due to a very low signal intensity in either samples or controls, causing erroneously small or large ratios, were prevented by the introduction of a cut-off value (Materials and Methods) for which the low values were substituted. Based on these criteria, 460 genes were identified as regulated by UVC radiation, which is ≈8% of transcripts.
detected as present. Of the regulated genes, 49 were induced, 409 repressed and two were either induced or repressed depending on the dose of UV radiation used (Fig. 2). Only five induced and 18 repressed transcripts were common to both low and high doses of radiation, which indicated highly unique sets of transcriptional targets.

To further compare the transcriptional responses to low and high doses of UVC radiation, we created average profiles of genes that underwent >3-fold changes. Induction of transcripts by the low dose was rapid and transient peaking at 12 h (Fig. 3A) and the mRNA levels were normalized by 24 h. In contrast, following irradiation with the high dose the induction of targets was slower and more sustained, peaking at 18 h. The downregulated targets (Fig. 3B) showed similar fast and transient kinetics after the low dose as the upregulated ones. Most prominent suppression was observed at 12 h followed by normalization of the mRNA levels by 24 h. On the contrary, after the high dose, downregulation of the responsive genes was slower and persistent (Fig. 3B). We further analyzed how regulation of the identified transcripts differed between the low and high doses of UVC radiation. Transcripts identified as induced or repressed by the low dose were not significantly regulated by the high dose and vice versa (Fig. 4). This further strengthens the notion that highly distinct sets of genetic responses are invoked by the high and low doses of DNA damage.

**Clustering of the transcripts regulated by UVC radiation**

Gene transcripts exhibiting similar expression patterns were grouped using the K-means clustering method. The algorithm used is designed to maximize inter-cluster similarity while retaining maximal intra-cluster diversity. The clusterings were performed separately for transcripts induced and repressed by low dose (29 and 68, respectively) and for transcripts induced and repressed by high dose (27 and 361, respectively). Clustering results are illustrated in Figure 5.

Four clusters were identified among the genes for which the expression was induced by the low dose of UVC radiation (Fig. 5A). Immediate UV-responsive transcripts form cluster a1 and show rapid (6 h) and transient induction. Cluster a2 includes transcripts with rapid, but more sustained kinetics of induction peaking at 12 h. Intermediate kinetics of induction followed by downregulation are found in cluster a3, whereas cluster a4 represents genes upregulated with late kinetics and shows normalization of the levels by 24 h.

Genes upregulated by the high dose of UVC radiation presented in four clusters (Fig. 5B). In contrast to the low dose clusters, however, clusters with immediate/early kinetics of induction were absent. Cluster b1 represents transcripts with intermediate, transient induction, while cluster b2 contains transcripts with peak induction at 18 h with some decline thereafter. Both clusters b3 and b4 represent slow and sustained inductions but peak at 18 and 24 h, respectively.

Transcripts repressed by the low dose of UVC radiation fall into five clusters (Fig. 5C). Two clusters showed immediate/early repression (clusters c1 and c2) differing in the kinetics of recovery of the targets. Genes with intermediate kinetics of repression are represented in cluster c3. Cluster c4 contains late repressed transcripts recovered by 24 h, and cluster c5 the remaining transcripts with very late repression.

Genes downregulated by the high dose of UVC radiation grouped into nine clusters (Fig. 5D). Clusters d1 and d2 represent transcripts with immediate/early response of repression in which recovery is evident only in d1. Clusters d3 and d4 represent genes with kinetically intermediate repression pattern. Genes with slow repression after the high UVC-damage belong to clusters d5, d6, d7 and d8, differing in their kinetics of repression. Cluster d9 is the only cluster showing very slow and sustained repression with no recovery, even after 24 h.
Functional classification of the UVC-regulated genes

The genes regulated by UVC radiation in our microarray analysis were classified into 28 functional categories. There were no strong correlations between the functional groups and the clusters although some functions were over represented in certain clusters. Fourteen functional groups were present amongst the genes induced by the low UVC-dose and 20% of the genes coded for proteins with unknown function (Table 1). Of the genes with known function, the main groups were genes involved in stress response and regulation of the cell cycle, both comprising 21% of the genes. Several transcripts belong to the signaling, transcription and metabolism groups. No proteolysis or vesicle trafficking genes were regulated by the
Figure 5. K-means clustering of transcripts regulated in response to UVC radiation. Each cluster is represented by a graph showing both the average expression profile (thick line) as well as the individual gene expression profiles (thin lines). Graph headings represent the cluster identifier and the number of transcripts (in parentheses). Transcripts induced by low (A) and high (B) doses of UVC radiation, and transcripts repressed by low (C) and high (D) doses of UVC radiation.
low dose of UVC. Immediate/early UV-responsive genes in cluster a1 include cell cycle regulatory genes TOB1 and cyclin E. Immediate/intermediate cluster a2 includes several cell cycle and stress responsive factors as well, such as cell cycle inhibitor p21 (CDKN1A), serum inducible kinase SNK and BTG2. Genes in this cluster seem to be mostly unaffected by the high dose of UVC radiation and are thus highly specific for the transient UV response. An exception in this group is ISG15, which is also repressed by the high dose. Genes with intermediate induction by the low dose in cluster a3 include heat shock proteins. Genes with late kinetics of induction in cluster a4, NOXA (PMAIP1), IL-11 and spermidine/spermine N1-acetyltransferase (SAT), are not functionally similar but are, interestingly, all also highly upregulated with the high dose of UVC radiation.

Transcripts upregulated by the high dose of UVC radiation are prominent in metabolism, nucleosome, signaling and transcription categories (Table 2), and include some apoptotic and proteolytic genes as well. Only two unknown genes and one stress responsive gene, GADD45, were induced by the high dose. Cluster b1 contains ISG15, which is similarly induced by the low dose. Cluster b2 contains genes characterized as apoptotic, such as NOXA and p8, in addition to structural proteins like alpha cardiac actin (ACTC) and collagen IIIA1. Cluster b3 is composed of transcripts such as Gadd45A, two splice variants of SAT and one histone (H2AFO). Cluster b4, induced late by the high dose, contains mostly genes involved in signaling and transcription as well as two histones (H2BFFH, H2BFK).

The functions, normalized array results and cluster identifiers for genes downregulated by UVC radiation are listed in the Supplementary Material (Tables S1 and S2, available at NAR Online). In brief, genes repressed by the low dose grouped into 18 different functional classes. Several intra- and intercellular signaling and metabolic molecules were downregulated. Interestingly, 22% of the known genes coded for structural proteins involved in cell adhesion and motility. The immediate/early response clusters (c1 and c2) of genes repressed by the low dose include growth factor signaling molecules, e.g. VEGF, IGF1-receptor, transcript for death domain containing protein CRADD, and several unknown genes (48% of cluster c2). Kinetically intermediate cluster c3 represents genes of several functional groups. Interestingly, most structural transcripts repressed by the low dose were in this cluster, such as collagens IVa2 and XIIIa1, ACTC and type 2 cadherin. Clusters of genes repressed late after low UV insult include signaling molecules, e.g. IGFBP5 (cluster c4) and some structural molecules, such as nucleosomal NAP1L1 and cytoskeletal dynein (DNCL2) (cluster c5).

<table>
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<th>Gene symbol</th>
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<th>Function</th>
<th>6 h</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
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<td></td>
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Table 1. Genes induced by 3-fold in response to low dose UVC radiation, cluster identifiers as in Figure 5A.
Table 2. Genes induced by 3-fold in response to high dose UVC radiation, cluster identifiers as in Figure 5B

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<td>COL13A1</td>
<td>collagen, type XIII, d1</td>
<td>M59217</td>
<td>structural/cell adhesion</td>
<td>-1.6 -1.4 3.3 2.4</td>
</tr>
<tr>
<td></td>
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<td>actin, alpha, cardiac muscle</td>
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<td>structural/cytoskeleton</td>
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</table>

All types of gene, except proteolytic genes, were represented amongst those downregulated by the high dose of UVC radiation. A large proportion of the genes were unknown or ESTs (27%). Well-represented groups were genes coding for cytokines, hormones and proteins involved in growth factor signaling, metabolism, transcription and ribosomal/RNA processing. Transcription seems to be significantly affected by the high dose as general transcription factors TFIB and TFIE are downregulated as well as elongation factors EF1E1 and initiation factor EIF4E. Immediate/early repressed genes included signaling and transcription factors such as E2F3, stress responsive phosphatase DUSP1, DNA repair protein TOPBP1 and cell cycle kinase WEE1 in cluster d1, and growth factor signaling transcripts like VEGF, transcriptional regulators, e.g. ID1 and ID2, cell adhesion molecules pisin and thrombospondin 1 as well as protein phosphatases such as DUSP5 in cluster d2. Gene clusters with intermediate repression pattern (clusters d3 and d4) included several structural proteins, e.g. nuclear lamin B1 and collin, cell cycle regulators such as cyclin B1 and CKS2, and transcription modifiers e.g. HMGI C, HMGI2 and ID3. Of the genes repressed by the high dose, >60% involved in either DNA repair (e.g. thymine- and uracil-DNA glycosylases) or chromatin modulation were in cluster d3. In cluster d4 proteasome-related (e.g. ubiquitin-specific proteases 1 and 10) and transport-related (e.g. exportin 1) transcripts were particularly frequent. Genes repressed late in the response (clusters d5 to d8) included serine-threonine protein kinases CDK7 and CDK8, and known UV-responsive factors such as junD and UVRAG. Several genes with sustained repression in cluster d9 were involved in cell cycle and stress response, including cyclin A, cyclin G and Hsp40 homolog DnaJB1.

Verification of microarray results by northern analysis

To confirm the results of the microarray analysis we performed northern analysis from a selection of genes, examples of which are shown in Figure 6. We selected mRNA transcripts of genes undergoing >3-fold regulation (CDKN1A, GADD45, MMP3, VEGFC, CCNB1) as well as non-regulated genes (c-Myc, IL-8) in the microarray. The gene expression profiles obtained with the different methods were concordant for all transcripts except IL-8. Although IL-8 was regulated by >3-fold according to the northern analysis, it was not identified as regulated in the array analysis because it did not fulfill the criteria of scoring present calls in at least two of the samples.

DISCUSSION

Recent developments in DNA-microarray methodology have enabled high throughput analysis of transcriptional responses of cells. We adopted this method to compare changes in transcription induced by different doses of UVC radiation. Recent microarray studies of UVB responses in keratinocytes...
and melanocytes (11–14) did not assess effects of apoptosis-inducing doses of UV damage. Here we compared the microarray profiles of UVC-induced skin fibroblast responses to low and high doses of UV radiation, which induce either a transient replicative arrest or apoptosis, respectively (8). To limit the damaging effects of the radiation mostly to DNA we chose to use UVC in the present study.

We found that the transcriptional response to UVC radiation is highly dependent on the dose of radiation inflicted on the cells. The low dose, which induces replicative cell cycle arrest, results in a transient transcriptional response in which rapid up- or downregulation of the genes is followed by a restitution to basal levels 24 h after the insult. The high dose, on the other hand, which induces apoptosis by 24 h, leads to a slower and persistent effect on transcription. Intriguingly, only 10% of the induced and 4% of the repressed genes in our analysis were common to both low and high doses. Thus low and high doses of UVC-induced DNA damage generated clearly distinct sets of genetic responses.

Fast and transient induction of transcripts by the low dose of UVC radiation may result from fewer damaged sites undergoing repair, enabling faster recovery from transcriptional repression. Still, gene-specific events are probably involved. A high dose of UVC radiation represses significantly more genes and functional groups than the low dose, indicating a more general downregulation of transcription. This is not surprising, as the high dose is sufficient to produce RNA polymerase II-inhibiting DNA bulks to every gene of the genome (15). Over 90% of transcripts detected by the array were, however, not significantly regulated by UVC. In addition, only a minority of transcripts repressed by the low dose were also repressed by the high dose of UV radiation. This argues that responses to both low and high doses are highly specific and do not represent a general shutdown of cellular transcription.

Transcription was profoundly affected by the downregulation of several basal transcription factors (including general, initiation and elongation factors) as a result of the high dose of UV radiation. Several sequence-specific transcription factor families were also downregulated in the apoptotic response to UV radiation. Id-family transcriptional repressors (Id1) (2,3), which negatively regulate cell cycle inhibitory effects by downregulating transcription of cell cycle inhibitors (p16 (Id1) (16) and overriding functions of retinoblastoma family members (Id2) (17,18), are downregulated by the high dose. High mobility group (HMG) proteins, functioning as ‘architectural transcription factors’ thereby promoting transcription by bending DNA without sequence specificity (19), are also downregulated in the apoptotic response. Interestingly, HMG1 protein has been shown to specifically bind UV-damaged DNA (20). Additionally, both subunits of the NF-Y transcriptional regulator were downregulated by the high dose of UV radiation. NF-Y regulates transcription of several damage- and cell cycle-associated genes like topoisomerase II B, hsp70, E2F1, cyclin A and cyclin B1 (21). It is possible that all the above factors are downregulated to prevent the cells from entering the cycle erroneously in the apoptotic response and to block transcription promoting survival. Interestingly, in the apoptotic response cyclin D (D1–3), functioning in G1-phase of the cell cycle, is not downregulated but mitotic cyclins (cyclin A2, cyclin B1) are, indicating inhibition of cell division rather than replication at the transcriptional level.

The transcriptionally-regulated events during the replicative cell cycle arrest induced by the low dose of UV radiation had several unique features. Several cell cycle-associated genes were induced and none was repressed. Some of these have an established role in the UV response, such as p21 WAF1/Cip1 and others are less well recognized, for example TOB1 and BTG2. These factors are bound to have a significant role in the fast and transient proliferation arrest response launched after a tolerable amount of UV-induced DNA damage, and ensure time for damage repair. Remarkably many of the genes upregulated in the transient UV response coded for proteins with unknown functions.

Several stress response factors were upregulated following the low dose of UV radiation. These include two main heat shock proteins, Hsp70 1A and 1B, that work as chaperones in stress involving protein misfolding. Hsp70 increases UVB resistance of cell lines (22) and lack thereof increases UVB-induced injury of murine skin (23). Proteins are chromophores for UV-light equal to and above UVB wavelengths, which create distortedly folded proteins recognized by chaperones. It

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Figure 6. Verification of mRNA expression by northern analysis. Northern analysis of indicated transcripts and quantifications of the signals are shown. GAPDH was used as a loading control. Fold inductions were calculated by comparing signals of 6 and 12 h UV-treated samples to the 6 h control, and signals of 18 and 24 h samples to the 24 h control. CDKN1A, p21; CCNB1, cyclin B1.
is not clear, however, why heat shock response is launched by UVC. Indirect evidence exists from Escherichia coli that chaperones maintain NER proteins in their proper folding, thus participating in the repair of UV-damaged DNA (24). Hsp70 induction may be required for the repair of UV-induced DNA damage also in human fibroblasts.

Sesto et al. (13) found generalized upregulation of basal transcriptional machinery by UVB-radiation. No similar response was detected in our arrays, most likely due to less protein damage and less need for overall protein turnover after UVC insult. However, subunit 4 (p52) of the multicomplex transcription factor TFIH was identified as a novel target induced by low dose of UVC. p52 is directly involved in transcription and NER (25), and therefore transcriptional upregulation of p52 may be necessary for UVC-induced DNA repair. UV radiation induces transcription of NER proteins p48/DDB2 (26) and XPC (27) required for global genomic repair. Accordingly, in our assay these proteins were induced by 2.7- and 2-fold, respectively, by low dose of UVC but not by the high dose (data not shown).

Only one histone, H2AFO, was upregulated in fibroblasts following the low dose of UV radiation as compared to five induced in keratinocytes (11). In addition, H2AFO and two H2B-family histones were upregulated late in the apoptotic response. Histones may take part in transcriptional events and nucleosome assembly, but also have a specific role in apoptosis, possibly changing the composition of nucleosomes to modify DNA accessibility to DNases. However, histones may also be involved in UVC-induced DNA repair, as found for H2B in Saccharomyces cerevisiae by Martini et al. (28).

Fibroblasts take part in the inflammatory response occurring in skin after UV exposure. Intercellular signaling by fibroblasts in response to low or high amounts of DNA damage was significantly different. Arresting cells induced specifically prostate differentiation factor (PLAB; also referred to as PDF, MIC1, GDF15) and Ephrin B1. PLAB is a member of the TGF-β superfamily of growth regulators and is associated with macrophage activation, wound healing and inflammation (29). Ephrin B1 transduces signals to activate integrin-mediated migration, attachment and angiogenesis (30). Skin healing from the UV-induced damage may also be promoted by several structural molecules related to cell adhesion and motility, which were downregulated by the low dose of UV radiation. N-cadherin (cadherin 2) suppresses cell growth by tightening cell–cell interactions and by promoting differentiation events (31,32). During repair events following low UV insult, cadherins may be downregulated to release growth suppressive signaling or to loosen cell–cell interactions to promote movement of the cells in skin.

An inflammatory response is launched in response to both doses of UV damage and includes factors such as IL-11 and ISG15. IL-11 is a secreted cytokine able to inhibit apoptosis and proliferation, e.g. in epithelial cells, and to stimulate tissue fibrosis, regulate B cell functions and inhibit macrophage cytokine production (33). Exogenous IL-11 can reduce apoptosis in UVB-irradiated mouse skin (34), probably serving as a protecting molecule to maintain tissue homeostasis after UV insult. ISG15 is a secreted ubiquitin-like covalent modifier of proteins that has immunomodulatory characteristics (35). The consequences of ISG15 conjugation are speculated to alter the biological activities of proteins but remain unclear. ISG15 produced by melanocytes can induce E-cadherin expression in dendritic cells (36). ISG15 may function in immunosignaling between cells in the UV responses and/or ISG15-conjugation may have a yet unidentified role in regulating cellular survival in DNA damage response.

We detected repression of several transcripts involved in the TGF-β signaling pathway (e.g. TβRII, SMAD3) in the apoptotic response occurring after the high dose of UVC radiation as well as downregulation of other cytokine signaling pathways (e.g. VEGF, FGF5, MET, IL4R, IFN-γ-R2). This possibly reflects shutdown of intercellular growth factor signaling for cells that are determined to undergo apoptosis and will similarly affect the neighboring keratinocytes and melanocytes. However, two inflammatory factors were specifically induced in the apoptotic response, namely diptheria toxin receptor (DTR) and follistatin like 3 (FSTL3). DTR is a heparin-binding (HB) growth factor homologous to epidermal growth factor (EGF) (37). It is produced as a membrane anchored precursor and cleaved to a soluble form proteolytically. These forms have separate activities: the membrane bound form acts in a juxtacrine manner in cell–cell contacts to inhibit growth and promote apoptosis, whereas the soluble form is mitogenic for fibroblasts and keratinocytes, and is involved in wound healing. Thus, after UV radiation DTR may work both in para- and autocrine fashion. Follistatin takes part in the inflammation reaction through inhibiting activins which act as inhibitors of proliferation and as apoptotic agents for immune cells (38). Follistatin is also able to induce angiogenesis (39). FSTL3 may take part in control of vascular integrity after UV-induced tissue injury, and several other angiogenic factors are downregulated as well (e.g. VEGF, Ang-1). Interestingly, activin can also block IL-11 action (38) and may boost IL-11 upregulation by UV in the more severely damaged cells.

Apoptosis-associated genes, with the exception of NOXA, were not specifically induced by the high dose of UVC radiation. Many UV-induced apoptosis initiation and effector cascades do not require de novo protein synthesis but function through modulation of existing pools of protein, e.g. translation and proteolysis (reviewed in 40). Thus, it can be argued that transcriptional events are not the main mode of regulation for initiation of UVC radiation-induced apoptosis. However, NOXA, which is characterized as a proapoptotic p53 target gene (41), was the most highly induced gene by the high dose in our microarray analysis. This may indicate a powerful role for NOXA in the apoptosis resulting from UVC-radiation-induced DNA damage. Spermidine/Spermine N1-acetyltransferase (SAT), a rate-limiting enzyme in polyamine catabolism was identified as a UV-responsive target in keratinocytes by Li et al. (11). Polyamines are involved in either promoting or inhibiting apoptosis in a cell type specific manner (42). We found SAT induced by both doses of UV radiation but more powerfully upregulated in the apoptotic response, indicating it may be fine-tuning the balance between survival and apoptosis induced by UV-type of DNA damage.

Several structural transcripts were regulated in UVC-induced apoptosis. Some proteolytic genes were induced, e.g. MMP-3 that contributes to breakdown of collagen matrix in photodamaged skin (43). Repressed genes include both lamin B1 and its receptor, functioning in the dynamic
regulation of nuclear structure (44). Degradation of lamin B by caspases has been suggested to participate in the activation of DNA fragmentation and nuclear apoptosis (45). Our results indicate that transcriptional downregulation of lamin B may be involved in the nuclear launch of UV radiation-induced apoptosis. It is worth noting that the two transcripts that are both induced and repressed, collagen XIIIa1 and α-cardiac actin, are both structural proteins. Both are downregulated during the transient cell cycle arrest, and upregulated late (18 h) in the apoptotic cells, perhaps suggesting their involvement in structural remodeling favoring apoptosis.

Several interesting UV-damage-associated genes were not significantly regulated after UVC radiation at the mRNA level. These include well-known UV-responsive factors such as p53 and NF-kB, which are both regulated mainly post-translationally (6). Still, the importance of these transcription factors in the UVC response is evident. In addition to the well-known UVC-induced p53 targets, including p21, Gadd45 and NOXA, several less well-characterized factors, e.g. ATF3, BTG2 and HB-EGF which are primary p53 targets (46,47), were regulated by UVC radiation in our assay.

With the completion of the present study, microarray analysis of UV damage responses has now been carried out in all major skin cell types. Previous reports using microarray methodology have concentrated on assessing UV-responsive groups of genes (11,13,14). We, on the other hand, aimed at identifying groups of genes responding either similarly or differently to doses of UVC-induced DNA damage resulting in biologically distinct events in the cells. We found similarities between our results and the previous studies, particularly with the low dose of UVC, which most closely resembled the conditions used in other reports (11,13,14). Similar functional groups of genes were affected in all studies, but the regulated transcripts varied between the assays. These variations are likely due to differences between UVB and UVC-induced events, and to inherent differences between keratinocytes and fibroblasts. Some inconsistencies between these findings are also explained by the dose-dependency of regulation of particular genes, underlining the importance of studying the cellular UV response in a more detailed fashion than as an on-off system. The detailed molecular studies of cellular UV responses in cell culture systems are valuable tools in determining the molecular mechanisms underlying skin cancer development.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at NAR Online.

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