Response of *Dictyostelium discoideum* to UV-C and involvement of poly (ADP-ribose) polymerase

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Abstract

Objectives: Radiation and chemical mutagens are direct DNA-damaging agents and ultraviolet (UV) radiation is frequently used in biological studies. Consequent to ozone depletion, UV-C could become a great challenge to living organisms on earth, in the near future. The present study has focused on the role of poly (ADP-ribose) polymerase (PARP) during UV-C-induced growth and developmental changes in *Dictyostelium discoideum*, a phylogenetically important unicellular eukaryote.

Materials and methods: *Dictyostelium discoideum* cells were exposed to different doses of UV-C and PARP activity, and effects of its inhibition were studied. Expression of developmentally regulated genes *yakA*, *car1*, *aca*, *csA*, *regA*, *ctnA*, *ctnB*, *gp24*, *hspD* and *dsn* were analysed using semi-quantitative RT-PCR.

Results: We report that the *D. discoideum* cells displayed PARP activation within 2 min of UV-C irradiation and there was increase in NO levels in a dose-dependent manner. UV-C-irradiated cells had impaired growth, delayed or blocked development and delayed germination compared to control cells. In our previous studies we have shown that inhibition of PARP recovered oxidative stress-induced changes in *D. discoideum*; however, intriguingly PARP inhibition did not correct all defects as effectively in UV-C-irradiated cells. This possibly was due to interplay with increased NO signalling.

Conclusions: Our results signify that UV-C and oxidative stress affected growth and development in *D. discoideum* by different mechanisms; these studies

could provide major clues to complex mechanisms of growth and development in higher organisms.

Abbreviations

Ax-2: axenic 2

FITC: fluorescein isothiocyanate

HA: hydroxylamine

iNOS: inducible nitric oxide synthase

NO: nitric oxide

PAR: poly-ADP ribose

PARP: poly(ADP-ribose) polymerase

PBA: phosphate-buffered agar

SB: Sorenson's buffer

G0, G1, G2, M, S: phases of the cell cycle

L-NIO: iNOS inhibitor

Introduction

Dictyostelium discoideum is a unicellular organism that feeds on bacteria and divides (under favourable environmental conditions) approximately every 4 h. It divides every 8–12 h in axenic media. During starvation, cells become chemotactically sensitive to cAMP pulses and initiate their developmental program to ultimately form a multicellular fruiting body, consisting of spores within a sorocarp, and a stalk. Spores germinate to form unicellular amoebae under favourable conditions. *Dictyostelium discoideum*, being a eukaryote that stands at the transition point of unicellularity and multicellularity, is an exceptional model system to study various signal transduction pathways (1) that can be extrapolated to mammalian systems. The unicellular stage is known to be highly resistant to DNA-damaging agents and oxidative stress (2,3).

Ionising radiation and chemical mutagens are direct DNA-damaging agents. Ultraviolet (UV) radiation, of a

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broad band of energy extending from 200 to 400 nm, is one of the frequently used types of radiation in biological studies. UV wavelengths of the solar electromagnetic spectrum can be subdivided into three regions: UV-C (200–280 nm), UV-B (280–315 nm) and UV-A (315–400 nm). Of these, UV-C is the most harmful although its rays do not reach the surface earth, due to the protective ozone layer. All UV-C and approximately 90% UV-B radiation is absorbed by ozone, water vapour, oxygen and carbon dioxide [WHO] as sunlight passes through the atmosphere. However, consequent to ozone depletion, UV-C could become a great challenge to living organisms in the future. Oxidative stress induced by ionizing radiation and alkylating agent accounts for DNA damage induced by these agents. UV-C can cause formation of thymine glycol (4) but the main lesions are caused by cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photo-products (5). In double-stranded DNA, these lesions lead to generation of SSBs (single-strand breaks) when they are repaired by NER (nucleotide excision repair) (6). They also lead to distortion in the DNA double helical structure which is sufficient to activate the nuclear enzyme, poly(ADP-ribose) polymerase (PARP) (7). PARP uses NAD⁺ as donor of poly(ADP-ribose) and catalyses poly(ADP ribosylation) (PARylation), of itself and of a variety of other proteins. Numerous substrates list PARP and link it to a wide range of physiological processes. Primarily, it is involved in chromatin remodelling, DNA repair and maintenance of genomic integrity (8–13). PARP contributes to cellular homeostasis under conditions of basal DNA damage, wherein it is involved in cell cycle arrest (14). During conditions of moderate/severe cell stress, PARP over-activation leads to cell death resulting in various pathological conditions (15). PARP inhibition during moderate/severe cellular stress is beneficial (16) and we have reported long-term consequences of PARP inhibition and down-regulation (17) in oxidative stressed *D. discoideum* cells (18,19). Also, staurosporine-induced cell death has been studied in *D. discoideum* (20). In the present study, the role of PARP in UV-C-induced growth and developmental changes in *D. discoideum* has been addressed by inhibiting its activity with benzamide, and also long-term effects of PARP inhibition for two successive generations and interplay with nitric oxide (NO) have been explored.

Materials and methods

Cell death after UV-C stress

Dictyostelium discoideum cells (Ax-2 strain) were grown in suspension in HL5 medium (21), with shaking at 3 g,

at 22 °C. All experiments were carried out with *D. discoideum* cells at mid log phase expansion, cell density 2.5×10^6 cells/ml. Cells were washed in 1× SB (1× Sorenson's buffer from 50× SB, 2 mM Na₂HPO₄, 15 mM KH₂PO₄, pH 6.4) by centrifugation, at 300 g for 5 min, then exposed to different doses of UV-C (254 nm) (10.4 J/m², 13 J/m², 65 J/m² and 130 J/m²). Cells were then resuspended in HL5 after pelleting, and incubated at 22 °C, with shaking at 150 rpm, for 24 h.

Growth curve studies

For growth curve studies after UV-C irradiation, 1% inoculations (50 µl from $\sim 2.5 \times 10^6$ cells/ml into 5 ml) were performed in HL5 medium, and cell viability was assayed using the trypan blue exclusion technique, every 24 h until death (22).

Cell cycle analysis

The cell cycle was analysed by flow cytometry using propidium iodide. Mid-log-phase cells were fixed by drop wise addition of 70% ethanol, and incubated at 4 °C overnight. Fixed cells were resuspended in staining solution (TritonX-100, DNase-free RNase and propidium iodide) and incubated for 30 min, followed by FACS analysis (23). Quantification was performed using flow cytometry, with FACS ARIA (BD Biosciences, San Jose, CA, USA). Data were analysed with FACSDiva software.

Developmental studies

2.5×10^6 cells were harvested and processed as described above for UV-C treatment (10.4 J/m², 13 J/m², 65 J/m² and 130 J/m²), then were resuspended in 100 µl 1× SB and spread on non-nutrient agar plates (2% agar in 1× SB) kept at 22 °C, and different stages of development were studied.

Chemotactic assay

Chemotactic studies were performed according to the method of Wallace and Fraizier (24). Exponentially growing cells were washed free of medium with 1× SB and starved for 5–6 h after treatment. Of the cell suspension, 5 µl were placed on 2% agar surfaces at a distance of 2 mm from wells containing 1 µM cAMP. Movement of cells towards wells was observed using phase contrast microscopy (Nikon TE-2000S, Tokyo, Japan) and was photographed. Relative number and distance moved by cells was an indication of their chemotactic activity.

cAMP pre-treatment. Exogenous (1 μ M) cAMP was added 2.5 h prior to UV-C treatment to *D. discoideum* cells in 1 $\times$ SB. Plates were then kept at 22 °C to observe different stages of development, or cells were harvested to study developmental gene expression, at various time points.

cAMP estimation in UV-C-irradiated cells

5×10^6 cells were harvested by centrifugation at 300 g/5 min/4 °C and subjected to UV-C irradiation and they were resuspended in Sorenson's buffer for 6 h. Cells were then collected, and extracellular cAMP was estimated in the buffer, using an ELISA kit method according to the manufacturer's instructions (Calbiochem, Gibbstown, NJ, USA). A total of 200 μ M L-NIO (iNOS inhibitor) pre-treated cells were also collected, and extracellular cAMP was estimated.

Nitric oxide estimation

NO generation was estimated according to the method of Green *et al.* (25). 5×10^6 cells were suspended in 1 ml 1 $\times$ SB and incubated at 22 °C for 20 min, to allow for accumulation of NO. 1 ml Griess reagent was added and mixed in well. Incubation was again carried out at 22 °C for 15–30 min. Absorbance was measured at 546 nm.

PARP activation under UV-C stress

Cells treated with different doses of UV-C were processed for PARP assay (19). Data were analysed using Image Proplus software to calculate mean intensity of fluorescence from different fields and ~50 cells were examined for each dose. PARP was inhibited by 12 h pre-treatment with 1 mM benzamide (Sigma Aldrich, St Louis, MO, USA), prior to UV-C irradiation.

Expression analysis of developmentally regulated genes, by RT-PCR

RNA extraction and cDNA synthesis. *Dictyostelium discoideum* cells were exposed to UV-C stress as mentioned above. After specific pre-treatment, cells were pelleted and washed in 1 $\times$ SB before finally being resuspended in 1 $\times$ SB. Total RNA was isolated from cells at two time points (as specified in figure 4) using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by 1.5% agarose gel electrophoresis and 260/280 absorbance ratio >1.95. RNA was treated with DNase I (Ambion Inc., Austin, TX, USA) before cDNA synthesis, to avoid DNA contamination. Reactions were performed by

reverse transcriptase using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions, in MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

Second strand synthesis. First-strand cDNA was used as a template with gene-specific primers to synthesize second strand DNA by conventional PCR. Expression kinetics of DYRK family protein kinase (*yakA*), cAMP receptor-1 (*car1*), adenylyl cyclase (*aca*), cell adhesion molecule–contact site A protein (*csA*), component of the counting factor complex–countin A (*ctnA*) and countin B (*ctnB*), calcium-dependent cell adhesion molecule-1 (*gp24*), cAMP phosphodiesterase (*regA*), a heat shock protein of Hsp90 family (*hspD*) and discoidin (*dsn*) genes were analysed. *m1A* was used as internal control (Table 1). Reactions were performed according to the manufacturer's instructions (Fermentas, Burlington, ON, Canada). DNA fragments were amplified for 24 cycles and signal intensities were analysed on 2% agarose gel stained with ethidium bromide. Densitometric analysis was performed using AlphaImager software, and mean density for respective genes with respect to *m1A*, was plotted.

Fate of spores formed under UV-C stress. Spores formed from cells irradiated with 10.4 J/m² UV-C in the presence and absence of benzamide (Sigma Aldrich)

Table 1. Primer sequences used for gene expression analysis.

Gene	Primer sequence
<i>yakA</i> Forward	5'-GCTTAGAGGACTTTCAACCAATTT-3'
<i>yakA</i> Reverse	5'-GATTTTTCATAACAAGCAGATCCA-3'
<i>cAR1</i> Forward	5'-TGTGGACTTTATGTCTTGCAATTAG-3'
<i>cAR1</i> Reverse	5'-CCAATACTGCTGAAATGCCC-3'
<i>aca</i> Forward	5'-GTGATACTGCCAATACCGCC-3'
<i>aca</i> Reverse	5'-ACCCAAGAGAGTTCCAGATAATTGG-3'
<i>csA</i> Forward	5'-ATAGTGCACATTCAGCTCC-3'
<i>csA</i> Reverse	5'-AAGAACCTTGCCATACCTTTGG-3'
<i>ctnA</i> Forward	5'-ATGAATAAAATTATTTTCATTAATTTTAG CTTTATTCCTTGTCACACTCCGC-3'
<i>ctnA</i> Reverse	5'-TTAAAATAAAGCAAAACCTGAACCTGA ACCAGAGGCGGCACC-3'
<i>ctnB</i> Forward	5'-GTGGTGCCGTTTGTTCATTACTCCC-3'
<i>ctnB</i> Reverse	5'-CCAGTTGGGTGAGTTACCATAACAGCAAC-3'
<i>gp24</i> Forward	5'-CCAGGAGCTTTTCAATGGGCAGTTGATG-3'
<i>gp24</i> Reverse	5'-GTGTAACAGTCATATCTTTGGGAATGTCTC-3'
<i>dsn</i> Forward	5'-CCACCCATTAACCTGGAATG-3'
<i>dsn</i> Reverse	5'-TGGTGGCATCAGTACAATCG-3'
<i>hspD</i> Forward	5'-ACATTCCAAGCTGAAATTAATCAGC-3'
<i>hspD</i> Reverse	5'-GTGTAAGAGTTTTGGCAGTCTTATC-3'
<i>regA</i> Forward	5'-AATTGTTGGGGATACTGAATCAGC-3'
<i>regA</i> Reverse	5'-ATAAAGTGGGTGATATTTTC-3'
<i>m1A</i> Forward	5'-TTACATTTATTAGACCCGAAACCAAGCG-3'
<i>m1A</i> Reverse	5'-TTCCCTTTAGACCTATGGACCTTAGCG-3'

were picked from different areas, with the aid of sterilized nichrome loops, and added to 5 ml HL5 medium. Flasks were kept shaking at 150 rpm/22 °C. After germination, cells were counted using a haemocytometer, every 12 h, for growth curve experiments.

Assessment of DNA damage in cells germinated from spores (26). Histones become phosphorylated during oxidative stress. Hence, presence of phosphorylated histone indicates oxidative stress-induced DNA damage. Anti-H2AX at 0.5 µg/ml, and anti-mouse IgG (whole molecule)–TRITC conjugate (Sigma, St. Louis, MO, USA), 1:200, were used to study presence of oxidative stress. Cells were pelleted and washed once in phosphate-buffered saline (PBS) pH 7.4, fixed in 70% chilled methanol for 10 min at –20 °C, then washed in blocking solution (1.5% BSA with 0.05% Tween 20 in PBS) and incubated for 1 h in primary antibody. After incubation, cells were washed 2–3 times in blocking solution and further incubated for 1 hr with TRITC-labelled secondary antibody. Then, they were washed 2–3 times in PBS and fluorescence was observed under 60× magnification. Data were analysed using Image Proplus software to calculate mean density of fluorescence and graphs were plotted using Graphpad prism software.

Results

Cell death and PARP activation after UV-C irradiation

Cell death was monitored after different doses of UV-C irradiation. UV-C dose (10.4 J/m² and 13 J/m²) led to less than 10% cell death (Fig. 1a). UV-C is a potent DNA damage inducer and is well reported to activate PARP (7,15). PARP was assayed at various time points post UV-C stress. 10.4 J/m² and 13 J/m² UV-C-treated cells had highest PARP activity at 2 min (Fig. 1b), which then declined, and reached basal levels after 5 min. Benzamide pre-treated cells had reduced activation of PARP (Fig. 1b).

Dictyostelium discoideum growth and development under UV-C stress

Growth analysis revealed dose-dependent increase in lag phase with UV-C dose. As can be seen from Fig. 2a, with increase in UV-C dose, there was increase in lag phase and consequently late entry into the log phase. Also, the stationary phase was achieved at lower density compared to control cells. Pre-treatment of cells with benzamide resulted in partial rescue in UV-C-induced changes in growth (Fig. 2a). Lag phase growth of UV-C-stressed cells can be clearly explained by cell cycle

Figure 1. Cell death and PARP activation in UV-C-irradiated cells. (a) UV-C induced dose-dependent cell death as monitored by the trypan blue exclusion method. Results are mean ± SE of four independent experiments. (b) Peak PARP activity induced by UV-C irradiation was intercepted by benzamide. Benzamide inhibited PARP activity at 2 min post 10.4 J/m² and 13 J/m² irradiation respectively. Data (mean ± SE) are from four independent experiments. **P* < 0.05 compared to control; ^a*P* < 0.05 compared to respective dose of UV-C irradiation, without benzamide pre-treatment.

analysis studies, wherein UV-C-stressed cells were seen to be arrested in G0/G1 (~77%) as opposed to untreated control cells (~68%), at 48 h growth (Fig. 2b). Also, marked reduction is seen in percentage of cells in S phase after UV-C irradiation at 10 J/m² (13.6%) and 13 J/m² (12.1%) compared to untreated control cells (22.1%) (Fig. 2b), explaining the slower growth in stressed cells.

Effects of UV-C on development also reflected dose dependency as seen in growth (Fig. 3); however, it was more drastic. Cells exposed to 13 J/m² (Fig. 3) and higher doses (65 J/m² and 130 J/m²) (data not shown) UV-C failed to undergo complete development. 10.4 J/m² UV-C-treated cells displayed development that was delayed compared to control cells. Delay in development induced by 10.4 J/m² was rescued when cells were pre-treated with benzamide (Fig. 3). Interestingly after 12 h, benzamide-pretreated cells exposed to higher doses (65 J/m²) of UV developed streaming structures and incompetence to form aggregates, compared to untreated control and UV-C-exposed cells.

Figure 2. Effect of PARP inhibition on UV-C induced growth changes and fate of spores in *Dictyostelium discoideum*. (a) Effect of PARP inhibition on UV-C induced growth changes Benzamide rescued UV-C-induced changes as shown in the growth curve. The stationary phase was achieved at higher cell density in benzamide pre-treated cells compared to respective control. Results are mean of four independent experiments. (b) Cell cycle analysis of UV-C-irradiated cells by propidium iodide staining. The 10.4 J/m² and 13 J/m² UV-C-treated cells had G0/G1 arrest, i.e. ~77% cells were in G0/G1 phase in contrast to ~68% untreated control cells. This figure is a representative picture of three independent experiments performed.

Figure 3. Development of *Dictyostelium discoideum* cells under UV-C stress. *Dictyostelium discoideum* cells after UV-C treatment were allowed to develop on nutrient-free agar medium and were observed at various time intervals. Benzamide pre-treatment restored the delay induced by 10.4 J/m² but did not lead the 13 J/m² UV-C-treated cells to complete development, though the cells entered it. Photographs taken with 4× objective. Scale = 100 µm.

Also, developing fruiting bodies formed after 10.4 J/m² UV-C stress were smaller and ~40% greater in number. Cells subjected to UV-C stress formed less compact aggregates (Fig. 3). This observation led us to assess expression profile of development-related genes such as *ctnA*, *ctnB*, *dsnI*, *hspD*, *csA* and *gp24*. These are associated with regulation of aggregate size (27–29). Expression profiles of *yakA*, *carI*, *aca* and *regA* were also studied as these genes are important for growth to differentiation transition and cAMP-mediated

signalling, respectively (1,30). As shown in Fig. 4a and 4b, UV-C affected levels of *ctnA*, *yakA*, *carI*, *aca*, *csA* and *regA* mRNA. Benzamide pre-treatment prevented UV-C-induced changes in expression of *ctnA* and *regA*; however, *yakA*, *carI*, *aca* and *csA* expression levels could not be restored. Altered expression levels of *yakA*, *carI*, *aca*, *csA* and *regA* during UV-C stress indicated that the finely tuned cAMP signalling network of development was affected, which was re-established by *yakA*, *carI* and *aca* restoration in presence of exog-

enous cAMP. To probe effects on cAMP signalling, chemotactic assay and cAMP estimations were undertaken. Control amoebae moved toward $1 \mu\text{M}$ cAMP wells, while UV-C-irradiated cells failed to sense cAMP in cups (Fig. 5a). These results justify effects of exogenous cAMP on altered expression profiles of developmentally regulated genes (Fig. 4a, b) and on developmental arrest (Fig. 5b). Also cAMP levels were found reduced in UV-C-irradiated cells (Fig. 6). Spores developed after 10.4 J/m^2 UV-C irradiation were delayed by around 27 h in revival ($111 \text{ h} \pm 6.0$) compared to control cells ($81 \text{ h} \pm 6.658$) (Fig. 7a). These spores also had longer lag phases compared to controls. However, benzamide pre-treatment had no effect on revival of spores formed after 10.4 J/m^2 UV-C exposures as shown in (Fig. 7a). Also this second generation of UV-C-exposed cells did not differ from that of control cells with respect to damage in DNA (Fig. 7b); ensuring that partial inhibition of PARP did not interfere with basal repair of the cells.

UV-C-induced developmental changes restored by iNOS inhibition

UV-C radiation, unlike oxidative stress, leads to increased NO generation which may further interfere with *D. discoideum* development. Hence, we monitored effects of inducible nitric oxide synthase inhibitor (L-NIO) on UV-C-induced developmental changes. UV-C dose of 13 J/m^2 caused arrested development. However, $200 \mu\text{M}$ L-NIO (iNOS inhibitor) pre-treated cells exhibited partial rescue in developmental delay due to UV-C treatment (Fig. 8a). These results were further confirmed by monitoring NO production under UV-C stress (Fig. 8b). Results clearly suggest that *D. discoideum* cells exposed to UV-C irradiation had dose-dependent increase in production of nitric oxide, and NO levels decreased significantly ($P < 0.05$) on benzamide pre-treatment. Also inhibition of iNOS partially restored cAMP levels (Fig. 6). Thus, increased NO generation affected signalling in UV-C-exposed cells, thereby impeding their development.

Figure 4. (a) Irradiation with UV-C affected mRNA expression of various genes involved in *Dictyostelium discoideum* development. Expression of *yaka*, coding for cell cycle arrest protein; cAMP receptor – *cAR1*, *aca* coding for adenylyl cyclase A, countin, coding for a protein involved in cell counting mechanism, *csA*, a cell adhesion molecule and *regA*, a phosphodiesterase were found to be reduced in UV-C-treated cells compared to internal control *mlA* (mitochondrial rRNA IG7). Benzamide pre-treatment restored expression levels of countin, however, it did not affect *yaka* and *cAR1* mRNA levels. Levels of countin (another protein of cell counting mechanism) mRNA were found increased in benzamide pre-treated cells. Exogenous cAMP restored *cAR1* without affecting *yaka* and *csA*. Expression of *dsn*, a gene coding for a protein which helps cells to adhere to the substratum, *hspD*, a heat shock protein and *gp24* (intercellular contacts and aggregation) were not affected. mRNA levels of *yaka*, *cAR1*, *aca*, *csA* and *regA* were assayed at 0 and 9 h, while the rest of the genes were assayed at 6 and 10 h of development induction. (b) Densitometric analysis of various genes involved in *D. discoideum* development. $^{***}P < 0.001$ compared to respective controls; $^{**}P < 0.01$ compared to respective controls; $^{*}P < 0.05$ compared to respective controls and $^{a,a+,s,##,p}P < 0.05$ compared to respective controls. For benzamide pre-treated and 13 J/m^2 UV-C-treated cells, $^{bbb}P < 0.001$, $^{bb}P < 0.01$ compared to 13 J/m^2 UV-C-treated cells (-benzamide/-cAMP). For cAMP pre-treated and 13 J/m^2 UV-C-treated cells, $^{qqq}P < 0.001$, $^{qq}P < 0.01$ compared to 13 J/m^2 UV-C-treated cells (benzamide/cAMP).

Figure 5. Effect of UV-C irradiation on chemotaxis in *Dictyostelium discoideum*. (a) UV-C-exposed *D. discoideum* cells failed to move towards cAMP. Wells were formed on PBA plates using a cup borer and were filled with 100 μ l of 1 μ M cAMP; cells were placed at a distance of 2 mm from wells. Photographs were captured 6 h after plating the cells at 4 $\times$ magnification. Results are representative of three independent experiments. (b) Exogenous cAMP resumed development of UV-C-exposed *D. discoideum* cells. Exposure to 13 J/m² UV-C blocked initiation of development; however supplementation with cAMP (1 μ M) restored development. Photographs were captured at 4 $\times$ magnification. Results representative of three independent experiments. Scale = 100 μ m

Figure 6. cAMP levels in UV-C irradiated *Dictyostelium discoideum* were reduced in NO-dependent manner. UV-C irradiated *D. discoideum* cells had reduced cAMP levels. Cells pre-treated with iNOS inhibitor, L-NIO, retained levels of cAMP even after UV-C exposure. Results are mean $\pm$ SE of three independent experiments. * P < 0.05, *** P < 0.001 compared to control (0 J/m²), ^a P < 0.05 and ^{aa} P < 0.01 compared to benzamide pre-treated cells and ^{pp} P < 0.01 compared to L-NIO pre-treated cells.

Discussion

PARP characteristics render it an ideal candidate for participation in cell responses to UV stress (31). UV-C

induces direct DNA lesions such as CPDs (e.g. T-T, T-C or C-C) or 6,4-photoproducts. These lesions distort DNA structure. DNA strand breaks generated during excision repair of such lesions activate PARP (32–35). Using elegant techniques that allow visualization of events occurring in UV-C-irradiated zone, Vodenicharov *et al.*, (36) demonstrated that UV-C causes immediate PARP activation within the first 15 s to 5 min. Our results corroborate these reports. UV-C treatment lead to both dose-dependent cell death and PARP activation within 2 min (Fig. 1). Cells exposed to higher doses of UV-C (130 J/m²) had a longer lag phase (~100 h) than at milder doses, which could be due to seized cell cycle (Fig. 2b). This is in accordance with the finding that UV-C lead to cell cycle arrest after activation of the repair machinery (37). PARP inhibition allowed cells exposed to 10.4 J/m² UV-C to enter the log phase earlier than UV-C-only exposed cells. These also attained higher cell density compared to 10.4 J/m² UV-C-only irradiated cells, suggesting that PARP played a role in cell cycle arrest and DNA repair.

Dictyostelium discoideum, during its unicellular vegetative phase, exhibits higher resistance to oxidative stress (3) and other DNA-damaging agents (2,20). Very high doses of hydroxylamine (4 mM) leading to ~90%

Figure 7. (a) Effect of PARP inhibition on fate of spores developed under UV-C stress. Spores of control cells revived within 81 h whereas spores formed after 10.4 J/m² UV-C stress exhibited ~27 h of delay in spore revival, which could not be rescued by benzamide pre-treatment. Data are mean of four independent experiments. (b) DNA damage monitored in second-generation *Dictyostelium discoideum* cells. No significant damage was observed in second-generation cells.

cell death, is required to block *D. discoideum* development (17,18). Surprisingly, the effect of UV-C is more drastic on *D. discoideum* development. Doses of UV-C equivalent to 13 J/m² (~6% cell death) or higher, completely arrested *D. discoideum* development at the loose aggregate stage, whereas 10.4 J/m² UV-C-irradiated cells completed development albeit delayed (Fig. 3). Benzamide pre-treatment of 13 J/m² UV-C-exposed cells had streaming structures indicating a definite role of PARP in development. This is supported by work previously published (19) from our laboratory, where we showed that both constitutive and slug stage-specific down-regulation of PARP lead to blocked or arrested development respectively. Protective effects of PARP inhibition were not seen at doses higher than 13 J/m², as cells died (38).

Azzam *et al.* (39) demonstrated cell type-specific effect of UV-C irradiation on expression of connexin 43 native isoform, in human fibroblasts and AG1522 cells. Experiments performed with UV-C irradiation in human skin fibroblasts (37) showed that UV-C reduced transcription of certain proteins involved in adhesion and motility. Hence, expression of certain genes crucial to various aspects of *Dictyostelium* development were assessed. Our results indicate that heat shock protein D, a cytosolic protein was required for early developmental stages. Discoidin-1, a marker of growth to differentiation transition that aids differentiating cells adhere to the substratum and gp24, required for filopodia formation and

Figure 8. Effect of UV-C on development of *Dictyostelium discoideum* under iNOS inhibition and nitric oxide generation. (a) Development of UV-C-irradiated cells pre-treated with iNOS inhibitor. L-NIO-treated cells developed like control untreated *D. discoideum* cells within 24 h. Photographs were captured at 4× magnification 24 h after plating the cells. Results are representative of three independent experiments. Scale = 100 μm. (b) Nitric oxide generation increased in UV-C-treated *D. discoideum* cells. NO generation was estimated 30 min after UV-C treatment by the Griess method and was found to increase with increasing doses of UV-C. Results are the mean ± SE of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to untreated control cells, ^a*P* < 0.05 compared to benzamide pre-treated cells and ^{###}*P* < 0.001 compared to L-NIO pre-treated cells.

aggregation, was not affected in UV-C-treated cells. Nevertheless, expression of *csA* and *ctnA* was markedly reduced without great alteration in *ctnB* mRNA levels (Fig. 4a, b). *csA* is involved in Ca^{2+} independent cell–cell adhesion during aggregation, and countin and countin 2 along with at least three other polypeptides form a > 450 kDa counting factor complex, involved in maintaining aggregate size. *ctn* null cells had increased group size (40) similar to larger aggregate size seen in UV-C-treated cells (Fig. 3), unable to progress to fruiting bodies. Such cells have been reported to have reduced cAMP-induced cAMP pulse, and decrease in cAMP-stimulated Akt/PKB membrane translocation and kinase activity, which in turn lower cell motility (40–42). This is substantiated by reduced expression of *cAR1* and *aca* (Fig. 4a, b) and lower cAMP levels (Fig. 6), quintessential for cAMP signalling. In addition to this, reduced *regA* expression underlay compromised motility of UV-C-irradiated cells (Fig. 5a), as the protein is a phosphodiesterase that regulates PKA activity *via* reducing cAMP levels during chemotactic aggregation. cAMP addition was capable of restoring UV-C-induced changes in *car1* and *aca* expression (Fig. 4a, b). This explains that some UV-C-induced developmental defects were *via* modulating cAMP signalling to alter expression of genes associated with growth to differentiation transition, chemotaxis and aggregate size regulation (43–45).

Interfering PARP activity partly rescued development of UV-C-irradiated cells. It restored countin levels to normal but failed to affect expression of *car1*, *aca*, *yakA*, crucial for regulating cell cycle exit and growth to differentiation transition, and *csA*. Also, expression of *gp24*, a cell adhesion protein, increased in the presence of benzamide. PARP activity has previously been associated with expression of adhesion proteins (46). As increased adhesion proteins are known to hinder chemotaxis (47), the effect of benzamide on the gp(s) may mask its effect on development *via* restoration of countin levels.

On the other hand, UV irradiation induces NO generation in keratinocytes; this serves as a signal for melanogenesis (48). NO functions as a signalling molecule for *D. discoideum* cells also. NO-treated cells transiently activate their adenylyl cyclase and produce pulses of cAMP when stimulated with exogenously applied cAMP (49). However, physiological or environmental conditions that enhance external NO levels delay initiation of cAMP pulses, which are essential for cell differentiation (50). Estimation of NO (Fig. 8b) during development with L-NIO, iNOS (inducible nitric oxide synthase) inhibitor, showed NO generation *via* iNOS in *D. discoideum* after UV-C irradiation. Also, this NO

production (Fig. 8b) was dependent on PARP. Activated PARP-1 up-regulates iNOS expression (51,52) and further iNOS byproducts may modulate PARP-1 enzymatic activity by nitration (53) making PARP activity and NO production interdependent. In *D. discoideum* cells, UV-C irradiation affects expression of certain developmentally important genes (*yakA*, *aca*, *car1*) and hence affects cAMP pulses *via* PARP activation and NO production. This has also been hinted at by increase in levels of cAMP with iNOS inhibition after UV-C exposure. An alternative sequence of events in which UV-C induces NO production prior to PARP activation is also possible. Further experiments need to be performed to pinpoint the affected pathways. Nevertheless, our results suggest interplay between PARP and NO with respect to regulation of gene expression during developmental defects induced by UV-C. Delayed revival of spores could be attributed to down-regulation of certain genes involved in spore revival, by UV-C. PARP may not be involved in increasing dormancy of spores induced by UV-C, as benzamide pre-treatment did not show any significant change in the revival of spores compared to UV-C-only treated cells. Cells germinated from spores did not show any significant damage (Fig. 7b) signifying that reduced activity of PARP (Fig. 1b) may be sufficient to repair DNA damage induced by 10.4 J/m² UV-C.

Interestingly, effects of PARP inhibition on UV-C-induced changes in *D. discoideum* growth and development also differed from our oxidative stress response results (17,18). Spores formed under oxidative stress exhibited delayed revival compared to benzamide-pre-treated cells suggesting that PARP inhibition during oxidative stress not only resumed delayed development but also retained normal spore revival (17). However, the present study suggests that PARP inhibition and UV-C treatment did not have any effect on spore development of second-generation cells. This, in addition to the observation that lower doses of UV-C caused developmental defects in *D. discoideum* cells, a few of which were unaffected by PARP inhibition, raises a question concerning therapeutic significance of PARP inhibition in various DNA damage-related diseases. Thus, the concept of PARP inhibition being beneficial in various DNA associated diseases should be considered cautiously.

On a different note, *D. discoideum* despite being a lower eukaryote shows differential effects of oxidative and UV-C stress on development and spore germination. Hence, this organism has the complex signalling machinery to deal with different stresses such as oxidative stress and UV-C, in diverse ways. This fact emphasizes the importance of signalling pathway studies in *D.*

discoideum, which is simple and easy to handle compared to mammalian cell lines. Proteases involved in *D. discoideum* cell death downstream of PARP have been identified in oxidative stress-induced cell death (54). Further work needs to be performed to explore downstream targets of PARP during *D. discoideum* development for understanding the role of this multifunctional enzyme, in developmental cell death.

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