cAMP mediated chemotaxis during UV-C induced *D. discoideum* developmental changes

7.1. Introduction

D. discoideum is a social amoeba whose life cycle consists of two distinct phases i.e., unicellular reproductive and multicellular developmental phases. When nutrients are abundant *D. discoideum* exists as unicellular amoeba, however under nutrient starvation conditions, cells shut down growth and cell division and enter a developmental program. During development the starving amoebae initiate cAMP pulses attracting the neighboring amoebae to migrate towards them. The moving cells in turn synthesize and secrete cAMP, ensuring that the signal spreads in the surrounding area. Extracellular cAMP acts through cAR1-4 receptors of neighboring cells (Mir *et al.*, 2007; Elzie *et al.*, 2009; Loomis, 2008; Jin *et al.*, 2000).

Thus, cAMP acts as both primary (chemo attractant) and secondary (stimulant of the stage-specific gene expression) messenger in the signal transduction pathway during development. Up to ~10^5 cells are involved in the chemotactic formation of one aggregate. Each aggregate then goes through a series of morphological changes (Loomis, 1982; Loomis, 2008) that are accompanied by cytodifferentiation finally giving rise to a multicellular structure consisting of dormant spores supported by a dead stalk. These spores get dispersed to favourable surroundings where they germinate, each releasing several amoebae.

Several reports are available on UV-C mediated toxicity in unicellular and multicellular phases of *D. discoideum* cells (Mauldin *et al.*, 1994; Garcia *et al.*, 2000; Garcia *et al.*, 2003), however UV-C mediated toxicity during differentiation is yet to be elucidated. Thus this system would be an excellent model to study radiation toxicity during differentiation and its therapeutic potential. Earlier we have reported the effect of oxidative stress on *D. discoideum* development (Rajawat *et al.*, 2007), and in this study we have made an attempt to investigate the effect of UV-C stress on signaling processes during differentiation, especially at the pre-aggregation stage.

7.2. Results

7.2.1. Effect of UV-C irradiation on number and size of the developing bodies of *D. discoideum*

D. discoideum cells exposed to the UV-C apart from showing delay in development (Fig. 6.5) also showed a decrease in the size and number of fruiting bodies. Table 7.1 shows a dose dependent effect of UV-C on *D. discoideum* development.

Table 7.1: Dose dependent effect of UV-C on *D.discoideum* development: Exposure to 13 J/m^2 and higher UV-C doses, blocked development completely while 10.4 J/m^2 delayed the development by ~11 hours.

UV-C (J/m^2)	**LA** (hours)	**TA** (hours)	**SF** (hours)	**FBF** (hours)
0	8	12	18	24
10.4	19	23	29	35
13	–	–	–	–
130	–	–	–	–

LA : Loose aggregate; TA: Tight aggregate; SF: Slug formation; FBF: Fruiting body formation.

7.2.2. Synergistic development by mixing UV-C treated GFP tagged *D. discoideum* cells with healthy cells

To evaluate whether the extracellular signaling is altered under UV-C irradiation, GFP tagged UV-C irradiated cells (13 J/m^2) were mixed with healthy cells and allowed to undergo development. Interestingly, normal development was observed (Table 7.2) and it was also found that UV-C exposed cells were more prone to stalk formation while healthy cells formed the spore as seen from GFP fluorescence (Fig. 7.1).

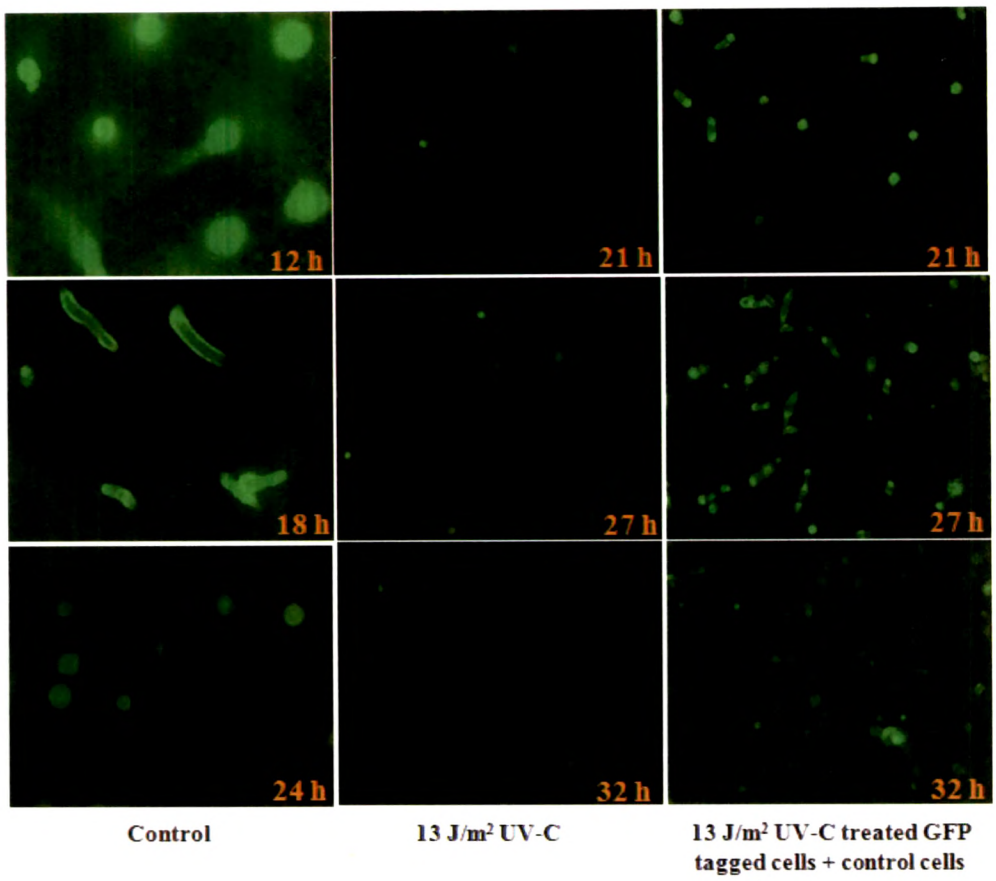

Figure 7.1: UV-C irradiated GFP tagged cells synergistically develop with healthy cells. Exposure to 13 J/m^2 UV-C prevents chemotaxis of (GFP tagged) *D. discoideum* cells. Mixing of these UV-C treated GFP tagged cells with healthy cells leads to synergistic development of the cells i.e., both the cell types participate in the development. Photographs were captured at 4X magnification. Data are representative of at least three independent experiments.

Table 7.2: Synergistic development of UV-C treated GFP tagged *D. discoideum* cells with healthy cells. Mixing healthy cells with UV-C treated GFP tagged cells prevented developmental changes in *D. discoideum* development.

2.5 x10^6 UV-C exposed cells					2.5x10^6 UV-C exposed cells (GFP tagged) + 2.5 x10^6 healthy cells				
UV-C (J/m^2)	LA (hours)	TA (hours)	SF (hours)	FBF (hours)	UV-C (J/m^2)	LA (hours)	TA (hours)	SF (hours)	FBF (hours)
0	8	12	18	24	0	8	12	18	24
10.4	19	23	29	35	10.4	12	16	22	28
13	-	-	-	-	13	17	21	27	32

7.2.3. Effect of cAMP on UV-C induced *D. discoideum* development

To explore whether the inefficiency of *D. discoideum* cells to develop under UV-C treatment is due to defect in cAMP secretion, extracellular cAMP levels of UV-C treated *D. discoideum* cells were estimated and found to be reduced by ~75% in UV-C (13 J/m^2) treated cells as compared to control cells (Fig. 7.3). To further confirm our results, cAMP addition experiment was carried out. Complete restoration of development was observed in cells exposed to UV-C for 10.4 J/m^2 and 13 J/m^2 (Table 7.3 and Fig. 7.2) with addition of 1μM cAMP. Moreover, the exogenous cAMP addition also resulted in an increase in the number of fruiting bodies.

cAMP addition and measurement experiments suggested a defect in cAMP secretion under UV-C treatment. To further probe this result, chemotaxis assay was performed with 1μM cAMP. The control amoebae moved towards 1 μM cAMP well, while UV-C irradiated cells failed to sense the cAMP (Fig. 7.4). This result along with the mixing (Fig. 7.1 and Table 7.2) and cAMP addition (Fig. 7.2 and Table 7.3) experiments results suggested that sensing of cAMP was compromised under UV-C treatment though not lost completely.

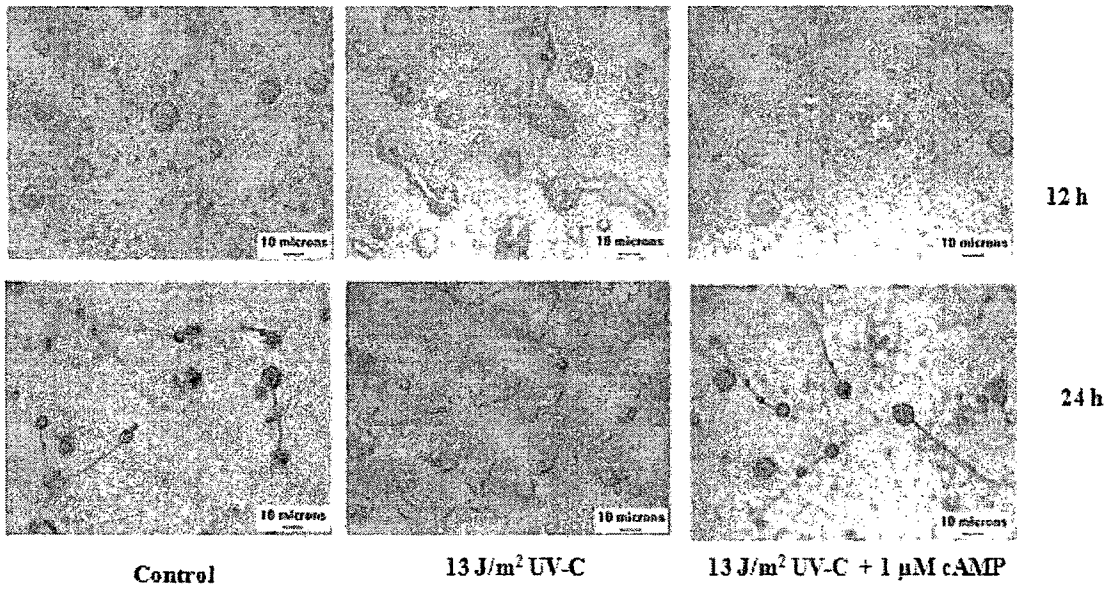

Figure 7.2: Exogenous cAMP resumes development of UV-C exposed *D. discoideum* cells. Exposure to 13 J/m^2 UV-C blocked the initiation of development however, supplementation with cAMP (1μM) restored the development. Photographs were captured at 4X magnification. Results are representative of three independent experiments

Table 7.3: Addition of cAMP (1 and 10 μM) resumes development of UV-C exposed *D. discoideum* cells.

UV-C					UV-C + 1μM cAMP				
(J/m^2)	LA (hours)	TA (hours)	SF (hours)	FBF (hours)	(J/m^2)	LA (hours)	TA (hours)	SF (hours)	FBF (hours)
0	8	12	18	24	0	8	12	18	24
10.4	19	23	29	35	10.4	8	12	18	24
13	-	-	-	-	13	10	14	20	26
13	-	-	-	-	13 J/m^2 +10μM cAMP	10	14	20	26

LA : Loose aggregate; TA: Tight aggregate; SF: Slug formation; FBF: Fruiting body formation.

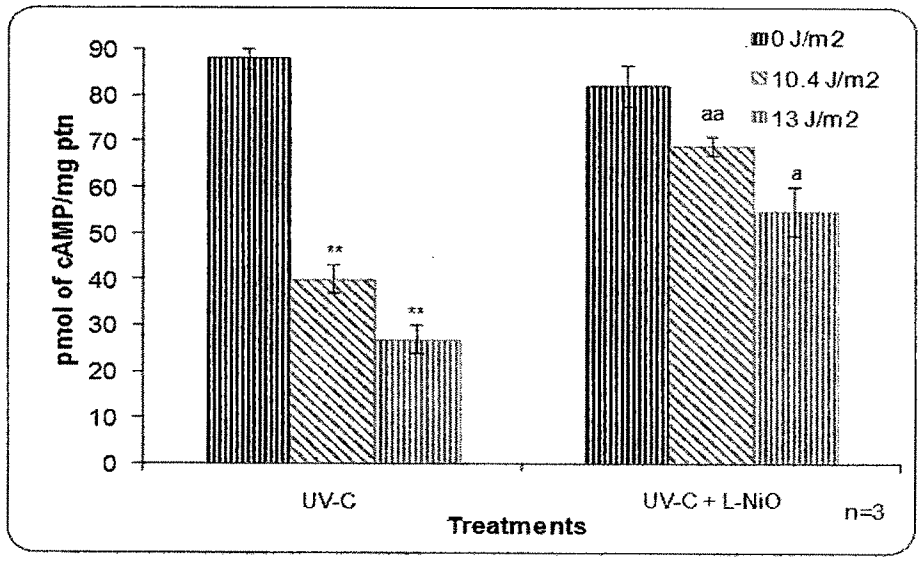

Figure 7.3: cAMP levels in UV-C irradiated *D. discoideum* were found to be reduced in NO dependent manner. Cells pretreated with iNOS inhibitor, L-NIO, could retain the levels of cAMP even after UV-C exposure. Results are the mean ± SE of three independent experiments. ** p value <0.01 as compared to control (0 J/m^2) and ^{a} p value <0.05 and ^{aa} p value <0.01 as compared to respective UV-C control.

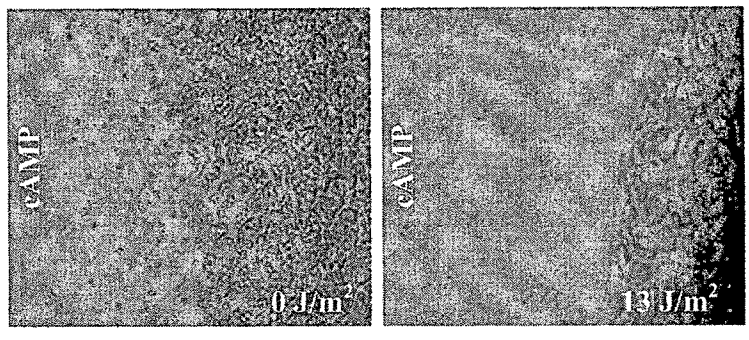

Figure 7.4: UV-C exposed *D. discoideum* cells failed to chemotax towards cAMP well. Well was formed on PBA plate using a cup borer and was filled with 100 µl 1µM cAMP and cells were placed at a distance of 2mm from the well. Photographs were captured at 4X magnification 6 hours after plating the cells. Results are representative of three independent experiments.

7.2.4. Expression kinetics of different genes after UV-C exposure

In order to better understand the cellular processes in response to DNA damage induced by UV-C irradiation, we next examined the gene expression pattern of pre-aggregative genes involved in cAMP production. The developmental expression pattern of *yak*A, *cAR*1, *aca, countin, countin*2, *gp*24, *gp*80, *discoidin*I *and hsp*D *genes* is shown in Figure 7.5. Expression of *yak*A, *cAR*1, *aca* and *countin* was found altered in a dose dependent manner after 10.4 and 13 J/m^2 UV-C exposure. These results suggest that expression of *yak*A, *car*1, *aca* and *countin* is down regulated without affecting other genes and *rnl*A-the housekeeping gene and hence we emphasize that the cAMP chemotactic signaling mechanism gets affected under UV-C stress. Interestingly exogenous cAMP restored *car*1 and *aca* without affecting *yakA* and *countin* expression.

7.2.5. UV-C induced developmental changes restored by iNOS inhibitor

UV-C radiation also leads to increase NO generation which will further interfere with *D.discoideum* development, hence we monitored effect of inducible nitric oxide synthase inhibitor (L-NIO) on UV-C induced developmental changes. 13 J/m^2 UV-C dose showed arrested development, however 200 µM L-NIO (iNOS inhibitor) pretreated cells exhibited partial rescue in the developmental delay caused due to UV-C treatment (Fig. 7.6). These results were further confirmed by monitoring NO production under UV-C stress and the results are shown in Figure 7.7.

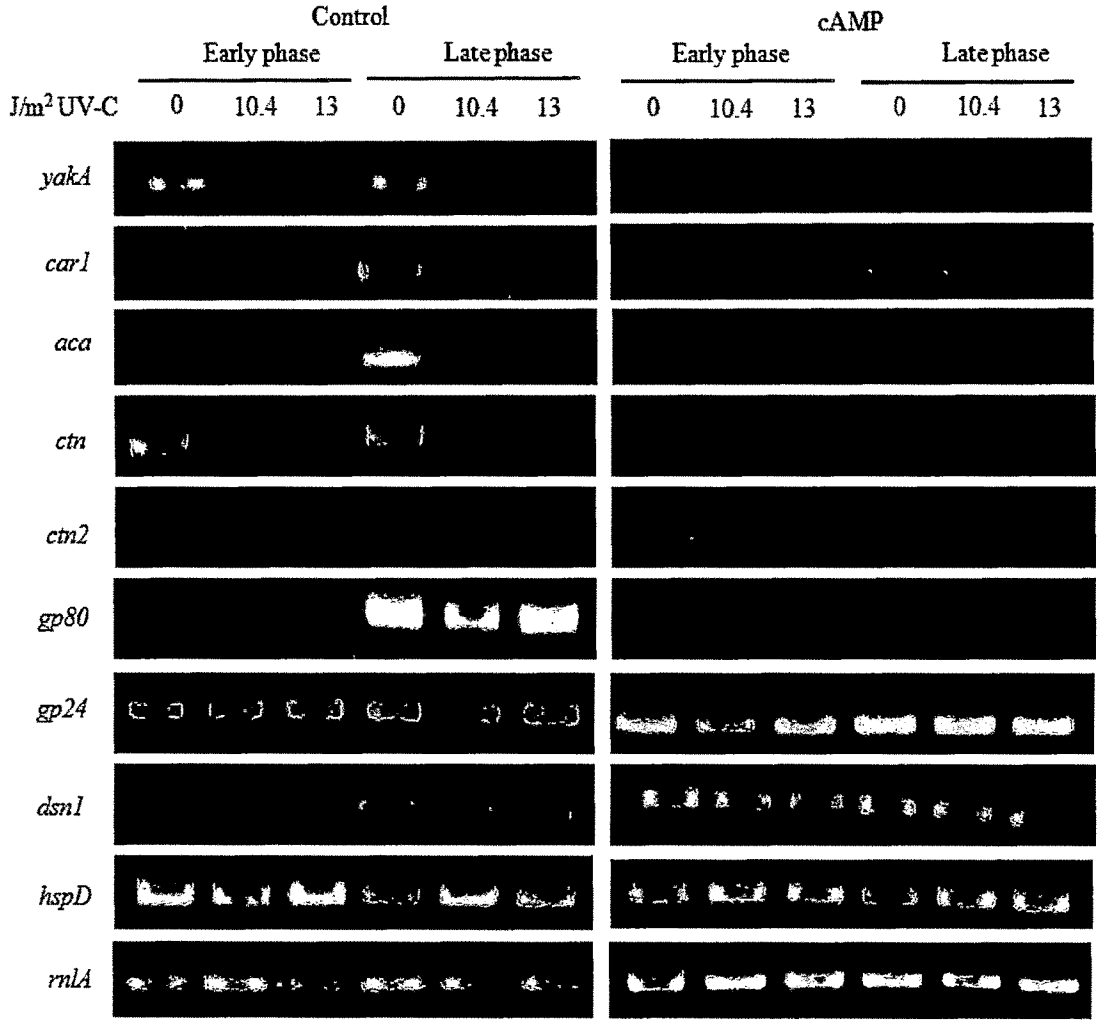

Figure 7.5: Irradiation with UV-C affects the mRNA expression of various genes involved in *D. discoideum* development. Expression of cAMP receptor- *car1*, adenylyl cyclase A- *aca* and *yakA* were found to be decreased in UV-C treated cells as compared to heat shock protein *hspD* the internal control rnlA (mitochondrial rRNA IG7). Exogenous cAMP restored *car1* and *aca* without affecting *yakA* and *hspD*.

The results clearly suggest that *D. discoideum* cells exposed to UV-C irradiation showed a dose dependent increase in the production of nitric oxide. Also inhibition of iNOS could restore cAMP levels partially (Fig. 7.3). Thus increased NO generation affects the signaling in UV-C exposed cells thereby impeding the development.

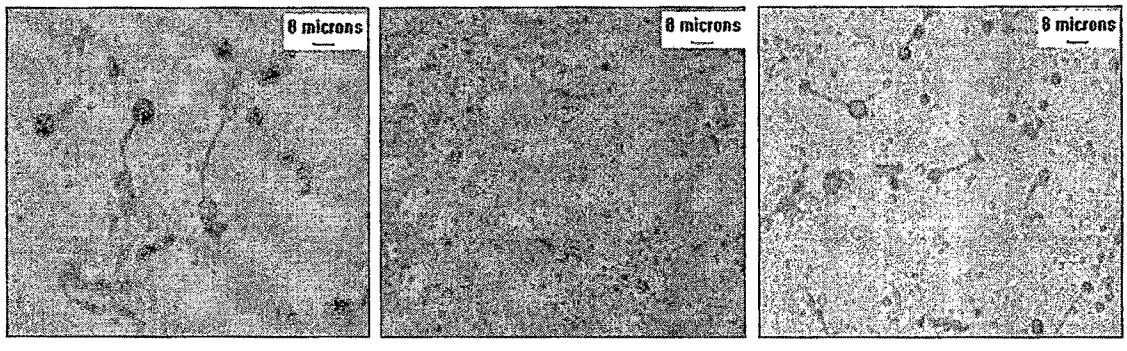

Figure 7.6: Development of UV-C irradiated cells pretreated with iNOS inhibitor. L-NIO treated cells could develop like control untreated *D. discoideum* cells within 24 hours. Photographs were captured at 4X magnification 24 hours after plating the cells. Results are representative of three independent experiments.

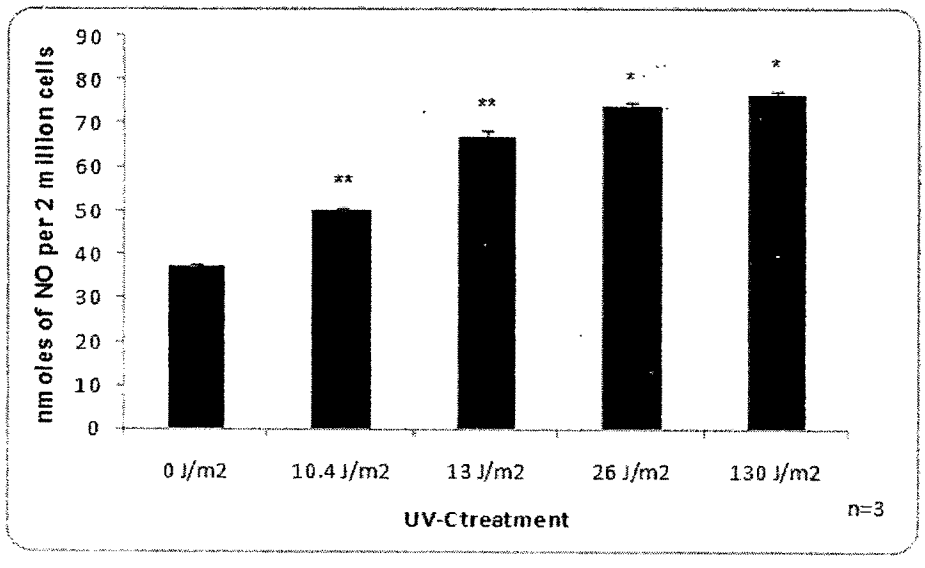

Figure 7.7: Nitric oxide generation increases in UV-C treated *D. discoideum* cells. NO generation was estimated 30 minutes after UV-C treatment by Griess method and was found to increase with increasing doses of UV-C. Results are the mean ± SE of three independent experiments. * p value <0.05, * p value <0.01, as compared to immediate lower dose.

7.3. Discussion

Signal transduction is involved in nearly all physiological processes, and defects in signal transduction pathways often lead to disease conditions. These processes are

relatively difficult to study in complex multicellular organisms; nevertheless some signaling pathways are conserved throughout the eukaryotic evolution (Parent and Devreotes, 1996). Counterparts of mammalian G protein-linked signal transduction events essential for chemotaxis, cell aggregation, morphogenesis, gene expression, and pattern formation are found in *D. discoideum* (Manahan *et al.*, 2004). Interestingly *D. discoideum* chemotaxis process resembles chemokine mediated chemotactic movement of leukocytes towards infected area (van Es and Devreotes, 1999). In this study an attempt to explicate UV-C mediated toxicity during development has been made.

Despite high resistance, *D. discoideum* cells exhibited complete block in the development (Table 7.1) when exposed to 13 J/m^2 UV-C. Although cells exposed to 10.4 J/m^2 develop, the number and size of the fruiting bodies is much less compared to control in addition to the observed delay. Mixing UV-C exposed cells with healthy cells evade the developmental block induced by irradiation (Fig.7.1 and Table 7.2). Recovery of development of UV-C irradiated cells in the mixing experiment notifies that UV-C induced developmental defect is probably due to impaired extracellular signaling where either the production or the response to the signal is being affected. Local cAMP and cGMP reciprocally regulate the differentiation process in axon and dendritic formations neurons as reported by Shelly *et al* (2010). Similarly in *Dictyostelium* development, cAMP being the key component of chemotaxis, controls developmentally regulated gene expression and hence development. Any defect in generation of cAMP pulses or its sensing would affect aggregation and thus leads to delay or arrest in development. This assumption is supported by the observed restoration of development of UV-C exposed cells with exogenous cAMP (Fig. 7.2 and Table 7.3). Our results are in accordance of Anjard *et al* (2000) who showed that the cells with disrupted *acaA* did not secrete cAMP and were unable to chemotactically signal each other unless pulsed with extra cellular cAMP or genetically engineered to over express the catalytic subunit of the cAMP-dependent protein kinase A (PKA) (Wang and Kuspa, 1997). Also, cAMP levels in UV-C treated cells are found to be decreased in a dose dependent manner (Fig. 7.3). These experiments confirm that UV-C affects development by impairing cAMP mediated signaling.

Chemotaxis experiment done using exogenous cAMP connotes that UV-C treated cells also failed to chemotax towards cAMP (Fig. 7.4). Gene expression studies showed that UV-C alters the expression of *car*1 and *aca* in addition to that of *yak*A and *countin*

(Fig. 7.5). The expression of *hsp*D, a heat shock protein, glycoproteins (*gp*24 and *gp*80) and *discoidin*I, a protein involved in adherence to substratum and *countin* 2 (another component of counting factor complex) were not affected suggesting the UV-C induced defects during developmental and gene expression are specific. It is documented that cAMP levels regulate expression of aggregation genes and adenylyl cyclase A (*aca*), as well as its activation and adaptation. These long term responses of cAMP are mediated *via* cAR1 receptor (Mann and Firtel, 1987; Mir *et al.*, 2007). YakA is a crucial protein for the growth to differentiation transition (GDT) (Mir *et al.*, 2007). Effect of UV-C on the expression of *aca* and *car*1 could be either direct or *via* altered YakA. Exogenous cAMP could restore *aca* and *car*1 expression however it does not affect decreased *yak*A that lies upstream to cAMP in the signaling pathway and that of *countin* which is known not to be affected by cAMP. Thus chemotaxis assay and gene expression studies imply that initial sensing and subsequent production of cAMP are getting affected under UV-C irradiation. Independent or cumulative effect of these two factors may seize the process of development.

UV-A and UV-B irradiation induces NO generation in keratinocytes which further stimulates the melanogenesis (Romero-Graillet *et al.*, 1997). Additionally, increased NO inhibits some isoforms of adenylate cyclases (especially AC5 and AC6) in mammalian cells (Freeman *et al.*, 2004). Interestingly probing the development of UV-C treated cells with iNOS (inducible nitric oxide synthase) inhibitor, L-NIO (Fig. 7.6) and estimation of NO (Fig. 7.7) during development supports the notion that UV-C induces NO generation via iNOS in *D. discoideum*. On the other hand it has been reported that *yak*A mutants are hypersensitive to NO generating compounds and oxidative stress (Taminato *et al.*, 2002). Tao *et al.*, (2006) reported that NO is a signaling molecule for *D. discoideum* cells and physiological or environmental conditions that enhance external NO levels will delay the initiation of cAMP pulses, which are essential for cell differentiation (Tao et al., 1996). Thus it could be concluded that UV-C irradiation affects expression of *yak*A, *aca* and *car*1 and hence cAMP pulses *via* increased production of NO (Fig. 7.8) thereby leading to a delayed or blocked development. Addition of cAMP corrects some of the changes and restores the development. cGMP is by itself a signaling molecule which is involved in the pseudopod formation during *Dictyostelium* development which is under cAMP regulation

In conclusion, these studies emphasize that despite high resistance of unicellular phase against oxidative stress, gamma and UV radiation, mild dose of UV-C was

sufficient to prevent differentiation in *D. discoideum* by interfering with the cAMP signaling pathway. UV-C brings about this defect in cellular signaling by affecting the expression of various developmentally regulated genes.

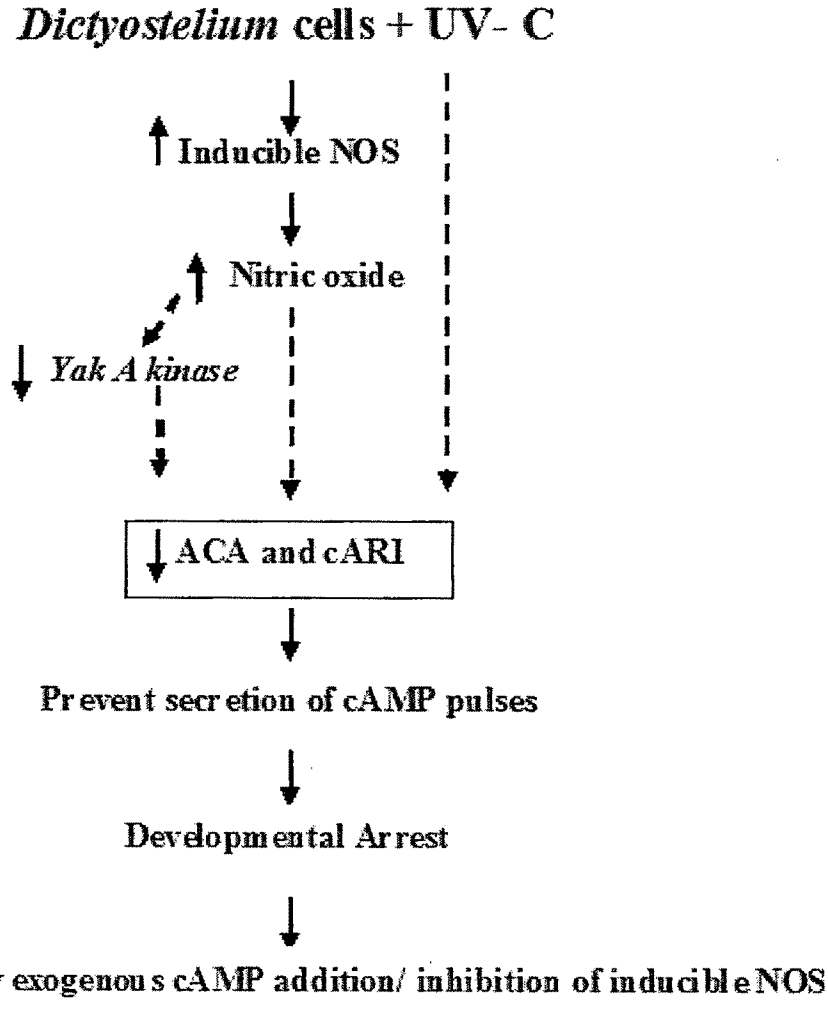

Figure 7.8: Mechanism of UV-C induced defects in *D. discoideum* development *via* cAMP signaling. UV-C increases NO generation *via* inducible NO synthase and affects expression of yakA. cAMP signaling gets affected due to reduced *car1* and *aca* expression caused by reduced YakA, increased NO or directly due to UV-C ultimately hampering *Dictyostlieum* development. Exogenous cAMP and iNOS inhibitor rescue UV-C treated *Dictyostelium* cells from these chemotactic defects.

7.4. References

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