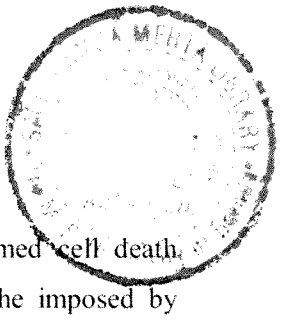
Synopsis



Introduction

Eukaryotic cell death has been classified into necrosis and programmed cell death (PCD). During necrosis cells die as a result of bioenergetic catastrophe imposed by external conditions. Apoptosis, a type of PCD is an evolutionarily conserved cell suicide program implicated in the removal of unwanted cells and is essential for development, maintenance and perpetuation of cellular integrity and tissue homeostasis. Apoptosis is characterized by certain biochemical and morphological changes which include among others mitochondrial membrane potential changes, early translocation of phosphatidylserine to outer leaflet of plasma membrane, caspase activation, oligonucleosomal DNA fragmentation and apoptotic body formation (Hengartner, 2000). However, occurrence of cell death upon caspase inhibition suggests that cells undergo death by employing certain caspase independent nonapoptotic cell death pathways. These alternate pathways are essential to protect the organism against potential harmful cells when caspase-mediated mechanisms fail. One such alternative non-apoptotic form of programmed cell death is paraptosis (Sperandio *et al.*, 2000). Paraptotic cell death is characterized by cytoplasmic vacuolization, mitochondrial swelling, absence of caspase activation as well as oligonucleosomal DNA fragmentation (Katoch *et al.*, 2002; Sperandio *et al.*, 2000). MAPK (Mitogen activated protein kinase) pathways are also reported to be involved in paraptotic cell death processes (Sperandio *et al.*, 2004). Interestingly, both types of cell death i.e., apoptosis and paraptosis are reported to exist in mammalian cells. Although the apoptotic cell death is well characterized, the biochemical and molecular aspects of paraptotic cell death are yet to be fully understood.

Cell death can be induced by a number of agents including reactive oxygen species (ROS) (Hasnain *et al.*, 1999; Mohan *et al.*, 2003; Sah *et al.*, 1999). ROS, among other effects cause DNA damage and thus act as potent apoptotic stimuli. Poly ADP-ribose polymerase (PARP), a nuclear enzyme gets activated in response to DNA damage and helps in DNA repair (Burkley, 2001). PARP-1, a 116 kDa protein (Smith, 2001) functions as a DNA damage sensor and gets activated consequent to DNA damage. Activated PARP cleaves its substrate NAD^+ and transfers ADP-ribose to several target proteins including PARP itself (Burkley, 2001). PARP activation/over activation may result in necrotic / apoptotic / paraptotic cell death, and the type of cell death depends on the cell type as well as nature of the stimulus (Virag, 2006). While PARP is sentinel in the regulation of cellular DNA repair processes and cell death under DNA damage, its

own function can be modulated by several means. The mechanism by which PARP activation leads to cell death appears to be linked with rapid utilization of cellular NAD^+ during formation of poly ADP-ribose (PAR) residues. PAR has a fast turn over rate, with a half life of around 1 minute due to its rapid degradation by poly (ADP-ribose) glycohydrolase (PARG). Such a regulation could play a very important role in deciding the fate of a cell. Role of PARP in apoptotic cell death in several eukaryotic systems is well characterized, but the role of PARP and PARG in paraptotic cell death is yet to be addressed.

Downstream to PARP activation, a cascade of events occurs wherein, mitochondria are known to play a central role (Hong *et al.*, 2004). Certain mitochondrial killer proteins such as apoptosis inducing factor (AIF) (Susin *et al.*, 1999) and endonuclease G have been implicated in the execution of paraptotic cell death (Wang *et al.*, 2004). The cell death pathways initiated by PARP-1 activation are mediated by AIF. Also neutralizing antibodies to AIF block the PARP-1 dependent cell death (Yu *et al.*, 2006). Taken together, these results indicate that AIF is an essential downstream effector of the cell death programs that are initiated by PARP.

Dictyostelium discoideum that lacks caspases (Olie *et al.*, 1998) has been used as a model system in this study to explore the role of PARP in paraptosis, necrosis and development. Nine potential PARP genes have been identified in *D. discoideum* (Otto *et al.*, 2005). Despite of its importance as a model organism for developmental studies and paraptotic PCD, information on the role of PARP in *D. discoideum* cell death and development is limited. Therefore, the present study describes the events during oxidative stress induced PARP mediated cell death and development in *D. discoideum* when the action of activated PARP is blocked with a potent PARP inhibitor benzamide as well as by PARP antisense.

Objectives of the present study

- Role of Poly ADP-ribose polymerase (PARP) and Poly (ADP-ribose) glycohydrolase (PARG) in oxidative stress induced cell death in *D. discoideum.*
- Proteases involved during oxidative stress induced paraptosis and necrosis in *D. discoideum.*
- Role of PARP in oxidative stress induced changes in *D. discoideum* growth and development.
- Effect of cAMP and glutathione on oxidative stress induced *D. discoideum* development.
- Down-regulation of PARP by antisense and its effect on oxidative stress induced cell death and development in *D. discoideum.*

Role of PARP and PARG in oxidative stress induced cell death:

Reactive oxygen species (ROS) at low doses act as signaling molecules, however, at high doses they are known to cause DNA damage that results in the activation of PARP. The role of PARP has been elucidated in *D. discoideum* subjected to oxidative stress induced by exogenous application of oxidant such as cumene H2O2 and in vivo generation of ROS by treatment of cells with hydroxylamine, a catalase inhibitor.

Interestingly, oxidative stress resulted in PARP activation within 5 minutes which is inhibited by benzamide. Dose dependent effect of hydroxylamine (HA) and cumene H2O2 was carried out to select paraptotic and necrotic doses. Annexin V-FITC and PI dual staining results suggested that 2.5 mM HA and 0.05 mM cumene H2O2 were necrotic as both phosphatidylserine (PS) exposure and Propidium Iodide (PI) staining were seen simultaneously, whereas 1 mM HA or 0.03 mM cumene H2O2 stress yielded paraptotic cell death as PS exposure was visible at 5 hours post HA or cumene H2O2 stress and plasma membrane integrity was lost after/at 12 hours. Thus HA or cumene H2O2 at different concentrations can induce different types of cell death in *D. discoideum.* Cellular ROS levels were monitored by fluorimetry using Dichloro fluorescein diacetate (DCFDA) dye and were found to be increased in a dose dependent manner within 5-10 minutes of HA and cumene H2O2 exposure. DNA damage observed by immunofluorescence indicates that oxidative stress induces γH2AX phosphorylation, which in turn leads to PARP activation. Peak PARP activity was observed at 10 minutes of 1 mM HA or 0.03 mM cumene H2O2 stress and at 5 minutes in 2.5 mM HA or 0.05

mM cumene H2O2. As a consequence of PARP activation, cellular NAD+ and ATP levels were reduced to 60% and 40% respectively within 1 hour of 1 mM HA and 0.03 mM cumene H2O2 stress; and a reduction up to 80% in the cellular NAD+ and ATP levels was seen within 1 hour of 2.5 mM HA or 0.05 mM cumene H2O2 stress.

Subsequent to PARP activation mitochondrial membrane potential (MMP) change was observed which was initiated at 3 hours and was almost lost by 5 hours post 1 mM HA or 0.03 mM cumene H2O2 stress. In necrosis the MMP change occurred at 2 hours, much earlier compared to paraptotic dose. MMP changes further led to AIF release which accounts for the nuclear changes during paraptosis. During paraptotic cell death, downstream to MMP change AIF release was observed at 5 hours. Furthermore, AIF upon translocation to nucleus caused large scale DNA fragmentation at 6 hours, a hallmark feature of paraptosis. Oxidative stress induced cell death in *D. discoideum* is caspase independent as no caspase activity was detected. Also cell death induced by oxidative stress was not prevented by caspase-3 specific inhibitor (DEVD-CHO) and broad caspase inhibitor (ZEVD-fmk). On the other hand, during necrotic cell death nuclear translocation of AIF was observed at 3 hours. Thus, like other cell death model systems *D. discoideum* cells also showed AIF translocation during paraptotic and necrotic cell death. Interestingly, *D. discoideum* exhibited formation of apoptotic vesicles during oxidative stress induced paraptotic cell death. These vesicles consisted of DNA surrounded by a membrane as they could be stained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) and DPH (1,6-Diphenyl-1,3,5-hexatriene). However, vesicles were not detected during necrotic cell death.

To elucidate the role of PARP during oxidative stress induced cell death in *D. discoideum*, PARP inhibition studies were attempted by using benzamide, a known PARP inhibitor. Pretreatment of *D. discoideum* cells with benzamide prevented the activation of PARP and rescued the NAD+ and ATP levels by ~30% and ~35% respectively at paraptotic dose, thus maintaining the cellular energy levels. This prevention in oxidative stress mediated alterations in energy levels by PARP inhibition indicates that PARP activation results in energy derangement. Benzamide also intercepted the loss of NAD+ and ATP during necrosis. The data suggests that PARP inhibition resulted in partial recovery of ATP and hence shifts the necrotic mode of cell death to paraptotic type. Our results showed that downstream to PARP activation with 1 mM HA or 0.03 mM cumene H2O2 stress MMP change was observed by 3 hours. Benzamide delayed the change in MMP by 7 hours which correlated with the delay seen

in PS exposure. Conversely, benzamide had no effect on MMP changes that occurred during necrotic cell death, suggesting multiple distinct cell death pathways are employed by the cell. Oxidative stress results in the activation of various kinases like MAPK (Sperandio *et al.*, 2004). Interestingly, PARP mediated paraptotic and necrotic cell death pathways in *D. discoideum* were found to be delayed upon treatment with the inhibitors of MEK-1 and MEK-2 kinases.

AIF is known to be an important executioner of caspase independent cell death. ROS mediated PARP activation is necessary for the AIF release from mitochondria (Kang *et al.*, 2004). Our findings support this hypothesis as PARP inhibition partially prevented the release of AIF from mitochondria and also delayed DNA fragmentation. PAR polymer itself, PARylated proteins or NAD+ depletion (Hong *et al.*, 2004) could lead to mitochondrial permeability transition linking PARP activation to AIF translocation. Benzamide is a potent rescuer of cell death induced by 1 mM HA or 0.03 mM cumene H2O2. Nevertheless, at 2.5 mM HA or 0.05 mM cumene H2O2 dose, inhibition of PARP hyperactivity switches the cell death to paraptotic type though some of the cell death parameters were not reversed. These results suggest that oxidative stress induced paraptosis is mediated by PARP. Interestingly, our results also suggest that poly (ADP-ribosyl)ation is not involved at the late stage in necrotic cell death; and lysosomes might be involved in causing MMP change and downstream events including cell dismantling in absence of caspases. Our study also highlights that necrosis occurs in a programmed fashion where proteases cause MMP changes followed by plasma membrane rupture and early loss of plasma membrane integrity.

Poly ADP-ribose glycohydrolase (PARG) regulates PARP activity (D' Amours, 1999) hence we have attempted to explore the involvement of PARG in PARP mediated cell death. Inhibition of PARG using gallotannin (15 μM) effectively reduced oxidative stress (1 mM & 2.5 mM HA; 0.03 & 0.05 mM cumene H2O2) induced *D. discoideum* cell death. Gallotannin pretreatment significantly reduced the activation of PARP, MMP changes and AIF translocation to nucleus. Cellular NAD+ levels were depleted during HA and cumene H2O2 treatments. Also PARG inhibition resulted in lower NAD+ consumption during oxidative stress induced cell death.

Inhibition of PARG has delayed paraptosis and also switched necrosis to paraptosis. These results support PARP inhibition data and also suggest that paraptosis which was

induced by oxidative stress can be intercepted by PARG inhibition. Our results suggest that PARG could influence PARP mediated cell death and PARG activity is protective in function during oxidative stress induced cell death.

Thus we conclude that PARG inhibitors could delay oxidative stress induced cell death, suggesting that PARG activity is necessary for PARP mediated cell death to occur.

Proteases involved in cell dismantling

a) During oxidative stress induced paraptosis:

Caspase activation is reported during oxidative stress induced cell death in all metazoans (Earnshaw *et al.*, 1999). However, caspase activation could not be seen in this study suggesting that HA or cumene H2O2 induced cell death in *D. discoideum* is caspase independent and is of paraptotic type. Absence of caspases led us to apprehend and characterize caspase independent cell dismantling. The role of proteases in MMP changes during paraptosis was studied using protease inhibitors. Protease inhibition results suggested that lysosomal proteases are not involved in the early phase of paraptotic cell death. Nevertheless, during the late stage of paraptosis, lysosomes might be involved in dismantling the cell. Cathepsin D, a lysosomal protease could be involved in dismantling the cell (Turk and Stoka, 2007) during oxidative stress induced paraptotic cell death. PARP activation also perturbs calcium homeostasis (Miller, 2004). Calpain inhibition with ALLN partially rescued the paraptotic cell death, MMP changes and AIF translocation to nucleus. However, Cathepsin D inhibition alone with Pepstatin A neither could delay the cell death nor showed any effect on MMP change. These results implicate that Calpains function upstream while lysosomal proteases function downstream to mitochondrial changes during HA or cumene H2O2 induced paraptotic cell death and their inhibition prevented dismantling the cell leading to delayed paraptotic mode of cell death. Furthermore, our results suggest that Calpains and Cathepsin D which are instrumental in dismantling the cell during paraptotic cell death act downstream to PARP. Thus PARP, AIF, Calpains and Cathepsin D are the key players in *D. discoideum* paraptotic cell death.

b) During oxidative stress induced necrosis:

Recent reports suggest that necrosis also occurs in a sequential way termed as necroptosis. Necroptosis is a programmed necrosis which involves extensive network of genes (Hitomi *et al.*, 2008). Our results in this study showed that necrosis occurs in a

programmed fashion where proteolysis involves Calpains and lysosomal proteases. Partial rescue in MMP was seen with protease inhibitor cocktail in necrotic cell death. No effect was observed on 2.5 mM HA or 0.05 mM H2O2 induced MMP changes by ALLN, a Calpain inhibitor alone. Calpain and Cathepsin D inhibition collectively prevented the MMP changes in necrotic cell death. Thus during necrosis lysosomal involvement is seen in the early stage also.

Role of PARP in oxidative stress induced changes in growth and development

Reactive oxygen species (ROS) at low doses act as signaling molecules, however, at high doses they are known to cause DNA damage that results in the activation of PARP. As *D. discoideum* exhibits multicellularity upon nutrient starvation and is a good model system for developmental studies, it would be of interest to study the role of PARP during its growth and development.

Oxidative stress (HA or cumene H2O2) showed dose dependent delay on *D. discoideum* growth which could also be partially intercepted by benzamide. PARP inhibition conferred protection against 2.5 mM HA (LD50) and 0.05 mM cumene H2O2 (LD50) induced delay in growth. The lag phase in benzamide pretreated cells was reduced from 60 to 50 hours, followed by a longer log phase.

Oxidative stress induced delay in *D. discoideum* development which was also partially intercepted by benzamide. Our results suggest that 2.5 mM HA / 0.05 mM cumene H2O2 delayed the development due to cell cycle arrest while at 4 mM HA dose due to 90% cell death, cell density was not sufficient for aggregation leading to complete developmental arrest. HA treated *D. discoideum* cells exhibited dose dependent decrease in the number and size of fruiting bodies as compared to control cells. Our results showed that *D. discoideum* exhibits basal PARP activity and its inhibition by benzamide (1-3 mM) did not affect the development. However, 4 mM benzamide treated *D. discoideum* cells were unable to differentiate properly and exhibited delayed development, especially during prespore and prestalk formation. These results suggest that lower doses of benzamide have no deleterious effects on *D. discoideum* development.

In higher eukaryotic cells, PARP contributes to cell homeostasis under mild stress conditions, and conversely, during conditions of moderate/ severe cellular stress, PARP over activation leads to cell death, which results in several disease conditions. Pharmacological inhibition of PARP during moderate/ severe cellular stress is beneficial; however, the consequences of such inhibition for genomic integrity are not yet

understood. Hence long term effects of PARP inhibition on *D. discoideum* development under oxidative stress was also studied, by reviving the spores and monitoring the growth and doubling time for the next two generations. It was found that PARP inhibition (1 mM benzamide) did not affect spore germination in normal cells. However, when the cells were allowed to develop after exposure to oxidative stress (2.5 mM HA), the spores remained dormant for longer time taking 56 hours to germinate as compared to the control cell spores. Conversely, in case of the cells that were allowed to develop after exposure to oxidative stress (2.5 mM and 4 mM HA) with PARP inhibition, the spores showed faster germination (32 hours and 60 hours) compared to cells exposed to oxidative stress alone (2.5 mM HA). Interestingly the amoebae thus formed due to spore germination (2.5 and 4 mM HA with and without PARP inhibition) exhibited normal development, suggesting that second generation cells have overcome the effect of oxidative stress. Thus, our results demonstrate that spores formed under HA stress exhibit significant delay in germination compared to benzamide pre exposed HA stressed cells. However, second generation cells showed normal development signifying that PARP inhibition has no deleterious effect on *D. discoideum* development (Rajawat *et al.*, 2007).

Effect of cAMP and Glutathione on oxidative stress induced *D. discoideum* development

Multicellular development in *D. discoideum* is initiated upon starvation and is controlled by a number of extracellular signaling molecules including cAMP (Mir *et al.*, 2007). As benzamide pretreatment could partially restore the oxidative stress induced delayed development, we proposed that HA and cumene H2O2 might affect the initial cAMP secretion. This was confirmed by monitoring cAMP levels and exogenous addition of cAMP. cAMP levels were found to be reduced in a dose dependent manner in cumene H2O2 stress while significant changes were not observed with HA. Exogenous cAMP treatment to the cells after oxidative stress resulted in restoration of development delayed by 2.5 mM HA and 0.05 mM cumene H2O2. 1 μM cAMP addition also resulted in an increase in the number of fruiting bodies. However, no effect was observed on developmental arrest caused by 4 mM HA. Gene expression studies revealed the changes in expression of certain genes (aca, car 1 and countin) involved in the early development during oxidative stress. Expression studies along with cAMP estimation and addition

experiments showed that oxidative stress leads to changes in the development of *D. discoideum* due to altered cAMP signaling and PARP activation.

Antioxidants are known to have crucial roles in the differentiation of *D. discoideum* (Choi *et al.*, 2006). Cells preexposed to antioxidants such as GSH (1 mM for 12 hour) and subjected to development, showed 6 hours delay at the loose aggregation stage. Antioxidant pretreatment rescued *D. discoideum* cells from oxidative stress induced cell death and growth effects, suggesting that HA is toxic to the cells via H2O2 generation. HA or cumene H2O2 induced developmental delay was partially rescued by glutathione treatment. Thus, our results suggest that moderate level of ROS are possibly acting as signaling molecules during *D. discoideum* development, whereas high levels of ROS (in situ generated H2O2 upon HA treatment) are toxic for its development.

Down-regulation of PARP by antisense

To support our results and to rule out any non specific effect of PARP inhibitor benzamide, we have attempted down regulation of PARP by antisense using inducible and constitutive promoters, and to study its effect on oxidative stress induced cell death in *D. discoideum*. PARP-1 consists of three domains i.e., N-terminal DNA binding domain, the auto-modification domain and the C-terminal catalytic domain. However, there is more than one type of PARP in *D. discoideum* (Olie *et al.*, 1995); they have a catalytic domain which is highly conserved. Hence, the PARP catalytic domain has been chosen as a target for the antisense RNA. The antisense clones under inducible and constitutive promoters (pVEII and pTX) were transformed in *D. discoideum* cells. PARP down-regulated cells with pVEII clone were induced with conditioned medium. The results on PARP down-regulated cells support our PARP inhibition studies with benzamide. Briefly, our results suggest that PARP activity was down-regulated in these cells upon exposure to different doses of oxidants, MMP changes were also restored, 60% rescue was observed in paraptotic cell death, and necrotic cell death was also switched to paraptotic type. Downregulation of PARP is confirmed by RT-PCR.

The present study demonstrates several important findings that help in understanding the sequence of events happening during oxidative stress induced PARP mediated *D. discoideum* cell death. PARP plays an important role in all types of metazoan cell death. Nevertheless, we have studied the role of PARP during oxidative stress induced cell death in *D. discoideum*, an organism which is at the crossroads of unicellularity and

multicellularity. Oxidative stress is a known inducer of cell death and severity of the stress determines whether cells undergo necrosis or apoptosis (Palomba *et al.*, 1996). Upon PARP inhibition during paraptosis, the cells show only partial rescue implying PARP is not the sole deciding factor. We have established *D. discoideum* as a model system for oxidative stress induced paraptotic and necrotic cell death, which can be extrapolated to higher eukaryotes. This is the first report where the involvement of PARP and the downstream events during oxidative stress induced cell death in *D. discoideum*, an ancient eukaryote, are established.

Thus we conclude that PARP and AIF are the major players governing *D. discoideum* cell death kinetics during paraptosis and necrosis induced by oxidative stress. Oxidative stress induced PARP mediated cell death can be intercepted by PARP down-regulation or by using PARP inhibitor benzamide. MEK signaling pathway is also involved in oxidative stress induced paraptotic and necrotic cell death. Calpains and Cathepsin D are the major proteases involved in cell dismantling during paraptotic cell death that serve as proxy to caspases. Our results provide insights for designing novel drugs to control diseases involving PARP.

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