# Concluding Remarks

Balance between cell division and cell death is of utmost importance for the development and maintenance of multicellular organisms. Disorders of either process have pathologic consequences and can lead to disturbed embryogenesis, neurodegenerative diseases, or the development of cancer. Successful therapy for prevention and treatment of such diseases entails a fine understanding of normal regulation of cell death and cell survival. Necrosis and apoptosis are two distinct and well characterized forms of cell death. During necrosis cell dies as a result of bioenergetic catastrophe imposed by external conditions. Apoptosis is an evolutionarily conserved cell suicide program for the removal of unwanted cells and is essential for development, maintenance and perpetuation of cellular integrity and tissue homeostasis. It is characterized by certain biochemical and morphological changes which include among others early translocation of phosphatidyl serine to outer leaflet of plasma membrane, caspase activation, changes in mitochondrial membrane potential, oligonucleosomal DNA fragmentation and apoptotic body formation (Hengartner, 2000). In addition to these well established cell death programs, there are programs that use common death machinery in specially defined, carefully choreographed way. The observation that cells do exhibit these alternate pathways to undergo cell death upon caspase inhibition has evoked interest in caspase-independent cell death pathways. These pathways are essential to protect the organism against potential harmful cells when caspase-mediated mechanisms fail. A novel caspase independent cell death i.e., paraptosis has been identified (Sperandio *et al.*, 2000) which 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; Wyllie and Golstein, 2001). Interestingly, both types of cell death i.e., apoptosis and paraptosis are seen in mammalian cells. Though the apoptotic cell death is well characterized, the biochemical and molecular aspects of paraptosis are yet to be fully understood. Present study aimed at deciphering the role of PARP and AIF during (a caspase independent model) *D. discoideum* cell death induced by different stress conditions.

## PARP and AIF during paraptosis in *D. discoideum*

In response to apoptotic stimuli Poly ADP-ribose polymerase (PARP), a nuclear enzyme gets activated within minutes and helps in DNA repair (Burkley, 2001; de Murcia *et al.*, 1994; Lautier *et al.*, 1993; Shall and de Murcia, 2000). Activated PARP cleaves its substrate $NAD^+$ and transfers ADP-ribose units to several target proteins including PARP

itself (Burkley, 2001; de Murcia *et al.*, 1994; Lautier *et al.*, 1993; Shall and de Murcia, 2000; Smulson *et al.*, 2000). PARP activation/over activation may result in necrotic/ apoptotic/ paraptotic cell death, and the type of cell death depends on the experimental conditions such as the cell type as well as nature of the stimulus (Virag, 2006). PARP mediated necrotic cell death is reported during ischemia reperfusion injury (Eliasson *et al.*, 1997; Endres *et al.*, 1997), inflammatory injury and reactive oxygen species induced injury (Szabo and Dawson, 1998). Downstream to PARP activation a cascade of events occurs wherein, mitochondria are known to play a central role (Hong *et al.*, 2004). Certain mitochondrial proteins such as cytochrome c, DIABLO (Fadeel and Orrenius, 2005) Apoptosis Inducing Factor (AIF) (Susin *et al.*, 1999) and endonuclease G have been implicated in the execution of cell death (Wang *et al.*, 2004). In addition to PARP we also focused on AIF during our study.

AIF is proposed to have a pivotal role in the execution of cell death. AIF, an evolutionarily conserved mitochondrial inter membrane space flavoprotein, is sentinel in the functioning of Electron Transport Chain (ETC) in addition to its contribution to cell death programs namely paraptosis and necrosis. AIF after translocation to nucleus induces large scale DNA fragmentation (Loeffler *et al.*, 2001; Joza *et al.*, 2001 ; Susin *et al.*, 1999). AIF performs critical cellular functions in a coordinated fashion, the mechanism of which still remains elusive.

*Dictyostelium discoideum,* the model organism does not have caspases (Olie *et al.*, 1998) which makes this organism a good model system to study paraptotic mechanism. Nine potential PARP genes have been identified in *D. discoideum* (Otto *et al.*, 2005). Despite its importance as a model organism for paraptosis, information on the role of PARP and AIF in *D. discoideum* cell death is limited.

Present study describes the events during *D. discoideum* cell death induced by different stresses such as UV-C, staurosporine, starvation and the interception by benzamide, a PARP inhibitor. Our results emphasize that cell death stimuli like UV-C and starvation lead to PARP activation causing depletion in the cellular $NAD^+$ and ATP levels. Subsequent to PARP activation mitochondrial membrane potential (MMP) changes occur followed by release of AIF from mitochondria. Released AIF translocates to nucleus where it leads to large scale DNA fragmentation, a hallmark feature of paraptosis. Hence, this study reinforces the earlier observation that PARP activation leads to translocation of AIF from the mitochondria to nucleus (Yu *et al.*, 2002). PARP

chemical inhibition, PARP genetic ablation, AIF knockdown, or neutralizing AIF using anti-AIF antibodies prevent AIF translocation to the nucleus and inhibit DNA damage mediated cell death in a variety of experimental paradigms (Lorenzo and Susin, 2007; Moubarak *et al.*, 2007; Xu *et al.*, 2006; Yu *et al.*, 2002). This indicates a pivotal role of mitochondrial AIF to translocate to nucleus and execute its function in PARP mediated cell death. Elucidation of this paraptotic pathway is of key importance, both in understanding the mechanism of PARP mediated cell death and also in identifying potential drug targets. However, involvement of PARP in paraptosis is not a universal truth. In other words, translocation of AIF does not require PARP activation. Our studies showed that PARP inhibition did not interfere with STS induced paraptosis. STS induced paraptosis is also characterized by AIF mediated DNA fragmentation which was not affected with PARP inhibition. Thus PARP is a dispensable player of paraptosis.

There are no known inhibitors for AIF. Bcl family of proteins regulate AIF release from mitochondria whereas Hsp70 and Scythe protein sequester AIF in cytosol after its release from mitochondria and prevent its nuclear translocation. To address the role of AIF in *Dictyostelium* paraptosis efforts were made to downregulate its expression. However, the dR cells showed survival defects probably due to its crucial role in the functioning of ETC. Hence further attempts should be made to study its paraptotic functions by moderate downregulation of AIF expression or by anti-AIF antibody transfection wherein its vital ETC functions would not be affected.

## PARP and differentiation in *D. discoideum*

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.

*D. discoideum* shows differential effects of oxidative and UV-C stress on development and spore germination as oxidaitive stress induced changes could be rescued by PARP inhibition, however many of the UV-C induced changes were not affected by PARP inhibition. This raises a question at the therapeutic significance of PARP inhibition in various oxidative stress and DNA damage related diseases, where PARP is known to be overactivated (Virag and Szabo, 2002). Hence the concept of PARP inhibition being beneficial in various DNA associated diseases should be considered cautiously. Also the

results point out that this organism in spite of being a lower eukaryote has the complex signaling machinery that deals with different stresses i.e., oxidative stress and UV-C in diverse ways. This fact emphasizes the importance of studies of signaling pathways in *D. discoideum* which is simple and easy to handle compared to mammalian cell lines.

## Links between PARP, AIF and cell death

PARP once activated leads to $NAD^+$ and ATP depletion (Virag and Szabo, 2002) and accompanied by changes in mitochondrial membrane potential (Cipriani *et al.*, 2005) followed by AIF release. However, the signal employed for nuclear and mitochondrial crosstalk has not yet been identified. Although studies indicate $NAD^+$ depletion, modified acceptor proteins and PAR polymer as potential candidates that could serve as the signals for induction of release of AIF from mitochondria, the results are conflicting (Hong *et al.*, 2004, Goto *et al.*, 2002). Yu *et al* (2007) showed PAR to be the signal leading to AIF release. Thus NAD depletion was considered to be of no significance for the AIF mediated DNA fragmentation however, recently it is reported that depletion in pyridine nucleotide post PARP activation is not only important in the release of AIF from mitochondria but also affects its association with DNA and hence influence its DNA cleaving activity (Sevrioukova and Churbanova, 2008). Interestingly DNA cleaving activity of AIF is mediated by NAD(P)H association with its oxidoreductase domain. This shows that there is link between PARP activation and AIF led DNA fragmentation as these two processes coordinate at multiple levels. Also the idea of the DNA binding domain and oxidoreductase domain being functionally independent or exclusive should be reconsidered.

The release of AIF is considered as a point of no return for paraptotic cell death which is independent of specific cysteine proteases i.e., caspases. It is reiterated that AIF is associated with large scale DNA fragmentation however, this would not be sufficient for the dismantling of the cells that are destined to die. Our calpain and cathepsin D inhibition studies show that these proteases could serve as surrogate caspases in paraptotic cell death mechanisms.

In addition to this there are certain important questions raised at the end of this study. In mammalian systems AIF release is known to be caused by mitochondrial destabilization which is regulated by Bcl family of proteins but interestingly *D. discoideum* does not have these proteins (Lam *et al.*, 2007). What alternate mechanism does this organism exploit to do the required function would be interesting to address.

Also PARP activation after DNA damage is mandatory, however in apoptosis and necrosis this is prevented by proteolytic inactivation of PARP. In STS induced cell death DNA is damaged although as a later event during the course of cell death; nevertheless, PARP seems to have no role in STS induced cell death. So it would be interesting to study the fate of PARP in STS induced cell death and also in paraptosis after the cell death signaling has been initiated from the nucleus. Third interesting issue is the half life and abundance of AIF in *D. discoideum*. Desmots *et al* (2008) have shown AIF to be a short lived protein. The survival defect of AIF dR cells was a daunting result; only if the turnover of the protein is fast could such results be obtained. This aspect could further be addressed.

In conclusion, present results provide insights for designing novel drugs to control diseases involving PARP and AIF, which are also involved in important cellular processes other than cell death. This study would help to identify better targets for inhibiting or inducing paraptotic cell death during diseased conditions. It also highlights the fact that *D. discoideum*, being a lower eukaryote, has different mechanisms to respond to different stresses similar to mammalian systems emphasizing the importance of this organism as a model for various studies.

## References

Burkley, A. (2001). Physiology and pathophysiology of poly(ADP-ribosyl)ation, *Bioessays. 23*, 795-806.

Churbanova, I. Y. and Sevrioukova, I. F. (2008) Redox-dependent changes in molecular properties of mitochondrial apoptosis-inducing factor, *J. Biol. Chem. 283*, 5622-5631.

Cipriani, G., Rapizzi, E., Vannacci, A., Rizzuto, R., Moroni, F., and Chiarugi, A. (2005) Nuclear poly(ADP-ribose) polymerase-1 rapidly triggers mitochondrial dysfunction, *J. Biol. Chem. 280*, 17227-17234.

de, M. G., Schreiber, V., Molinete, M., Saulier, B., Poch, O., Masson, M., Niedergang, C., and Menissier de, M. J. (1994) Structure and function of poly(ADP-ribose) polymerase, *Mol. Cell Biochem. 138*, 15-24.

de, M. G. and Menissier de, M. J. (1994) Poly(ADP-ribose) polymerase: a molecular nick-sensor, *Trends Biochem. Sci. 19*, 172-176.

Desmots, F., H. R. Russell, D. Michel, and P. J. McKinnon, (2008) Scythe regulates apoptosis-inducing factor stability during endoplasmic reticulum stress-induced apoptosis, *J. Biol. Chem. 283,* 3264-3271.

Eliasson, M. J., K. Sampei, A. S. Mandir, P. D. Hurn, R. J. Traystman, J. Bao, A. Pieper, Z. Q. Wang, T. M. Dawson, S. H. Snyder, and V. L. Dawson. (1997). Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia, *Nat. Med. 3*, 1089-1095.

Endres, M., Wang, Z. Q., Namura, S., Waeber, C., and Moskowitz M. A. (1997) Ischemic brain injury is mediated by the activation of Poly(ADP-ribose)polymerase, *J. Cereb. Blood Flow Metab. 17*, 1143-1151.

Fadeel, B. and Orrenius, S. (2005) Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease, *J of Internal Medicine 258*, 479–517

Goto, S., Xue, R., Sugo, N., Sawada, M., Blizzard, K. K., Poitras, M. F., Johns, D. C., Dawson, T. M., Dawson, V. L., Crain, B. J., Traystman, R. J., Mori, S., and Hurn, P. D. (2002) Poly(ADP-ribose) polymerase impairs early and long-term experimental stroke recovery, *Stroke 33*, 1101-1106.

Hengartner, M. O. (2000) The biochemistry of apoptosis, *Nature 407*, 770-776.

Hong, S. J., Dawson, T. M., and Dawson, V. L. (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling, *Trends Pharmacol. Sci. 25*, 259-264.

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death, *Nature 410*, 549-554.

Katoch, B., Sebastians, S., Sahdev, S., Padh, H., Hasnain, S.E., and Begum, R. (2002). Programmed cell death and its clinical implications, *Indian J. Exp. Biol. 40*, 513-524.

Lam, D., Levraud, J. P., Luciani, M. F., and Golstein, P. (2007) Autophagic or necrotic cell death in the absence of caspase and bcl-2 family members, *Biochem. Biophys. Res. Commun. 363*, 536-541.

Lautier, D., Lagueux, J., Thibodeau, J., Menard, L., and Poirier, G. G. (1993) Molecular and biochemical features of poly (ADP-ribose) metabolism, *Mol. Cell. Biochem. 122*, 171-193.

Loeffler, M., Daugas, E., Susin, S. A., Zamzami, N., Metivier, D., Nieminen, A. L., Brothers, G., Penninger, J. M., and Kroemer, G. (2001) Dominant cell death induction by extramitochondrially targeted apoptosis-inducing factor, *FASEB J. 15*, 758-767.

Moubarak, R. S., Yuste, V. J., Artus, C., Bouharrour, A., Greer, P. A., Menissier-de, M. J., and Susin, S. A. (2007) Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis, *Mol. Cell Biol. 27*, 4844-4862.

Olie R.A., Durrieu F., Cornillon S., Loughran G., Gross J., Earnshaw W.C. and Golstein P. (1998) Apparent caspase independence of programmed cell death in *Dictyostelium*, *Curr Biol. 8*, 955-958.

Otto, H., Reche, P. A., Bazan, F., Dittmar, K., Haag, F., and Koch-Nolte, F. (2005) In silico characterization of the family of PARP-like Poly(ADP-ribosyl)transferases (pARTs), *BMC Genomics. 6*, 139-161.

Shall, S. and de, M. G. (2000) Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model?, *Mutat. Res. 460*, 1-15.

Smulson, M. E., Simbulan-Rosenthal, C. M., and Boulares, A. H. (2000) Roles of Poly(ADP-ribosyl)ation and PARP in apoptosis, DNA repair, genomic stability and functions of p53 and E2F-1, *Adv. Enzyme regul. 40*, 183-215.

Sperandio, S., de Belle, I., and Bredesen, D. E. (2000) An alternative, nonapoptotic form of programmed cell death, *Proc. Natl. Acad. Sci. 97*, 14376-14381.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor, *Nature 397*, 441-446.

Szabo, C. and Dawson, V. L. (1998) Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion, *Trends Pharmacol. Sci. 19*, 287-298.

Virag, L., and Szabo, C. (2002) The therapeutic potential of poly (ADP-ribose) polymerase inhibitors, *Pharmacol Rev 54*, 375-429.

Virag, L. (2006) PARP-1 and the shape of cell death, *Springer Sciences* 141-152.

Wang, Y., Li, X., Wang, L., Ding, P., Zhang, Y., Han, W., and Ma, D. (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death, *J. Cell Sci. 117*, 1525-1532.

Wyllie and Golstein, (2001) More than one way to go, *Proc. Natl. Acad. Sci. 98*, 11-13.

Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor, *Science 297*, 259-263.