Programmed Cell Death in Procyclic Form *Trypanosoma brucei rhodesiense* - Identification of Differentially Expressed Genes during Con A Induced Death

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Trypanosoma brucei rhodesiense *can be induced to undergo apoptosis after stimulation with Con A. As cell death in these parasites is associated with* de novo *gene expression we have applied a differential display technique, Randomly Amplified Differential Expressed Sequence-Polymerase Chain Reaction (RADES-PCR) to the study of gene expression during Con A induced cell death in these organisms. Twenty-two differentially displayed products have been cloned and sequenced. These represent the first endogenous genes to be identified as implicated in cellular death in trypanosomatids (the most primitive eukaryote in which apoptosis has been described). Evidence for an ancestral death machinery, 'proto-apoptosis' in single celled organisms is discussed.*

Key words: *Trypanosoma brucei rhodesiense* - programmed cell death - apoptosis - differential gene expression - proto-apoptosis

Programmed cell death (PCD) and apoptosis comprise a well established genetically controlled cellular "suicide" mechanism in eukaryotes which leads to elimination of unnecessary or damaged cells. Originally described in mammals and later in *Caenorhabditis elegans*, a controlled death program is considered essential for the proper development and functioning of a cell system, organ, or for survival of the organism as a whole. Recently PCD/apoptosis has been described in several lower organisms including the slime mould *Dictyostelium discoideum* (Cornellion et al. 1994) and several single celled members of the parasitic protozoa: *Trypanosoma cruzi* (Ameisen et al. 1995); *T. brucei rhodesiense* (Welburn et al. 1996); *Leishmania L. amazonenis* (Moiera et al. 1996) and *Plasmodium falciparum* (Picot et al. 1997).

The demonstration of apoptosis in three members of the trypanosomatids has implications for our understanding of the origins of PCD in eukaryotic cell survival as these organisms represent the most primitive eukaryotes in which it has been described. The identification of genes that regulate cell suicide in these unicellular organisms is essential to address issues such as the social control of cell survival and whether unicellular eukaryotes share the same or similar regulators of cell suicide programmes with multicellular organisms (for reviews see: Ameisen 1996, Welburn et al. 1997).

It is well established in metazoan organisms that cell death is often an active process triggered by signals that induce crude biochemical changes in target cells resulting in many cases in changes in patterns of gene expression (Baudet et al. 1998). Several genes have been found to play a critical role in the apoptosis pathway in metazoan cells with their activation triggering or leading to the progression of cell death. In mammalian cells these include the proto-oncogene C-myc (Evan et al. 1992), nuclear regulatory factors encoded by the p53 gene (Lowe et al. 1993) and several non-nuclear components such as members of the bcl/bax gene family (Larsen 1994) or the Fas/apo-1 receptor (Trauth et al. 1989); in *C. elegans* these include the cell death genes ced 3, ced 4 and ced 9 (Hengartner 1997). It is clear that these genes are not acting in isolation and that the pathways leading to cell death are complex; similarly in lower organisms the process is likely to involve many genes.

We have shown that trypanosomes can be induced to undergo apoptosis after stimulation with Con A and that the process of death is associated with *de novo* gene expression (Welburn et al.1996, Murphy & Welburn 1997, Welburn & Murphy

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1998). To provide an overview of the genetic changes occurring during the execution of the cell death program in *T. b. rhodesiense*, we have used a differential display method [Randomly Amplified Developmentally Expressed Sequences-PCR (RADES-PCR)] for the identification of genes which are differentially expressed in cells which have been induced to die. These differentially expressed genes and their encoded products may be implicated directly or indirectly in the cell death mechanism.

MATERIALS AND METHODS

Trypanosomes - Bloodstream form *T. b. rhodesiense* (stock OBWANG isolated from a patient during a sleeping sickness epidemic in S. E. Uganda, 1990) were transformed to procyclic (insect form) parasites by transmission through tsetse flies. Trypanosomes were dissected from tsetse guts and cultured at 27°C in Cunningham's medium (CM) supplemented with 20% foetal bovine serum (Cunningham 1977).

Induction of apoptosis - Log-phase trypanosomes (10^6/ml) were incubated with Con A type IV (Sigma, UK) at 1-10 mg/ml in procyclic culture media (Cunningham's medium supplemented with 17% foetal bovine serum) and harvested 0, 2, 4, 7, 24, 48 and 72 hr after treatment as previously described (Welburn et al. 1996, Welburn & Murphy 1998).

Preparation of RADES template - Total RNA was prepared from parasites at each time point of Con A treatment (0, 2, 4, 7, 24 and 48 hr) and analysed by gel electrophoresis. RNA was enriched for polyadenylated transcripts and conversion to cDNA was effected using Moloney murine leukemia virus (M-MLV), reverse transcriptase and an oligo (dT) primer (TAG GCG CGC CTT TTT TTT TTT TTT TTT TTT). After removal of oligo d(T) primer and dNTPs via a Centricon 30 column (Amicon), ds cDNA was generated using a specific primer for the conserved 25 nt at the 3' end of the miniexon spliced leader of all trypanosome mRNAs (TAG GCG CGC CTA GAA CAG TTT CTG TAC TAT ATT G) containing nt 16 to 39 of the miniexon sequence and the oligo (dT) primer. PCRs contained 100 ng cDNA /10 ml 10X *Taq* buffer [10mM TrisHCL (pH8.3)]/ 2mM MgCl /ml/ 4 ml 5mM dNTPs/ 12.5 units *Taq* DNA polymerase/ 1 ml (100 ng/ml) each of the oligo(dT) primer and miniexon primer in 100 ml total volume. Cycling conditions were 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for 40 cycles followed by a 5 min extension at 72°C. Buffer, primers and dNTPs were removed by ultrafiltration in a Centricon 30 column (Amicon, Inc.). cDNAs were collected and diluted to 20 ng/ml in TE.

RADES-PCR - Double-stranded cDNA served as the template for arbitrary primers in the subsequent RADES-PCR fingerprinting reactions. PCR reactions were carried out at two template concentrations to check the reproducibility the fingerprints. PCR reactions were carried out using single arbitrary 10 mer primers (Murphy & Pelle 1994, Murphy & Welburn 1997, Welburn & Murphy 1998). PCRs contained 20 ng or 2 ng of target cDNAs and the volume brought to 10 ml with water /10mM Tris-HCL (pH8.3)/ 50mM KCl/ 3mM MgCl / 0.05% (v/v) NP40 / 0.05% v/v Tween 20/ 200 mM of dNTPs / 0.6 mM primer/ 0.5 units of Taq (*Thermus aquaticus*) DNA polymerase (Promega) in a volume of 20 ml, and were performed on a programmable thermal cycler. Cycling conditions were 94°C for 45s, 40°C for 1 min and 72°C for 1 min for 40 cycles followed by a 72°C 5 min extension. Amplification products were analysed by electrophoresis in 3% (w/v) MetaPhor® agarose gel and detected by UV illumination following staining with EtdBr.

Re-amplification and cloning of RADES products - RADES products were gel extracted and re-amplified using the PCR conditions described above. An A-tailing procedure was performed to increase the efficiency of cloning of differentially expressed cDNAs, (two units *Taq* DNA polymerase and dATP added to a final concentration of 0.6 mM, incubated at 70°C for 15 min). Amplified cDNAs were identified by electrophoresis as above and subsequently cleaned by gel purification (Geneclean II kit, Bio 101, Inc). A-tailed cDNAs were ligated into pGEM®-T vector (Promega) and successfully transformed into *Escherichia coli* strain JM109 high efficiency competent cells (Promega) by use of the Promega heat-shock method. Blue/white screening was used to identify recombinant plasmids, which were subsequently purified using the plasmid preparation method of Qiagen. Inserts carried by the clones corresponding to differentially expressed RNAs were analysed by automated sequencing. Sequences were compared to the GENBANK/EMBL sequence databases using BLAST (basic local alignment search tool) programme (Altschul et al. 1990) and Gapped BLAST programmes (Altschul et al. 1997).

Electron microscopy - Trypanosomes were cultured as described above and treated with Con A. Cells were examined 3 hr after treatment with Con A to examine morphological evidence of apoptosis. Cells were washed in PBS then fixed in 3% cacodylate buffered gluteraldehyde (pH 7.3 containing 0.01% calcium chloride) after centrifugation (10 min 500 x g) and processed for electron microscopy.

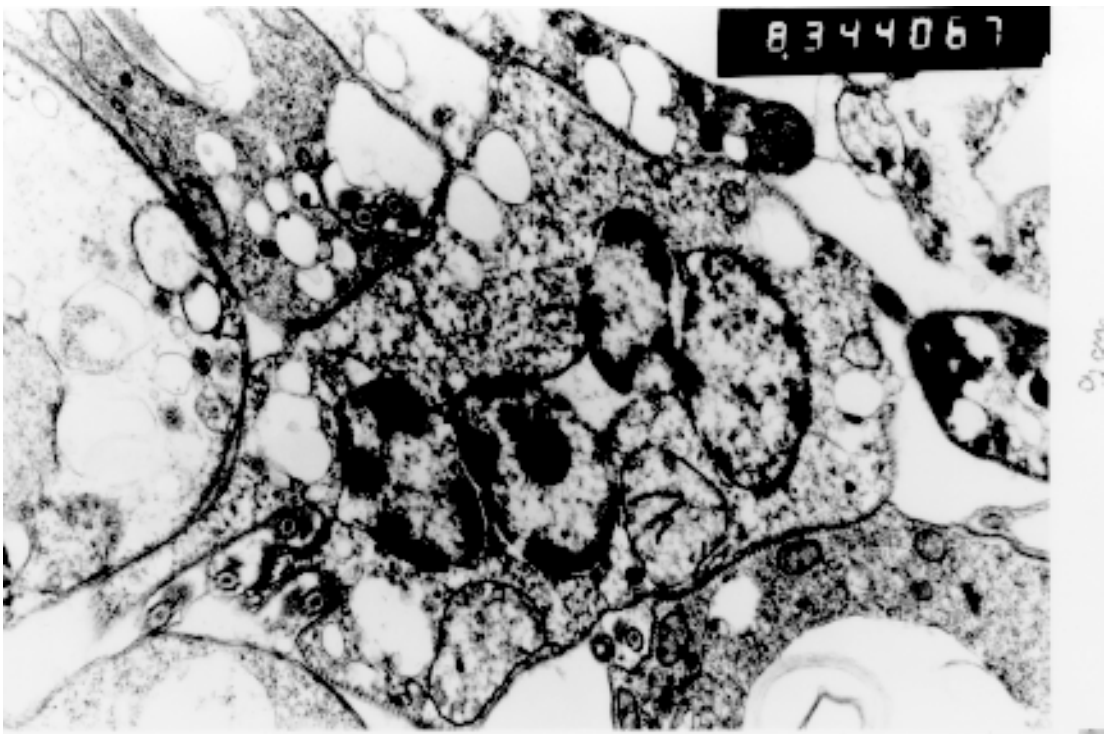
RESULTS

Concanavalin A induced death in trypanosomes - Procyclic trypanosomes when treated with the lectin Con A die by a process essentially similar to the process of apoptosis in mammals (Welburn et al. 1996). Electron micrographs of procyclic trypanosomes treated with Con A showed the characteristic phenotype of apoptotic cells which includes surface membrane vesiculation and chromatin condensation (Wyllie et al. 1980). Three hours post-treatment with Con A trypanosomes exhibit condensation and marginalisation of chromatin to the periphery of the nuclear membrane (Figure). A proportion of treated cells appear multinucleate. These results confirm that treatment with Con A triggers a cell death mechanism in *T. b. rhodesiense* similar to the process of apoptosis described in metazoa which may be described as 'proto-apoptosis'.

It is not unusual for a lectin to induce apoptosis in metazoan cells, indeed apoptosis is classically initiated by binding of a cellular or cytoplasmic receptor which generate second messengers (Arends & Wyllie 1991). Several lectins have been shown to trigger the activation of programmed cell death in tumour cells resulting in DNA fragmentation (Janssen et al. 1993, Kim et al. 1993). Con A has been shown to induce apoptosis in macrophages (Kong et al. 1996) and in synchronised T cell hybridomas in which activation-induced cell death resulted in all cells entering apoptosis shortly after reaching G2/M (Fotedar & Fotedar 1995). Susceptibility of cells to apoptosis often depends on their state of differentiation and cell-cycle checkpoints may also serve to link the cell cycle to apoptosis. Although cell-cycle components may not be involved in all forms of apoptosis, in many instances cell proliferation and cell death share common pathways (King & Cidlowski 1995).

Cross-linking of membrane receptors with Con A has also been shown to trigger programmed cell death in neurons which also exhibited an increase in c-Jun, an intermediate early gene product correlated with activation-induced programmed cell death in lymphocytes (Cribs et al. 1996, Azizeh et al. 1998). Similarly, Con A- induced CTLA-4 cross-linking on the surface of activated murine CD4(+) T lymphocytes leads to death of a substantial fraction of the cells whereas in resting CD4(+) T cells the same stimulation induces cell cycle arrest without apoptosis (Scheipers & Reiser 1998).

The predominant cell surface glycoprotein of procyclic form trypanosomes, PARP (Richardson et al. 1988), is rich in mannose residues which would provide an ideal ligand for Con A. Cell death *in vivo* in the tsetse fly vector can be pre-



Electron micrographs showing morphological changes in Con A-treated procyclic *Trypanosoma brucei rhodesiense*. Agglutinated multi-nucleate procyclic trypanosomes 3 hr after Con A treatment showing condensation and migration of chromatin to form crescents at the periphery of the nuclear membrane of the cell (x 16,600).

vented by feeding purified PARP in the diet (Welburn & Pearson, man. in prep.) Taken together these results suggest that death in procyclic parasites may be effected through Con A binding to PARP on the trypanosome surface.

Gene expression during ConA induced death of trypanosomes - To provide an overview of the genetic changes occurring during the death programme we have used an approach based on the identification of genes that are differentially expressed in parasites committed to die. As cell death in these parasites is associated with *de novo* gene expression (Welburn et al. 1996, Murphy & Welburn 1997) these genes may be implicated directly or indirectly in the death mechanism. Although it is a reasonable assumption that a cell death programme is directly controlled by the induction of several genes which are expressed at a very low level, it should not exclude the possibility that cell death might ultimately result from changes in the expression of some housekeeping genes (Baudet et al. 1998).

Overview of differentially expressed genes - Using RADES-PCR for differential display of cDNAs we have been able to identify genes which are differentially expressed during ConA induced death in *T. b. rhodesiese.* Twenty-two cDNAs have been cloned and sequenced. The majority of cDNAs which are differentially expressed after the parasites have been signalled to die are novel in trypanosomes but some have homologues in mammals, *C. elegans* or yeast. The differentially expressed cDNAs identified during Con A induced death in *T. b. rhodesiense* fall into several categories. Of the cloned cDNAs 13 clones carried inserts whose sequences are homologous to mammalian or invertebrate genes of which three have already been identified in typanosomatids and five clones represented homologues to ribosomal proteins. One clone carried an insert unique to trypanosomes. Eight clones carried inserts carried inserts with novel sequences. Five of the cDNA clones with homologues to mammalian or invertebrate genes represent ribosomal genes (two 40s and three 60s ribosomal proteins) and another is homologous to a peptide chain release factor. Genes relating to cell signalling and cellular communication include TRACK, a receptor for protein kinase C (Welburn & Murphy 1998), a serine threonine protein kinase and a leucine zipper protein. Genes related to cell cycle control include prohibitin (Welburn & Murphy 1998). Mitochondial involvement in ConA induced cell death is implied by the identification of a cDNA with homology to a mitochondrial RNA splicing protein, a mitochondrial transporter and cytochrome c1. A homologue to the histone H3 has also been identified.

DISCUSSION

The accumulating evidence for PCD/apoptosis in single celled organisms is suggestive of of an ancestral death machinery (Fraser & James 1998). The prediction that a single core molecular mechanism of PCD emerging in evolution prior to the postulated multiple emergences of multicellularity would dictate that some of the molecules involved in the core PCD mechanism of even phylogenetically very distant organisms should be related (Cornillion et al. 1994).

The genes described here represent the first endogenous genes to be identified as implicated in cell death in unicellular organisms. While it is evident that some of the genes involved in cellular suicide in trypanosomes are conserved in higher organisms, with the exception of cyctochrome c1 (upstream of cytochrome c in the electron transport chain) none of the genes described here have previously been directly implicated in either apoptosis or PCD in mammals. Cytochrome c is a prime initiator of apoptosis in mammalian cells acting downstream of bcl-2 and being released from mitochondria during apoptosis (Garland & Rudin 1998).

Two further genes which are up-regulated late during Con A induced cell death in *T. b. rhodesiense* which have homologues in multicellular organisms and which may have an indirect role in apoptosis in metazoan systems are prohibitin and TRACK (Welburn & Murphy 1998).

Prohibitins are a family of highly conserved nuclear encoded mitochondrial proteins which interact with a homologous protein BAP37, these proteins are implicated in cell cycle regulation, senescence and tumour suppression in mammals and in determining yeast replicative lifespan - the double knockouts showing an 80% reduction in mitochondrial membrane potential (Coates et al. 1997). Expression of prohibitin RNA in *T. b. rhodesiense* is relatively low in normal actively dividing cells and only enhanced after stimulation with Con A, with maximum expression at 24 hr. Trypanosomes also possess a BAP37 homologue, recently identified by the trypanosome genome sequencing project. Prohibitins have not previously been associated with cell death in multicellular organisms although mutants in the Cc gene in *Drosophilla* fail to develop past the embryonic stage (Eveleth & Marsh 1986).

A role for prohibitins in mitochondrial inheritance and in the regulation of mitochondrial morphology has recently been suggested since, in *S. cerevisiae*, phb1 or phb2 mutations were lethal when combined with a mutant for any of three mitochondrial inheritance components of the mitochondrial outer membrane, Mdm12p, Mdm10p,

and Mmm1p. In addition loss of function of either prohibitin in cells with mitochondrial DNA deleted led to altered mitochondrial morphology (Berger &Yaffe 1998). Although prohibitins have not been implicated in either apoptosis in mammalian cells or PCD in the nematode worm, evidence is accumulating that the nuclear features of apoptosis are preceded by alterations in mitochondrial function and structure (Petit et al. 1997).

TRACK is a member of the RACK gene family which are homologues of the beta sub-unit of G proteins and which are intracellular receptors for activated protein kinase C. TRACK RNA increased during cellular death of procyclic *T. b. rhodesiense* with maximal expression 48 hr after Con A treatment (Welburn & Murphy 1998), suggestive of a role for protein kinase C in cell death in trypanosomes. In other systems RACK1 has been shown to be an Src tyrosine kinase binding protein which can inhibit the kinase activity of Src and Lck tyrosine kinases. Cells over-expressing RACK1 exhibit decreased tyrosine phosphorylation on many cellular proteins with cellular growth being depressed by up to 50%. RACK1 over-expressing cells do not show an increased rate of necrosis or apoptosis but exhibit a prolongation in G0/G1 (Chang et al. 1998). Despite the high level of conservation between the genes implicated in cell death in trypanosomes and their mammalian homologues, neither gene described here has been previously directly implicated in PCD in mammals although an indirect role for protein kinase C has been proposed.

In this study a high percentage of differentially expressed cDNAs correspond to ribosomal proteins and it is unclear whether ribosomal protein genes play an active role in the death programme or whether this reflects metabolic disturbance of the dying cell. A similar percentage of ribosomal proteins were identified in a differential screen of rat glioma cells after induction of apoptosis and this was considered indicative of a possible involvement of the cellular translational apparatus in the death process (Baudet et al. 1998).

Several studies in yeast do suggest that an evolutionarily conserved metazoan PCD pathway may operate in single-celled organisms (Fraser & James 1998). Genes which affect developmental PCD in *C. elegans*, and apoptosis in mammals have been shown to function in yeast which lack such endogenous genes. Bax (which drives a mammalian cell towards death) and Bcl-2 (which suppress programmed cell death) have both been shown to be functionally active in yeast cells, either driving the cell towards death or protecting the yeast cell from death (Ink et al. 1997, Jurgensmeier et al. 1997). The principal genes involved in PCD in *C. elegans* also appear to function in a similar manner in *Schizosachromyces pombe*. Induction of wild-type ced-4 expression in *S. pombe* resulted in rapid focal chromatin condensation and lethality. Co-expression of ced-9 (which prevents the death-promoting actions of ced-4 in *C. elegans*) similarly prevented ced-4-induced death in yeast (James et al. 1997).

In conclusion, it is evident that several genes which affect apoptosis in mammalian systems and PCD in *C. elegans* function in a similar manner in single celled yeast which does not possess endogenous homologues for such genes. Whether members of the Trypanosomatidae and other single-celled organisms would be able to respond to these principal effectors and supressors of mammalian and invertebrate PCD/apoptosis remains unclear at present. The identification of novel genes that regulate cell suicide in these unicellular organisms (the most primitive eukaryote in which PCD has been described) is essential to establish whether unicellular eukaryotes do share the same or similar regulators of cell suicide programmes with multicellular organisms. An understanding of cell death processes in these parasites may also offer new possibilities for controlling parasitic disease (Ameisen 1996, Welburn et al. 1997). The extent of the overlap between effectors and regulators of PCD between trypanosomatid and mammalian host PCD pathways may determine whether or not the pathways leading to cell suicide in these parasites may be exploited as a parasite control strategy (Welburn et al. 1997).

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