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APOPTOSIS IN BRAIN AND GUT TISSUE OF MICE FED A SEED PREPARATION OF THE CYCAD *LEPIDOZAMIA PEROFFSKYANA*

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SUMMARY: Apoptosis (programmed cell death) was identified in histological sections of brain and gut tissue of adult mice fed seed preparations from the cycad *Lepidozamia peroffskyana*. This form of cell death was also found at high levels in brain tissue from neonatal mice born from a cycad-fed mother. The discovery was made during re-appraisal of archival tissue from a study of toxic properties of *L. peroffskyana*. Ingestion of appropriately prepared food or medicine derived from another cycad, *Cycas circinalis*, is thought to be associated with several motor neurone and other neurodegenerative disorders of some Pacific island inhabitants. Apoptosis is cell death under gene control. From the present study, presence of apoptosis in brain tissue after cycad toxicity may provide a link between cycad ingestion and development of neurodegenerative disorders and may provide a novel explanation for localization of some neurodegenerative disorders, as some inhabitants may have a genetic susceptibility to apoptosis induced by cycad toxicity.

There is renewal of interest in etiology and pathogenesis of amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC), a neurodegenerative disorder found in a proportion of the population of Guam and its adjacent islands, and other Pacific regions (1-3). An environmental cause of ALS-PDC, rather than an inherited one, is again finding acceptance. Flour prepared from the highly toxic cycad plant *Cycas circinalis*, now considered to be part of the *C. rumphii* complex, has been proposed as one environmental factor that may be involved (2). Several years ago, an experimental study using adult mice was undertaken to look at the toxic effects of a preparation made from seeds or leaves of another cycad, *Lepidozamia peroffskyana* (4). Toxicity of this plant, found along the central east coast and hinterland region of Australia, had not been reported previously. Although *L. peroffskyana* belongs to a different family of cycads (Zamiaceae) from *C. circinalis* (Cycadaceae), it demonstrated some toxic properties similar to those recorded for other cycads. Of most note was its hepatotoxicity, with some degenerative effects also recorded in the gut. Other tissues collected and studied in the initial experiments were the kidney and brain (cerebellum), and at first assessment, little change was apparent in these two tissues.

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Brain tissue had been collected for study because other plants of the Zamiaceae were thought to be associated with "Zamia staggers", incoordination of the hind limbs of cattle feeding in regions where these plants are common (5). The condition is irreversible, and an association was thought to exist between this condition in cattle and ALS-PDC in humans.

Recently, new investigation of archival paraffin-embedded tissues collected during the initial experiments was carried out. Degenerative changes in the gut were identified as apoptosis, and this same form of cell death was found in as yet unidentified cells of the brain. Apoptosis is known to occur after low levels of other toxins (6). It is proposed that results from the present study will be relevant to analysis of the pathogenesis of ALS-PDC and other neurodegenerative disorders.

MATERIALS AND METHODS

Feeding experiments have been described previously (4).

Preparation of cycad food. Mature fruiting cones of *L. peroffskyana* were collected in a wet sclerophyll forest near Brisbane, on the central east coast of Australia. Seeds were taken from the cones, dehusked, and the fine seed coat removed. They were then minced rapidly, mixed with the extruded juice, and immediately freeze dried under vacuum. The dried powder was stored over silica gel until mixed with moistened crushed standard pelleted animal diet in proportions of plant material of 5, 10, 15, 30%. These mixtures were made into "biscuits", air dried, and fed to groups of mice.

Animals. Adult Quackenbush mice (male, 30-35g) were used. They were housed in laboratory cages in experimental groups and allowed cycad "biscuits" and water *ad libitum*. Appropriate control groups were fed normal pelleted diet. The mice were inspected daily and where possible two from each group were killed by cervical dislocation at regular intervals (2, 4, 7 days, 2, 3, 4, 5, 6 weeks). Any mice that became distressed or moribund as a result of the feeding experiments were killed immediately with an overdose of Nembutal. Tissue from the liver, gut, kidney and brain were fixed in 10% phosphate buffered formalin or in Bouin's fixative for routine histological preparation. Tissue was also fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for electron microscopy. One of the cycad-fed mice had been incorrectly sexed at separation of males and females on weaning, and became pregnant. She bore a litter from which the selected tissues were collected at birth, and fixed and analysed using routine techniques for electron microscopy.

Retrieval and preparation of archival tissue. Paraffin blocks of selected tissues from the experiments were sectioned using routine histological methods. Some sections were stained with haematoxylin and eosin (H&E). Resin-embedded tissue for electron microscopy was also prepared routinely.

Assessment of cell death. The stereotyped morphological characteristics of apoptosis and necrosis (6) were used to assess modes of cell death, using light microscopy under oil (x100 objective), or electron microscopy. Sections of brain tissue were also treated using in situ hybridization techniques (7), with the antisense and sense preparations of the clusterin riboprobe labelled with ^{35}S-UTP. Clusterin has been found in association with apoptosis in other instances (7). Use of this technique in archival tissue depends on the preservation of the target RNA in the original tissue fixation, on the length of time the tissue remained in fixative before paraffin embedding, and on the availability of the target RNA to the probe by use of suitable prehybridization and hybridization conditions.

RESULTS

Light and electron microscopy: Control tissues appeared normal. In tissue from mice fed *L. peroffskyana*, most toxic effects were seen in the acute time (2 and 4 days), high dose (15 and 30%) cycad meal concentrations. Kidney tissue appeared normal. Liver tissue was most affected, with necrotic patches around the portal veins. Relationship between concentrations of cycad meal, length of time of feeding, and liver lesions, was positive, and has been reported previously (4). On re-examination of gut tissue sections, lesions that had been described previously as "degenerative changes", a description often synonymous with necrosis, proved to be apoptosis (Figs 1 a, b). The H&E-stained sections of brain tissue, on re-examination, appeared to have greatly increased apoptosis in animals from cycad feeding experiments (Figs 2 a, b, c). Bouin's fixative produced the best fixation of brain tissue for histological viewing. The type of brain cell undergoing apoptosis was not identified, and further experiments are planned to identify these cell types. Several apoptotic cells showed

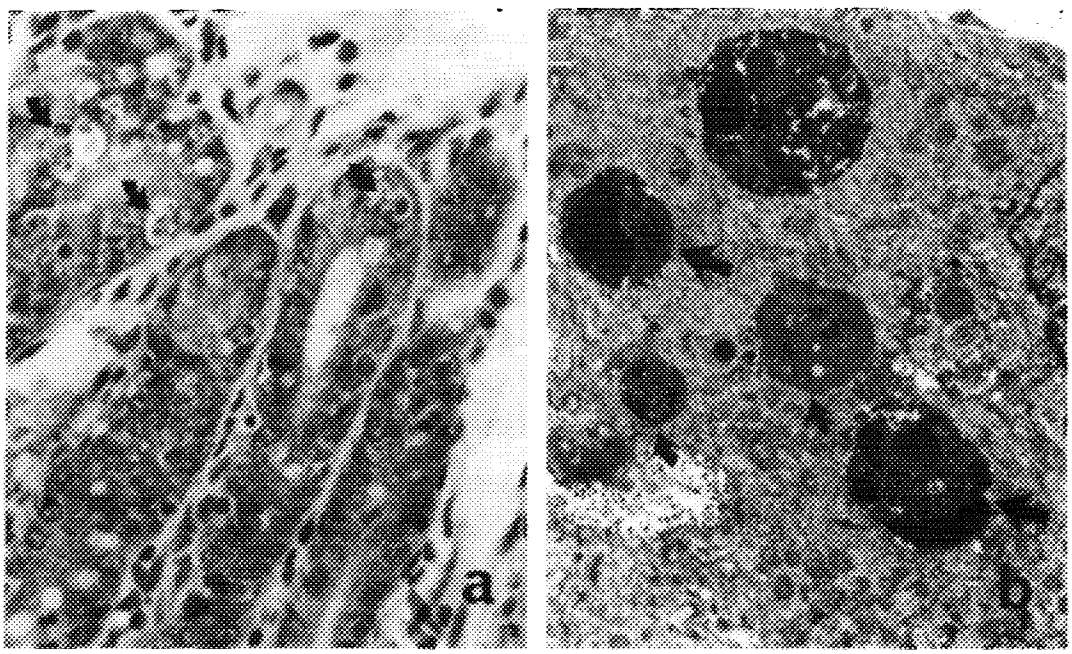

Fig. 1. (a) Apoptosis in epithelial cells of gut from mice fed 15% cycad meal for 2 days. Note the shrunken cells and abundance of apoptotic bodies (arrows). H&E, x306. (b) Ultrastructural characteristics of apoptosis were identified in gut tissue. Apoptotic bodies with condensed cytoplasm and marginated and condensed nuclear chromatin (large arrows), apoptotic bodies without nuclear chromatin (small arrows) and bodies that were undergoing digestion by the phagocytic cell (centre top) were found. x8640.

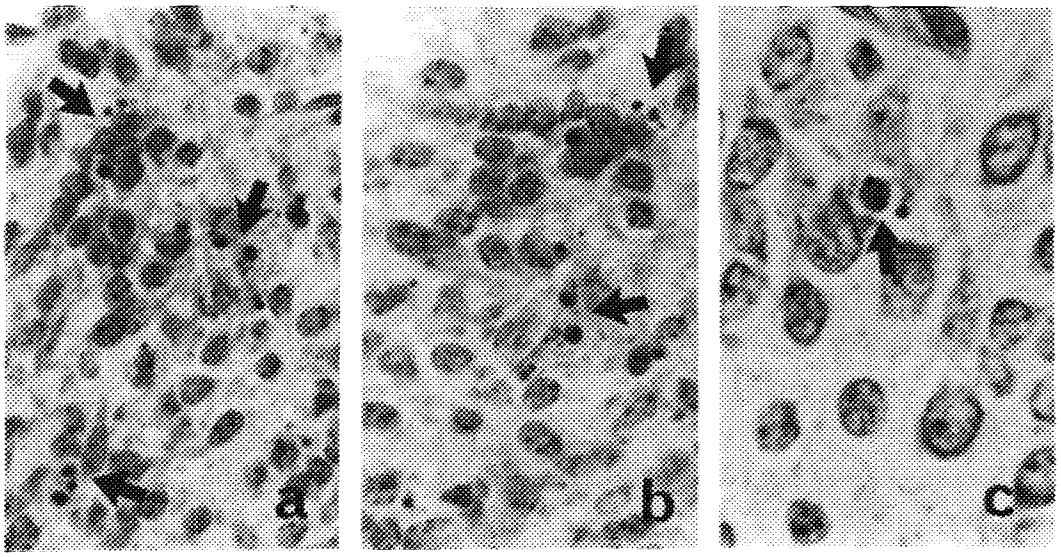

Fig. 2.a,b,c. Apoptotic cells and bodies (arrows) were identified in brain tissue from cycad fed mice (15% meal, 4 days). a,b x612; c x720.

dendritic features (Fig. 2b). Brain tissue collected from the neonates born to the cycad-fed mother, and studied under the electron microscope, demonstrated high levels of apoptosis (Fig. 3).

In situ hybridization techniques using antisense radiolabelled riboprobe of the clusterin gene did not show any specific labelling of brain tissue in which apoptosis had been identified. Lack of label may, however, have been related to earlier treatment of fixed tissue, with loss of target mRNA's.

DISCUSSION

A putative relationship exists between consumption of cycad (*C. circinalis*) seeds, albeit prepared in such a way as to minimize toxicity, and the neurodegenerative disorders noted at abnormally high levels in some Western Pacific regions. This relationship initiated further investigation of archival paraffin-embedded tissues collected during study of the toxic properties of another cycad, *L. peroffskyana*, in adult mice (4). The degenerative changes in the gut have now been identified as apoptosis, and this same form of cell death has been found in as yet unidentified cells of the brain.

Many past studies discuss "cellular hypoplasia", "degeneration", and also necrosis, in describing the neurotoxic effects of cycad derivatives (3, 8, 9). Apoptosis and necrosis are

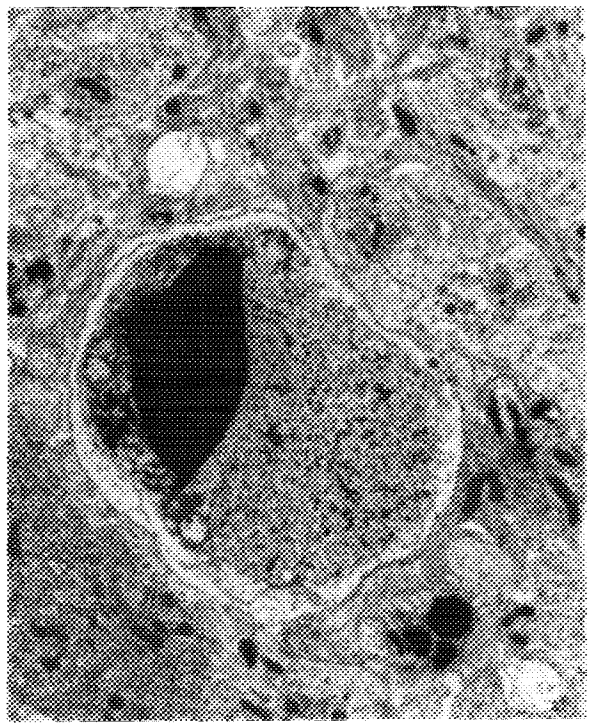

Fig. 3. Electron micrograph of apoptosis in brain tissue from neonatal mouse born to cycad-fed mother (15%, 7 days). Nuclear chromatin is condensed, mitochondria are visible, and the granular cytoplasm is condensed within the membrane of the apoptotic body. x12000.

quite distinct, both morphologically and biochemically. After noxious stimuli, such as toxins, one would expect to see necrosis, a pathological response usually affecting many cells, in which the cells swell and lyse. Apoptosis, on the other hand, has been identified in many physiological and pathological circumstances, including normal embryogenesis and adult tissue homeostasis, tissue atrophy after hormone withdrawal, regression of tumours induced by radio- and chemotherapy, immune cell killing, to name a few (6, 10). The process was originally defined on the basis of its morphology (11), with shrinkage and blebbing of cells, condensation and margination of nuclear chromatin, and formation of membrane bound apoptotic bodies that are phagocytosed by adjacent tissue cells or macrophages. The stereotyped histological and ultrastructural characteristics remain the most reliable method of its identification (10). It is an active process of cell death, requiring expression of new mRNA's and proteins in many instances, and able to be selectively regulated by products of certain genes, notably p53, bcl-2, and Fas (12).

There is speculation about the role that apoptosis may play in neuronal cell death, especially with regards Alzheimer's and Parkinson's diseases (13, 14). As far as the author can ascertain, there has been no unequivocal record of the occurrence of apoptosis of neurons in adult brain tissue, *in vivo*. Pender et al (15) demonstrated apoptosis in the spinal column of rats in experimental allergic encephalomyelitis. Sloviter et al (16) described hippocampal granule cell degeneration as apoptosis, in adrenalectomy-induced changes in rats. The photomicrographs of the latter publication do not, however, show typical ultrastructural morphology of apoptosis. Apoptosis has been recorded with low doses of toxins in tissues other than the brain (6), and has been identified in *in vitro* experiments, for example, in cultures of immature rat cerebellar granule neurons treated with a neurotoxin, methyl-phenylpyridinium (17), in sympathetic neurons after withdrawal of nerve growth factor (18, 19), a process that can be prevented by the bcl-2 proto-oncogene (19). Seawright et al (20) have also recorded degenerative changes (necrosis) in the cerebellum of young adult rats fed an acute high dose of synthetic L-beta-aminoalanine, but did not identify apoptosis.

Although the evidence supporting a link between neurodegenerative disorders and ingestion of cycad meal remains tenuous, the results of the present study have provided new important information, and further experiments will now be done to analyse this hypothesis further. Presence of apoptosis in brain tissue of cycad-fed mice may also provide a novel explanation for the discrepancies in proportions of populations of different countries or regions presenting with increased numbers of neurodegenerative disorders. The inhabitants of Guam who use the cycad-prepared flour have an abnormally high incidence of these disorders, yet many other populations using similar preparations do not seem to be so affected. These findings have been used as an argument away from the "cycad hypothesis". However, apoptosis is an active form of cell death under gene direction, and should it be found to be involved in the pathogenesis of ALS-PDC, its gene control may ultimately explain why some, but not all, people are affected in regions using the cycad-derived foods and medicines. For example, these (usually localized) people may have a genetic predisposition to apoptosis induced by cycad toxicity.

Identification of apoptosis in adult brain tissue, notably neurones, is of utmost importance. Should its presence be verified in association with cycad toxicity, this will provide further evidence for a role for cycad ingestion in the pathogenesis of the motor neurone and associated neurological diseases of some Pacific island inhabitants. However, identification of the occurrence of apoptosis of adult neurones has much wider importance and significance. There is currently doubt as to whether mature neurones are capable of undergoing apoptosis,

just as they probably do not undergo mitosis. On the other hand, it has been proposed that neuronal apoptosis is involved in the development of such important neurodegenerative diseases as Alzheimer's disease and Huntington's chorea. Such hypotheses need careful consideration.

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