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PROGRAMMED CELL DEATH IN THE OVARY : DEVELOPMENT TO DISORDER

In the body of an eukaryotic animal a cell either proliferates by mitosis or remains in a non proliferative stage. During the development and growth of the animal the ratio of cell proliferation to non proliferation is high, in adulthood it decreases while in aged individuals the ratio is inversed. Non proliferation can lead to cell death which can either be accidental (necrosis) or programmed (apoptosis). Kerr and co-workers[1] first coined the word apoptosis which in Greek means 'falling off of leaves from trees or petals from flowers'.

Researchers and clinicians have now realised that a proper balance between cell proliferation and apoptosis is essential for the maintenance of normal body function. An imbalance may lead to diseases such as acquired immune deficiency syndrome (AIDS), in which apoptosis of the immune cells is enhanced, or cancer, where apoptosis is inhibited (increased cell proliferation).

Morphological changes during apoptosis involve cell shrinkage changes in nuclear chromatin followed by nuclear deoxyribonucleic acid (DNA) fragmentation (apoptotic bodies). The apoptotic bodies can be phagocytosed by macrophages or neighbouring cells. Biochemically, increase in intracellular Ca^{++} can cause stimulation of endonucleases which cleave the internucleosomal DNA to form fragments of DNA~180 bp or its multiples. This is the basis for detecting apoptosis by agarose gel electrophoresis of the extracted DNA from cells. Ladder like bands of multiple base pairs of 180 to 2300 are formed which can be visualized after staining with fluorescent dyes such as ethidium bromide. Different investigators have reported various techniques for detecting apoptosis. These include morphological changes observed by staining of cells by dyes such as acridine orange, flow cytometry analysis by staining DNA with 4,6 diamidino-2-phenylindole (DAPI) or propidium iodide. In addition to the agarose gel electrophoresis; DNA ladder formation, microelectrophoresis (comet assay) has also been used to detect DNA fragmentation. Recently, *in situ* dUTP 3′ nich end labelling (TUNEL) of DNA fragments has been used to localize apoptotic cells in an organ with heterogenous population of cells while cellular DNA fragmentation enzyme linked immunosorbent assay (ELISA) has been employed for quantitation of apoptosis.

A number of genes have been identified that promote or inhibit apoptosis *eg.* interleukin 1β converting enzyme (ICE) or bcl-2[2,3], respectively.

Apoptosis during Development

In the human ovary, about 7 x 10^6 oogonia are present at 20 weeks of gestation, 2 x 10^6 oocytes are present at

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birth which are further reduced to 4 x 10^5 at the time of puberty. Thus, there is a significant reduction in the number of oocytes from the foetal to the adult ovary due to degeneration. In fact, of 7 x 10^6 oogonia present during the gestation period, only 450 oocytes can ovulate while the remaining 99.9 per cent are destined to die *in situ*.

Though it is well documented that the pituitary gonadotropins, FSH and LH stimulate follicular maturation and ovulation, 99.9 per cent of the follicles undergo atresia in the presence of gonadotropins. Studies are underway to elicit information on the factors responsible for oocyte degeneration, and on their origin–intraovarian or otherwise.

Intraovarian factors

It is well established that oestrogens secreted by the ovarian follicles stimulate follicular growth while high androgen levels lead to follicular atresia. It is suggested that inhibins/activins regulate folliculogenesis either by their regulatory action on pituitary FSH or by direct autocrine/paracrine effect on granulosa cell steroidogenesis[4].

Scientists at the Institute of Research in Reproduction (IRR), Mumbai, have purified a peptide that inhibits binding of FSH (FSHBI) to granulosa cells *in vitro*[5]. *In vivo*, the peptide suppressed ovulation and induced follicular atresia in cycling mice[6]. Experiments were further planned to understand whether in these animals the granulosa cells, due to non availability of FSH (binding inhibited) underwent apoptosis. Immature mice were injected with pregnant mares' serum gonadotropin (PMSG) and autopsied 48 h later. Another group was injected with PMSG and 20 µg FSHBI on the following day and autopsied 48 h after PMSG (24 h after FSHBI injection). The granulosa cells, punctured and collected from ovarian follicles, were studied, for apoptosis using 4 parameters *viz* (i) morphological changes by staining with acridine orange for fluorescent microscopy; (ii) DNA staining with DAPI for flow cytometry; (iii) DNA fragmentation (a) agarose gel electrophoresis, and (b) TUNEL technique; and (iv) DNA cellular fragmentation ELISA. All the parameters revealed that FSHBI is responsible for apoptosis in granulosa cells[7]. Recent studies have shown that a number of intraovarian factors which include growth factors and cytokines[8] may be responsible in programming cell death in ovarian granulosa cells. It is therefore suggested that a cocktail of intraovarian factors may bring about apoptosis in granulosa cells of ovarian follicles. A number of genes/proteins reported to be involved in granulosa/luteal cell apoptosis are listed in the Table. All the genes, except bcl-2 possibly alone or together induce apoptosis in granulosa/luteal cells to cause follicular atresia or luteal regression in rodents and primates.

Table: Genes involved in ovarian cell apoptosis

Genes/Proteins	Cell Type	Authors
c-myc	granulosa cells	Li *et al*[9]
c-myc	luteal cells	Fraser *et al*[10]
bcl-2	granulosa cells	Tilly *et al*[11]
p53	granulosa cells	Tilly *et al*[12]
Fas	granulosa cells	Quirk *et al*[13]
Fas/Fas ligand	granulosa cells	Harkuno *et al*[14] Sakamaki *et al*[15]
bax, ICE	granulosa cells	Rueda *et al*[16]

ICE–interleukin 1β converting enzyme.
Superscript nos. refer to the sl. nos. in the reference list.

In an ovarian follicle the granulosa cells are the major cell type which undergoes proliferation of apoptosis to decide the fate of the follicle/oocyte for ovulation or atresia. After ovulation the egg may be fertilized in the oviduct or may degenerate, if not fertilized. Recently, Takase *et.al*[17], have reported that degenerative changes in the unfertilized oocyte are apoptotic and are enhanced 32 h after ovulation.

The follicular cells, granulosa and theca, are luteinized after the release of the ovum. The corpus luteum is functional and short lived if the pregnancy does not occur. Prostaglandins are known to cause luteolysis in cattle but not in rodents and primates. Changes in luteal cells during luteal regression are apoptotic[10]. The granulosa cells in the follicle are devoid of blood supply while luteal cells are flooded with capillaries. Possibly, growth factors such as tumour necrosis factor and cytokines *eg.* interleukin, locally regulate luteal maintenance or regression in rodents as well as primates.

Apoptosis in Diseases

Polycystic ovary syndrome

Polycystic ovary syndrome (PCO) is a heterogenous clinical entity characterized by distinct symptoms, elevated androgen concentration and polycystic ovaries. Hyperandrogenaemia, a crucial etiological factor in PCOS

is caused by increased androgen production by the ovary or adrenal or both under the influence of elevated serum LH concentration. The arrest in follicular maturation and function of polycystic ovaries is not due to absence of aromatase activity, the enzyme responsible for the conversion of androstenedione to estradiol, as observed in *in vitro* and *in vivo* studies. An abnormal regulation of the activity of the enzyme 17 ∝ hydroxylase in PCOS[18] leads to high androgen concentration in the follicular fluid causing apoptosis of granulosa cells which in turn leads to the arrest of follicular maturation. Multiple small antral/cystic follicles observed in PCOS are viable[19] but they may or may not ovulate for being apoptotic.

Hypogonadism

In hypogonadism the gonadotropic levels are low and follicular development is arrested. Hypogonadism may occur due to lack of hypothalamic secretion of GnRH, due to deletion of GnRH gene, or mutation of GnRH receptor gene observed during puberty[20]. Mutation of LH β gene or LH receptor gene can also lead to ovarian dysfunction.

Premature ovarian failure

In premature ovarian failure (POF) the follicles may or may not be present and the levels of FSH are high. Where follicles are present it is not clear whether these follicles lack FSH receptors[21] or are apoptotic? A decrease in the number of follicles at an early age (<35 years) in POF patients possibly is due to some local factors responsible for the ageing/apoptotic effect on the follicle/oocyte.

Truncated point mutation of FSHβ, or change in FSH glycosylation can fail to activate FSH receptor thereby leading to POF. FSH receptor (mutation of DNA sequence) defect is also observed in some of the patients with POF. These patients have characteristic early age ovarian dysfunction, elevated serum gonadotropins and low estradiol concentration due to apoptosis in granulosa cells of the follicles.

Enlarged hypoanaemic ovaries have been observed in patients lacking estrogen responsiveness due to disruption of estrogen receptor gene in granulosa cells[22] possibly due to programmed cell death.

Autoimmunity

In patients with hypergonadotropin, hypogonadotropic amenorrhoea elevated levels of FSH failed to stimulate follicles (referred to as resistant ovary syndrome). In these patients circulating immunoglobulins interfere with FSH binding to its receptor[23]. Recently in some individuals with POF antibodies against multiple steroidogenic enzymes were found in circulation[24], specifically to the key enzyme, $\triangle^5$ 3 β hydroxysteriod dehydrogenase (βHSD)[25] required for conversion of pregnenolone to progesterone. Mutation of CYP19 aromatase gene can elevate serum gonadotropin concentrations inducing formation of ovarian cysts[26]. Growth hormone-insulin like growth factor (GH-IGF-1) system has been shown to be crucial for intraovarian regulation of steroid synthesis and follicular development[27]. A mutational defect in the system leads to ovarian failure.

Ageing

As the body ages, reproductive organs also simulate ageing effect. Fujino *et al*[28] have observed apoptotic changes in the oocyte of ageing mice.

Ovarian tumours

Inhibins and activins belong to a large group of transforming growth factors-β. Inhibins and activins exist as heterodimers composed of ∝ and β chains. The β subunit is common to inhibin and activin while ∝ is specific for inhibin. Interestingly, deletion of inhibin ∝ gene in embryonic stem cell increases ovarian tumours in mice[29]. Gonadotropins modify the action of inhibin[30] indicating that local factor as well as gonadotropin together are responsible for regulating ovarian tumours.

In conclusion, the granulosa cells, the oocytes and the luteal cells undergo apoptosis during normal ovarian function. The dysregulation of the ovarian function by alteration in the concentration of gonadotropins, local factors such as steroids, growth factors, or their receptors cause ovarian disorders[31] by enhanced apoptosis in these cell types.

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This write-up has been contributed by Dr. T.D. Nandedkar, Deputy Director, Institute for Research in Reproduction, Mumbai.

ABSTRACTS

Some Research Projects Completed Recently

Chronic calcific pancreatitis: A prospective study of response to surgical treatment

Patients with a clinico-radiological diagnosis of chronic calcific pancreatitis (CCP) were studied to evaluate the effect of surgical intervention on pain and the exocrine and endocrine functions.

Of the 126 patients (104 males and 22 females; mean age 36.98 years), 40 had alcoholic pancreatitis, 81 tropical calcific pancreatitis, 3 had carcinoma of the pancreas initially presented as chronic pancreatitis and 2 had pancreas divisum. One hundred and twenty patients presented with abdominal pain (of pancreatitis) with an average duration of 4.63 years, 33 had diabetes mellitus, 19 had pancreatic steatorrhoea, 23 obstructive jaundice and 10 patients had left sided portal hypertension (L-PHT).

Pancreatic calcification was observed in 37 of the 126 (29.4%) patients on plain x-ray of the abdomen, while ultrasonography showed dilated pancreatic duct and calcification in 49 (33.6%) patients. Endoscopic retrograde cholangio pancreatography (ERCP) done on 50 patients revealed dilated duct and/or calcification in all 50 patients.

All patients with pain were initially managed conservatively with abstinence from alcohol, small frequent carbohydrate rich diet, oral pancreatic enzymes and H_2 receptor blockers. Sixty nine of the 126 patients not responding to conservative treatment underwent surgery–modified Puestow's operation in 56, Duval's operation in seven and distal pancreatectomy in six. Internal drainage for symptomatic pancreatic pseudocysts was carried out in 17, biliary enteric anastomosis for obstructive jaundice in 22 and splenectomy for L-PHT in 10 patients. A total of 50 patients could be followed up for periods ranging upto 5 years. Postopertive relief of pain was recorded in 45 (90%) patients. Pain recurred in 4 (8%) patients at the end of the 5 years. Five of the 11 (45.4%) patients with overt diabetes mellitus improved in terms of insulin requirement and normalisation of blood glucose values. It is thus concluded that the drainage operations for CCP do result in a consistent long-term improvement in abdominal pain and diabetes mellitus.

Quantitative estimation of inorganic and organic constituents of 8 pancreatic stones showed that Ca^{2+} (31.37 ± 4.07 mg/dl) and oxalate (5.06 ± 2.18 mg/dl) were the major inorganic constituents and proteins (83.0 ± 13.5 µg %) and mucopolysaccharides (6.96 ± 1.77 mg %) the major organic constituents.

Rakesh Tandon
Department of Gastroenterology
and Human Nutrition,
All India Institute of Medical Sciences,
New Delhi

Publications:

1. Sidhu, S.S., Shah, P., Srikant, S. and Tandon, R.K. Ketosis resistant diabetes of the young: A profile of its exocrine and endocrine pancreatic dysfunction. *Indian J Physiol Pharmacol 38*: 289, 1994.
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c-myc localization in premalignant and malignant lesions of human colon

The correlation of the expression of c-myc oncogene with the degree of differentiation of premalignant and malignant lesions of the colon was examined immunohistochemically using monoclonal antibodies against c-myc protein and by morphometry. The colonic lesions studied included polyps with dysplasia, well differentiated, moderately differentiated and poorly differentiated carcinomas, signet ring carcinoma, mucinous carcinoma and adenosquamous carcinoma. Paraffin sections from normal colonic mucosa served as controls.

Sections representing the normal colonic mucosa showed the highest nuclear c-myc positivity (84%). The percentage of nuclear positivity declined with the increase in histologic grades–the highest (59%) and lowest (2%) nuclear c-myc positivity being recorded in cases of well differentiated carcinoma and adenosquamous carcinoma respectively. There was a significant increase in the cytoplsmic positivity of the c-myc oncoprotein in various

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lesions (except for signet ring carcinoma which showed zero cytoplasmic positivity) as compared to controls.

The nuclear diameter was found to be a reliable cytological variable in distinguising severe dysplasias with well differentiated carcinomas. Moderately and poorly differentiated carcinomas and adenosquamous carcinoma showed significantly higher values for nuclear diameter compared to controls and well differentiated carcinoma. There was a significant decline in nuclear volume with an increase in the histological grade. With an increase in nuclear volume the nuclear expression of c-myc oncoprotein declined. Well differentiated carcinomas showed nearly equal distribution of c-myc positivity in the nucleus and cytoplasm. Moderately differentiated carcinomas showed predominantly cytoplasmic c-myc positivity. Poorly differentiated carcinomas showed very weak nuclear but intense cytoplasmic positivity.

It may be concluded that cytoplasmic c-myc over expression is related to the aggressive behaviour of the tumour as it is a growth regulating protein. Hence the assessment of c-myc over expression by immunocytochemistry is an additional prognostic indicator. Combining immunocytochemistry of c-myc oncogene with morphometric analysis will give new dimensions in predicting the prognosis of colonic neoplasms. However, a large number of cases need to be studied for a more reliable assessment of c-myc expression.

J. Suresh Durai
K. Baba Krishnan
Department of Pathology,
Dr. A.L.M. Postgraduate Institute of
Basic Medical Sciences,
Chennai

ICMR NEWS

Participation of ICMR Scientists in Scientific Events:

Dr. V.P. Sharma, Director, Malaria Research Centre, Delhi, participated in the V Neem Awareness Workshop at Mbite, Kenya (June 15-24, 1997).

Dr. G.V. Satyavati, Director-General, ICMR, participated in the XX session of the Joint Coordinating Board of the TDR/WHO at Geneva (June 23-24, 1997).

Dr. G.V. Satyavati delivered the Key-note Address on "Opportunities for Women and Biomedical Sciences" at the Silver Jubilee symposium of the Indian Women Scientists' Association at Mumbai (June 27, 1997).

Dr. Jayashree Nandi, Senior Research Officer, National Institute of Virology, Pune, participated in the workshop on AIDS in Africa at Dar-es-Salaam, Tanzania (June 25-28, 1997).

Dr. P.K. Das, Director, Vector Control Research Centre (VCRC), Pondicherry and Dr. V. Kumaraswamy, Dy. Director, Tuberculosis Research Centre (TRC), Chennai, participated in the UNDP/World Bank/WHO Special Programme for Research and Training on Tropical Diseases meeting on Rapid Assessment of Lymphatic Filariasis at New Delhi (July 3-5, 1997).

Dr. P.K. Das, Director, Dr. S.P. Pani, Dy. Director and Dr. K.D. Ramaiah, Research Officer, VCRC, Pondicherry; Dr. A.P. Das, Dy. Director and Dr. B.V. Babu, Sr. Research Officer, Regional Medical Research Centre, Bhubaneswar; and Dr. V. Kumaraswamy, TRC, Chennai, participated in the UNDP/World Bank/WHO Special Programme for Research and Training on Tropical Diseases meeting on Protocol Development for Community Directed Treatment of Lymphatic Filariasis at New Delhi (July 7-11, 1997).

Dr. M.D. Gupte, Director, Institute for Research in Medical Statistics, Chennai and Officer-in-Charge, CJIL Field Unit, Avadi, Chennai, participated in the meetings of the (i) WHO Task Force on Monitoring and Evaluation of Elimination of Leprosy (July 14, 1997), (ii) WHO Steering Committee on Special Action Projects (July 15, 1997); (iii) Leprosy Elimination Advisory Group (July 16-17, 1997); and (iv) Joint ILEP/WHO Workshop on Reaching Undetected Leprosy Patients in Endemic Countries (July 18-19) at Geneva.

Dr. N.V. Giridharan, Sr. Research Officer, National Centre for Laboratory Animal Science, Hyderabad, participated in the VI International workshop on Lessons from Animal Diabetes at Bagsvaerd, Denmark (July 14-17, 1997).

ICMR AIDED SYMPOSIA/SEMINARS/WORKSHOPS/COURSES/CONFERENCES

Symposium/Seminar/Workshop/ Course/Conference	Date & Place	Contact Address
Workshop on Intellectual Property Rights	August 13, 1997; (at Ahmedabad)	Dr. H.N. Saiyed, Director, National Institute of Occupational Health, Ahmedabad-380016.
Symposium on Recent Trends in Tropical Disease Research	September 5-6, 1997; (at Sevagram)	Dr. M.V.R. Reddy, Organising Secretary of the Symposium, J.B. Tropical Disease Research Centre and Department of Biochemistry, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha-442102.
III National Conference of the Alzheimer's and Related Disorders Society of India, Hyderabad	August 31, 1997; (at Hyderabad)	Mr. K.R. Gangadharan, Organising Secretary of the Conference, Alzheimer's and Related Disorders Society of India, 7-1-59/4 & 8, Ameerpet, Hyderabad-500016.
National Symposium-cum-Workshop on Leptospirosis	September 11-13, 1997; (at Port Blair)	Dr. V.G. Rao, Organising Secretary of the Symposium, Regional Medical Research Centre, Port Blair-744101.
International Symposium on Recent Trends in Mental Health	September 19-20, 1997; (at Sevagram)	Dr. Prakash D. Behere, Organising Secretary of the Symposium, Department of Psychiatry, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha-442102.
IV National Conference of the Society of Biomaterials and Artificial Organs on New Horizons in Biomaterials Research	October 3-4, 1997; (at Hyderabad)	Dr. P.R. Chatterjee, Convenor of the Conference, Indian Institute of Chemical Technology, Hyderabad-500007.
Seminar on Current Status of Thalassemia in India and National Workshop on Carrier Detection and Antenatal Diagnosis of Thalassemia	November 13 and 14-15, 1997; (at Lucknow)	Dr. Sarita Agarwal, Organising Secretary, Department of Medical Genetics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow-226014.
International Conference on Advances in Reproductive Medicine	November 15-18, 1997; (at Calcutta)	Dr. Sudersan Ghosh Dastidar, Organising Secretary-ICARM'97, Infertility Clinic and I.V.F. Centre, 208, Rash Behari Avenue, Calcutta-700029.
Asia-Pacific Congress on Head and Neck Cancer	December 11-14, 1997; (at Mumbai)	Dr. Ashok R. Mehta, Chairman of the Congress, Department of Surgery, Tata Memorial Hospital, Mumbai-400012.

INDIAN COUNCIL OF MEDICAL RESEARCH

Grant-in-aid for organising Seminars/Symposia/Workshops

The Council provides partial financial assistance for organising Seminars/Symposia/Workshops. Applications for grant of financial assistance (complete in all respects in the prescribed proforma), will be considered only if furnished **at least four months before the date of commencement of the Seminar/Symposium/Workshop,** *etc.*

COUNCIL'S TRAINING PROGRAMMES FOR 1997-98

Leprosy

At the Central JALMA Institute for Leprosy, Agra:

- Training Course on National Leprosy Eradication Programme for Medical Officers (September 15-26, 1997).

Reproductive Biology

At the Institute for Research in Reproduction, Mumbai:

- Workshop on Genotoxicity (August 18-29, 1997).
- Workshop on Basic Molecular Biology for Clinicians (December 2-11, 1997).

Endocrinology

At the National Institute of Nutrition, Hyderabad:

- Annual Certificate Course on Endocrinological Techniques and their Applications (August 1-September 15, 1997).

Nutrition

At the National Institute of Nutrition, Hyderabad:

- Postgraduate Certificate Course in Nutrition (December 1, 1997-February 28, 1998).

Occupational Health

At the National Institute of Occupational Health, Ahmedabad:

- Orientation Course on Occupational Health for Industrial Medical Officers (September 30-October 11, 1997).
- Training Course on Environmental Hygiene Practice (November 10-14, 1997).

Laboratory Animal Technology

At the National Centre for Laboratory Animal Science, National Institute of Nutrition, Hyderabad:

- Training Course for Laboratory Animal Supervisors (September 1-November 29, 1997).

Haematology

At the Institute of Immunohaematology, Mumbai:

- Training Course in Transfusion Medicine for Blood Bank Medical Officers (August 27-October 24, 1997).
- Training Course in Blood Group Serology and Blood Bank Methodology for Blood Bank Technicians (August 27-September 26, 1997).
- Training Course in Advanced Haematology and Immunohaematology (October 6-24, 1997).

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