REVIEW

The Role of Apoptosis in Normal and Abnormal Embryonic Development

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Programmed cell death or apoptosis is a widespread biological phenomenon. Apoptosis is characterized by typical cell features such as membrane blebbing, chromatin condensation, and DNA fragmentation. It involves a number of membrane receptors (e.g., Fas, TNFR) and a cascade of signal transduction steps resulting in the activation of a number of cysteine proteases known as caspases. Disordered apoptosis may lead to carcinogenesis and participates in the pathogenesis of Alzheimer disease, Parkinson disease, or AIDS. Programmed cell death plays an important role in the processes of gamete maturation as well as in embryo development, contributing to the appropriate formation of various organs and structures. Apoptosis is one of the mechanisms of action of various cytotoxic agents and teratogens. Teratogen-induced excessive death of embryonic cells is undoubtedly one of the most important events preceding the occurrence of structural abnormalities, regardless of their nature. Therefore understanding the mechanisms involved in physiological as well as in disturbed or dysregulated apoptosis may lead to the development of new methods of preventive treatment of various developmental abnormalities. The present review summarizes data on the mechanisms of programmed cell death and concentrates on apoptosis involved in normal or disturbed gametogenesis and in normal and abnormal embryonic development.

KEY WORDS: apoptosis; gametogenesis; embryogenesis; maldevelopment.

INTRODUCTION

The death of live cells can occur due to one of two mechanisms. It may be a response to external damaging insults or may be a predetermined event in their developmental program ["programmed cell death" (PCD) or "apoptosis"]. PCD seems to play an important role in mammalian reproduction and development. Apoptosis first appears in the 32- to 64-cell embryo and can be demonstrated during the whole embryogenesis, when it plays an essential role in virtually all of the stages of development necessary to produce a normally developed newborn.

In recent years evidence has accumulated that the formation of inborn anomalies or intrauterine death, induced by different developmental toxicants, result from distortions of the normal pattern of PCD in the embryo (1). Various chemical agents and physical factors have been shown to exert their effect by disturbing the apoptotic process occuring during gametogenesis (2). This review outlines the role of apoptosis in gametogenesis and embryogenesis and its role in determining responses to gametotoxic agents and developmental toxicants.

MORPHOLOGY AND MECHANISMS OF PHYSIOLOGICAL APOPTOSIS

Stereotypical morphological changes occur in almost all cells undergoing apoptotic death. The signs of apoptosis include cell shrinkage, membrane blebbing, nuclear condensation, and fragmentation, and, finally, the formation of separated vesicles called "apoptotic bodies" (3). The major biochemical event associated with apoptosis is DNA fragmentation by different nucleases (4). Gel electrophoresis shows the formation of a specific picture called the DNA "ladder," consisting of DNA fragments of a certain molecular weight.

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Apoptosis is regulated at three levels (Fig. 1). At the membrane level, there are specific membrane receptors mediating death signals. At the nuclear level, the genome contains genes which are transcribed as a response to apoptotic process initiation. At the cytoplasmic level, there are signal transduction pathways. The initial pathways leading to signal transduction are different for each receptor, however, the final stages are similar in most cases and involve cysteine proteases called "caspases" (5).

The main death-mediating membrane receptors are those of the tumor necrosis factor receptor (TNFR) family including the surface complex Fas. Receptors of the TNFR family are widely expressed on various cell types, both normal and malignant and in different parts of the embryo (6). Binding to the specific ligand results in the formation of the specific molecular association on the inner surface of the membrane known as the "death-inducing signaling complex," including procaspase-8 (7). As a result, activated caspase-8 dissociates from the membrane and goes to the cytoplasm, where it stimulates other caspases (first of all caspase-3) and, together with them, mediates apoptotic changes in cell nucleus.

Among the apoptosis regulating genes, there are two important gene families: the p53 tumor supressor gene, a molecular responder to DNA damage (8), and the Bcl-2 gene family. The P53 gene is involved in various events including cell cycle arrest, stimulation of DNA repair, and apoptosis (9). When cell DNA is damaged, p53 mediates a temporal arrest of the cell cycle in the G1 phase, giving the cell sufficient time to repair its DNA (10). If the DNA damage is too severe to be repaired, p53 initiates cell death by stimulating the Fas receptor complex and Bax (proapoptotic member of the Bcl-2 family of genes) on the mitochondrial membrane, leading to caspase-3 activation (11). Thus, p53 prevents the further development and reproduction of a cell with a damaged genome.

The Bcl-2 family of genes is another important system regulating the apoptotic response. The Bcl-2 genes and their corresponding proteins may inhibit the apoptotic process or propagate it (12). Antideath factors of the Bcl-2 family prevent the release of cytochrome *c* from mitochondria, thus stabilizing mitochondrial membrane potential. However, proapoptotic members of the Bcl-2 family propagate membrane permeabilty and ionic imbalance (13), leading to the release of cytochrome *c*. In the cytosol cytochrome *c* activates caspase-9 in the presence of a protein called apoptosis activating factor (14). Caspase-9 in turn stimulates caspase-3 playing a central role in the execution of apoptosis (15).

The above data demonstrate that apoptosis is a very complicated process. Therefore, it is clear that various damaging agents acting at the different steps of apoptotic machinery are capable to alter normal course of apoptotic cell death, thus disturbing processes in which apoptosis is involved.

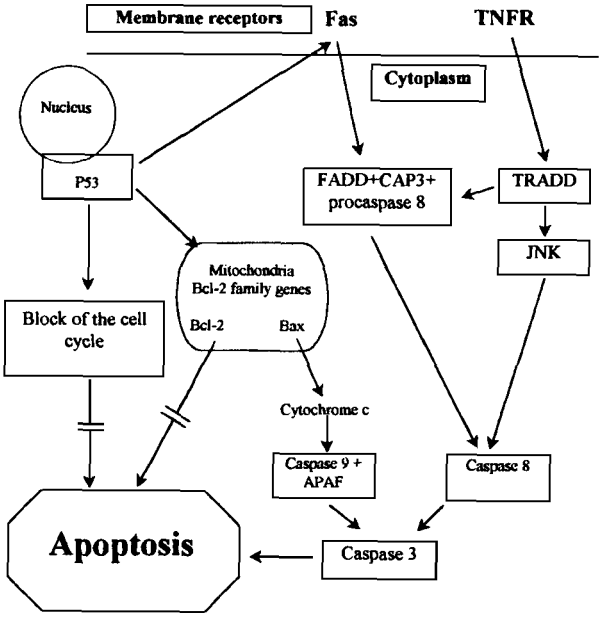

Fig. 1. Basic pathways of the apoptotic process.

APOPTOSIS IN GAMETOGENESIS

Apoptosis in Normal Gametogenesis

Apoptosis plays an essential role in spermatozoid and oocyte maturation in physiological conditions (16). It serves as a "guardian," controlling the quality of developing and differentiating cells and removing those which have undergone mutations or DNA damage which may be deleterious. On the opposite, any disturbance in normal pattern of apoptotic cell death causes marked abnormalities in gametogenesis. Normal gonadogenesis is characterized by a specific pattern of apoptotic cell death coinciding with certain periods in germ cell development. There are two peaks of spermatogenic cell apoptosis in mice—at 13 day postcoitum and around 10–13 days after birth—the first corresponding to the period of primordial germ cells immigration into gonads (17) and the second corresponding to the period when spermatogonia undergo active proliferation. The stable recurrence of these apoptotic waves indicates their importance in gametogenesis. In physiological oogenesis apoptosis

contributes to the degeneration of the majority of ovarian germ cells both during prenatal and reproductive life (18). Apoptosis involves two main phases of the meiotic process: an earlier one concerning the oogonia and oocytes in the preleptotene stage and a later one that concerns mainly the oocytes in the pachytene stage (19).

In addition to the germ cells themselves, apoptosis occurs in other cells, contributing to sperm maturation. The Sertoli cells also participate in the differentiation of the gametes (17). Recent works have also revealed that Sertoli cells are involved in apoptotic death (20).

Germ cells have been shown to possess the apoptogenic "machinery" described above. These cells have Fas as a main membrane death-mediating receptor, p53 gene as a protector of further development of damaged cells and antiapoptotic mechanisms including the Bcl-2 gene family. Flow cytometry and immunohistochemistry have demonstrated the expression of Fas on germ cells including spermatogonia, spermatocytes, spermatids (21), Leydig cells, Sertoli cells, and epithelial cells in the epidydimal duct (22). Expression of both Fas and the Fas-ligand (FasL) has also been detected in various kinds of tumors associated with the reproductive system (e.g., seminomas, carcinomas, yolk sac tumors) (23). Using immunohistochemistry Fas was localized to germ cells and Fas ligand to Sertoli cells (24). The expression of Fas was elevated after application of two germ cells apoptosis inducers so that the Sertoli cells seem to control the quality of germ cell output during spermatogenesis. Recent investigations identified DNA encoding a special death domain (called DEFT; death effector domain-containing testicular molecule) which is expressed in germ cells and may be important in the regulation of apoptosis during spermatogenesis (25). P53 mRNA is found in early spermatocytes (26) and p53 protein has been detected in mature spermatocytes in mice (27) and rats (28). Apoptosis is reduced and cell numbers increased in tetraploid germ cells in p53-negative mice (29). Morphological examination has revealed an elevated percentage of abnormal forms of spermatozoids in p53-knockout mice. Moreover, p53-deficient mice sired fewer offspring than p53-positive mice when both groups were mated with p53-positive females.

Along with the apoptogenic pathways, mechanisms confining apoptosis also exist in the gametes. It is uncertain whether the main apoptosis-inhibiting gene, Bcl-2 is expressed in germ cells. Bcl-2 products have been reported to be absent in the mammalian testis (30). There are reports suggesting that different members of the Bcl-2 family of genes are expressed in gonadogenesis. Bax is a potent inducer of apoptosis and Bax knockout mice are sterile (31). Mice lacking Bcl-w are viable, healthy, and normal in appearance, but the males are infertile (32). Both Sertoli cells and germ cells are reduced in number and the most mature germ cells are the most severely damaged. Consequently, the Bcl-2 family of genes seems to play an important but not completely understood role in gonadogenesis.

Apoptosis in Abnormal Gametogenesis

The apoptosis of normal gametogenesis can be altered by different factors. Dysregulation of apoptotic cell death resulting from the action of different pathogenic agents leads to abnormal germ cells development. Various stimuli may disorder the essential cellular systems, making the cell potentially dangerous or merely inviable. Such cells undergo apoptotic cell death.

The whole diversity of deleterious stimuli can be classified according to the mechanisms involved in modification of the apoptotic response. Agents influencing DNA integrity act chiefly via P53. Indeed, radiation (which is capable of killing the great majority of differentiating spermatogonia) was shown to activate p53 due to its DNA-alterating effect (33). Carcinogenic process in germ cells is also associated with disturbances in the regulation of apoptosis by p53 (34). At the same time abnormal apoptosis caused by environmental toxicants and some typical pathological processes leading to germ cells maldevelopment is often mediated whereby the Fas–FasL system. The Sertoli cell toxicant, mono-(2-ethylhexyl) phthalate, induces excessive germ cell death through overexpression of Fas and FasL (2). Such clinically important process as ovary atresia is based on dysregulation of Fas-mediated apoptosis (35). Detuning of the Fas-associated pathway participates at least partially in the formation of cryptorchidism in experiment (36).

Antiapoptotic mechanisms can prevent, to some extent, the excessive apoptosis caused by damaging agents. For example, overexpression of Bcl-X_L, which is an antiapoptotic member of the Bcl-2 family, inhibits apoptosis induced by etoposide (37), whereas Bcl-2 itself participates in regulation of apoptotic cell death induced by androgen withdrawal (38).

Therefore, it seems that PCD is necessary at different stages of germ cell development. Some pathogens alter the normal apoptotic pattern, leading to hypertrophic cell death. The excessive apoptosis caused by different deleterious stimuli (drugs, radiation, environmental

toxicants) which cannot be compensated by antiapoptotic mechanisms results in severe disorders in the process of germ cell maturation.

APOPTOSIS IN EMBRYO DEVELOPMENT

PCD is present in mammalian blastocysts, and its normal pattern is crucial for further development. Both sections of the blastocyst (inner cell mass and trophoectoderm) undergo apoptosis during normal development (39). However, both have different sensitivities to apoptosis-inducing factors (40). Distortions of apoptosis in the blastocyst result in compromise of future maturation and may lead to either early embryonic death or the formation of anomalies in the fetus (41).

At the later stages of normal embryo development, apoptosis plays a key role in the formation of the extraembryonic structures and the embryo itself. Thus, apoptosis has been demonstrated in fetal membranes (42). Electronic microscopy revealed ultrastructural changes in the amniotic epithelium and chorionic trophoblast cells consistent with apoptosis such as condensation of chromatin along the periphery of the nucleus and nuclear shrinkage. Mechanistic studies have revealed the involvement of Fas-mediated signaling pathways in the rearrangement of fetal membrane architecture during gestation.

Human trophoblast has been reported to undergo apoptosis under physiological conditions (43). The Bcl-2 gene and the Fas receptor have been shown to be involved in the regulation of this apoptotic process (44, 45). Therefore, apoptotic cell death may also play a key role in trophoblast turnover and renewal.

Apoptosis in postimplantation embryos is involved in processes such as eliminating abnormal, misplaced, nonfunctional, or harmful cells, sculpting structures, eliminating unwanted structures, and controlling cell numbers (46).

Formation of the preamniotic cavity has been shown to occur due to the death of the ectodermal cells in the core of the developing embryo. Apoptosis contributes to the formation of vesicles and tubes (e.g., neural tube) when epithelial sheets invaginate and tissue inside has to be eliminated (46). Neurons and oligodendrocytes which are overproduced during the development of the nervous system are also eliminated by apoptosis (47).

One example of the role of apoptosis can be seen in the hand plate. Here, cells which develop between the fingers are eliminated through apoptosis (48). The digits themselves begin to form as condensations of initial mesenchymal tissue. These condensations are the primary signs of future digit location (49). Apoptosis normally proceeds in zones which do not undergo condensation and is confined to strictly determined areas of mesenchyme. Further apoptosis expands into the whole interdigital mesenchymal tissue (50). Certain embryonic structures are removed during apoptosis, e.g., the Mullerian duct degenerates in males and the Wolffian duct degenerates in females. In both sexes, the pronephric tubes in mammalian embryos also disappear (17,51).

The above findings demonstrate the important role of apoptosis in the normal development. But what happens if the normal course of apoptosis in the embryo is disturbed? During the last decade evidence has accumulated that the majority of inborn birth defects induced by developmental toxicants are realized through distortions of the correct spatial and temporal pattern of apoptosis.

ABNORMAL APOPTOSIS LEADS TO ABNORMAL DEVELOPMENT

At present, between 3 and 6% of neonates have a major congenital anomaly (52). The pathogenesis of most of these anomalies is unknown. Nevertheless, it has been known for many years that excessive death of embryonic cells is one of the most important events preceding the occurrence of structural anomalies, regardless of their nature (53). Further studies have revealed that the effect of most of chemical and physical teratogens is associated with the induction of apoptosis in target organs, e.g., maternal hyperthermia has been shown to be a potent teratogen in experimental animals (54) and is also teratogenic in humans (55). Embryos of heat-shocked rodents exhibit anomalies such as exencephaly, microcephaly, microphthalmia, etc. (54). Hyperthermia has been shown to induce its teratogenic effect by activating the apoptotic process in target embryonic tissues. In rat embryos exposed to a teratogenic dose of heat (56), DNA fragmentation (a hallmark of apoptosis) was found as early as 2.5 hr after heat shock. The increased number of apoptotic nuclei was observed in the prosencephalic neuroepithelium in the area encompassing the optic cup. Our studies (57) have also demonstrated increased apoptosis in murine embryos after exposure to a teratogenic dose of heat. FACS analysis has shown an accumulation of apoptotic nuclei which reaches a maximum by 19 hr and declines by 24 hr after heating.

Apoptosis is also involved in the formation of structural anomalies induced by DNA damaging teratogens such as ionizing radiation and alkylating agents (58). Studies performed in our laboratory have demonstrated a strong correlation between the degree of apoptosis in target organs such as the limbs and the head and the severity of limb and craniofacial anomalies in embryos treated with cyclophosphamide (59).

Retinoic acid (RA) is necessary for normal embryonic development (60). However, retinoids also have a teratogenic potential [e.g., accutane (13-*cis*-RA), which is used for treating acne, is a human teratogen]. In the embryos of mothers treated with teratogenic doses of retinoids, the increased apoptosis in the neural crest cells and in cells located in the limbs correlated with the incidence of neural tube defects and limb malformations (49). Vitamin A-deficient embryos also exhibit a wide spectrum of anomalies and the spectra of malformations caused by retinoid deficiency and excess overlap. Accordingly, excessive apoptotic cell death of neural crest cells was also observed in the embryos of females deprived of adequate retinoids. (61).

Finally, diabetes-induced teratogenesis has also been associated with distortions in the regulation of apoptosis in the embryo. The occurrence of neural tube defects in the embryos of streptozotocin-induced diabetic mice correlated with an increased concentration of apoptotic cells in the mid- and hindbrains (62). Apoptotic cellular changes and overexpression of Bax (a death-promoting member of the Bcl-2 family of proteins) have been reported in blastocysts of diabetic mice (63). Maternal diabetes was accompanied by an increased number of apoptotic yolk sac cells. This effect may be responsible for the resorption of severely malformed embryos (64).

MODIFICATION OF APOPTOTIC PROCESS IN EMBRYO MAY INCREASE ITS RESISTANCE TO TERATOGENS

The above data suggest that apoptosis triggered by teratogens may be one of the basic mechanisms responsible for the formation of structural anomalies. Apoptotic cell death is an active process regulated by different genes in a tissue (cell)-dependent fashion. Therefore, new opportunities will arise to investigate the embryo's response to teratogens and to modulate sensitivity to developmental toxicants.

Thus, it has long been known that maternal nutritional deficiencies and excesses and metabolic and endocrine imbalances may not only result in the occurrence of malformed offspring but also modify the embryo's response to external teratogens (65). Mechanistic studies have revealed that maternal factors may modify the teratogenic response by influencing maternal metabolism of a teratogen or its placental transfer toward the embryo (65). Besides, evidence is beginning to accumulate that maternal factors may also be involved in the regulation of apoptosis induced by teratogens in embryonic cells. Thus, we (66) have shown that a manipulation of the maternal immune system significantly changes the incidence of malformations induced by the DNA-damaging teratogen—cyclophosphamide. Stimulation of maternal immune responses also has a protective effect against other teratogens and factors inducing embryonic death such as X-rays, urethane, and *N*-methyl-*N*-nitrosourea (67), the bacterial lipopolysaccharide (68), and restraining and ultrasonic stresses (69). Our recent studies have shown that immunostimulation significantly decreases the number of litters with malformed embryos in streptozotozin-induced diabetic mice (70) and increases the resistance of murine embryos to the teratogenic insult induced by heat shock (57).

Further studies have shown that teratogenic insult induced by cyclophosphamide, heat shock, or diabetes mellitus was associated with massive apoptosis in embryonic organ systems (66) and that immunopotentiation of female mice increases the resistance of murine embryos to induced teratogenesis and results in decreased apoptosis in target organ systems (71).

The precise mechanisms whereby maternal immune responses may influence the apoptogenic process in embryonic cells remain to be elucidated. It has been observed (72) that induced apoptosis in embryos followed by the formation of craniofacial anomalies was accompanied by the accumulation of tumor necrosis factor-α (TNF-α) mRNA, TNF-α protein, and TNF-α receptor (TNFRI) mRNA in cells situated in target embryo organ systems. Immunopotentiation of females while decreasing a level of induced apoptosis in cells was also followed by a clear decrease in the level of TNF-α mRNA and TNF-α protein (72).

Maternal immunostimulation has been shown to modify the expression of TNF-α and cytokines such as TGFβ2 and CSF-1 at the fetomaternal interface of mice with an increased level of spontaneous and induced postimplantation pregnancy loss. Immunostimulation induced decreased expression of TNF-α (73) and increased the expression of TGFβ2 (74) at the fetomaternal interface. This cytokine pattern was

accompanied by the improvement in their reproductive performance.

The above findings indicate that modification of the maternal immune response may be a powerful factor modifying the embryo's resistance to various developmental toxicants and suggest that its effect is realized by alterations in the apoptotic machinery. The question to be asked is what other means may be used to modify apoptotic response? One of the approaches may be realized by a variety of inhibitors of some key molecules involved in apoptotic machinery, e.g., blockers of main caspases. Therefore, further studies of the apoptotic mechanisms underlying this phenomenon and a search for agents capable of modifying some of the steps in the apoptogenic pathways might lead to new methods to prevent teratogen-induced birth defects.

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