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Apoptosis in the Embryo and Tumorigenesis

G. Prindull

INTRODUCTION

PROGRAMMED CELL DEATH (apoptosis) as a targeted active metabolic process leading to the demise of individual cells is an inherent property of rapidly proliferating cell renewal systems. It does not require prior arrest of the mitotic cycle, but occurs during G_1 and/or S phases [1–3]. Several distinct "death pathways" appear to be available to physiological and neoplastic cell systems [4, 5]. Apoptosis is a response to environmental growth factors and antibodies. The propensity to apoptosis is counterbalanced continuously in the cell by genes stimulating cell survival and proliferation. Genes inducing apoptosis include *p53*. *BCL-2* counteracts apoptosis, and *C-MYC* can promote both cell proliferation and apoptosis.

Apoptosis is the terminal event in the natural history of cell differentiation. In segmented neutrophils, high levels of DNAse activity have been found [6–8]. Environmental factors may trigger apoptosis at earlier stages of cell differentiation. It then becomes a physiological means of eliminating "unwanted" cells, such as auto-reactive cells [9–12], "hyperactive" T-lymphocytes [13], and neoplastic cells [14–16]. Tumour suppressor genes inhibit malignant cell growth by inducing apoptosis. A marked inherent propensity to apoptosis is displayed by hyperdiploid leukaemias, and may account for their relatively good prognosis [17]. Apoptosis is also a key mechanism in antitumour therapy. A number of cytostatic drugs, including the anthracyclins and glucocorticoids [18–20], kill cells by activating apoptotic pathways [14, 16], a process that in some leukaemias can be influenced by cytokines [21]. It should be emphasised at this point that not all forms of individualised cell death necessarily are apoptotic: tumour associated CD8+ lymphocytes and NK cells predominantly induce neoplastic cell death directly by osmotic lysis of cell membranes and cytoplasm [22].

We have pointed out repeatedly that some paediatric neoplasms can be considered as resulting from abnormal processes during embryonal ontogeny [23, 24]. The embryo must develop protective mechanisms against the generation and expansion of neoplastic cell clones, similar to the elimination of auto-reactive lymphocytes. Apoptosis is a key mechanism of eliminating such

Correspondence to G. Prindull at the Department of Paediatrics, Robert-Koch-Strasse 40, 37075 Göttingen, Germany.
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abnormal cells. This requires expression of the appropriate genes, membrane receptors and cytokines. In the event of failure of these mechanisms, chains of events may become permissive, eventually leading to neoplastic transformation of cells, terminating in the generation of congenital paediatric neoplasms. For example, failure to eliminate embryonal cells by apoptosis from the adrenal medulla, or in metanephrogenic kidney, will permit their persistence into post-natal life as part of the pathogenesis of neuroblastoma and Wilms' tumour, respectively [25, 26]. In this review, we will try to correlate our present knowledge of the time schedule for expression of genes, cell membrane receptors, and cytokines, with the ability to regulate apoptosis in embryonic and neoplastic cells.

APOPTOSIS IN NORMAL EMBRYONAL DEVELOPMENT: EARLY OBSERVATIONS

Targeted, individual cell death—as distinguished from random death in tissue necrosis—has long been recognised as a mechanism of normal human development [27–29]. It plays a major morphogenetic role in the formation of tubular structures, fashioning of limbs and interdigital clefts [30], formation of facial structures, including the palate [31, 32], morphogenesis of the heart [33], and involution of phylogenetic vestiges [34]. For example, differentiation of metanephrogenic mesenchyme into epithelial tissue (in the rat embryo) is accompanied by apoptosis of supernumerous mesenchymal cells. However, a select group of *BCL-2*-expressing mesenchymal cells is rescued from apoptosis [35], and is pushed into epithelial differentiation through activation of protein kinase C. The inducer of this process is the ureteric bud [36].

Developmentally programmed, specific cell death occurs as early as the stage of blastogenesis during embryonal ontogeny [37, 38]. In mouse blastocytes, 95 h after conception, dead cells with electron microscopic (EM) features of apoptosis are part of normal development [39]. Extracellular hydrogen peroxide found in the blastocoele fluid causes apoptosis. The inner cell mass generates hydrogen peroxide by oxidation of extracellular polyamines by an amine oxidase, and in this way specifically eliminates those cells that have trophoblastic potentials before formation of germ layers [37, 40, 41]. Blastocystic trophoectoderm exerts strong environmental influences by which embryonal teratocarcinoma cells lose their properties of neoplastic growth, and participate in normal embryonal development [42, 43].

In cultures of normal human 16 cell blastomeres, multinucleate cells have been described that appeared arrested in their development. Subsequently, they were excluded from the cyst and eventually sequestered [44]. Although these cells had some EM features of apoptosis, multinucleation is not usually a characteristic of the classic apoptotic pathway. Phagocytosed pycnotic nuclei, which may represent apoptotic cells, have also been observed in the human yolk sac from 3 weeks of gestation in connection with erythropoietic maturation and yolk sac regression [45–49].

SOME GENES EXPRESSED IN APOPTOSIS

Genes, such as *p53*, *BCL-2*, and *C-MYC*, assume central roles, not only as regulators of apoptosis, but also of neoplastic transformation. All are transcribed during embryogenesis, but only limited data exist on human embryology. In the nematode, *Caenorhabditis elegans*, two genes, that is *ced-3* and *ced-4*, are required for, and one gene, i.e. *ced-9* is protective of, apoptosis. The *ced-9* gene appears to be regulated by the activities of the other two genes [50].

Physiology

Wild type (wt) *p53* is a regulator of gene stability and of cellular responses to DNA damage during cell cycle progression. It functions both, as transcription activator and suppressor. Wt *p53* induces mitotic arrest and apoptosis in many normal and neoplastic cell systems. Other apoptotic pathways, such as those induced by glucocorticoids, are *p53*-independent [51, 52]. Cells in G_0 steady state express low levels of mRNA and protein p53. Stimulated, they increase their transcription rates to maximal levels in late G_1 [53–56]. The half-life of p53 mRNA in resting blood mononuclear cells is approximately 1 h [57]. Homologous null *p53* thymocytes are resistant to those forms of apoptosis that are induced by agents causing DNA strand breakage [51, 52]. *p53* can overcome the differentiation blockade by downregulating *C-MYC* mRNA levels [58].

BCL-2 encodes a mitochondrial membrane protein promoting cell survival [9]. In factor-dependent cell lines, this prolongation does not obviate the need for growth factor stimulation [59]. *BCL-2* opposes the induction of cell death by genes *p53*, *C-MYC* and by growth factor withdrawal [60]. *BCL-2* independent apoptotic pathways also exist [5, 59, 61]. Alone, *BCL-2* does not block the entry of cells into the mitotic cycle, and may not overcome p53 mediated proliferation inhibition [58, 62–65]. Transcription of *BCL-2* is induced by IL-2 [66].

BCL-2 is expressed in the lymphopoietic system by resting and memory B cells, and by medullary thymocytes, protecting them from apoptosis [9, 67, 68]. Characteristically, most cortical thymocytes, preparing for apoptosis, do not express *BCL-2*. Thus, it is a gene involved in clonal selection of thymic censorship of self-reactivity [12, 69], particularly under conditions of *C-MYC* repression [70]. *BCL-2* is also expressed by very immature (CD34+, CD33−) haematopoietic precursor cells [71]. Its levels decrease during differentiation to granulocytes. Overexpression of *BCL-2* delays apoptosis of differentiated cells and prolongs their survival [72].

C-MYC is able to stimulate cell proliferation, but is also a potent inducer of apoptosis. *C-MYC* "kills" any cell that encounters growth limiting conditions in its environment, perhaps on the basis of its proliferation promoting properties [73, 74]. Enforced expression of *C-MYC*, in particular in co-expression with *BCL-2*, will inhibit *p53*-induced apoptosis [58].

Embryology

Virtually no information exists on *p53* expression during human embryogenesis. In the mouse, *p53* mRNA is expressed strongly in all tissues of the early embryo, including haematopoietic cells of the yolk sac, liver, and thymus [75], but is downregulated at later stages of development [76]. It was surprising to learn that the offspring of *p53* knock-out mice have a normal morphogenetic appearance and post-natal development. However, these animals are susceptible to spontaneous tumour development suggesting that mutant oncogenic forms of *tp53* may not be obligatory in the genesis of some neoplasms [51, 52, 77].

As with *TP53*, there is no information on *BCL-2* expression during human embryonal development. In the mouse embryo, *bcl-2* is transcribed in many tissues including the nervous system, liver epithelium, metanephrogenic tissues, intestine, and the haemato-lymphopoietic system. In general, *bcl-2* expression decreases as the tissues mature and becomes restricted to progenitor cells with regenerative capacities. *bcl-2* -/- knock-out mice complete their embryonic development, but show growth retardation, immaturity, and early post-natal mortality. Haema-

topoiesis, including lymphocyte differentiation, is initially normal in these mice, but later shows massive apoptotic cell death with involution of thymus and spleen [35].

Members of the myc family are transcribed early in human embryos [78, 79]. Mitogenic stimulation rapidly induces strong expression of *C-MYC* in quiescent (murine) embryonic stem cells, and anti-myc antibodies arrest the development of murine embryos at the morula stage [80].

Neoplastic transformation

The property of wt *p53* to induce mitotic arrest and apoptosis is a control mechanism of neoplastic cell transformation and clonal expansion. In tumour cells, wt *p53* promotes spontaneous and drug-induced [81] tumour regression [82]. Wt *p53* induces apoptosis of human CSF-dependent acute myeloblastic leukaemia (AML) cells upon growth factor deprivation [83]. It also delays DNA repair after radiation damage and in cells acquiring resistance to alkylating agents [84, 85]. Wt *p53* has an important pathogenetic role in tumorigenesis of ataxia telangiectasia by promoting defensive DNA mismatch repair [86, 87].

Functional loss of *p53* results from alterations of both alleles on chromosome 11p13 [88]. Different mutant p53 proteins in cancer cells vary in their ability to inhibit transcriptional transactivation and specific DNA binding of wt p53 [89]. They seem to provide additional functional gains to the cell, such as escape mechanisms, to avoid apoptosis in the presence of cytotoxic drugs [67]. Since simultaneous (relative) overexpression of proliferation promoting genes, such as *BCL-2*, facilitates neoplastic transformation of these cells, mutant *p53* may exert oncogenic effects [9, 53]. However, recent studies suggest that functional loss or mutation of *p53* appears to be a late event in the natural history of a neoplasm, occurring with tumour progression and with changes to a more malignant phenotype [90]. *p53* mutations are one of the most common gene alterations, occurring in 50–80% of human cancers [91]. In paediatrics, they are found in lymphomas and leukaemias [92–94], blast cell crisis of CML [95, 96], rhabdomyosarcomas, Wilms' tumours, PNETs, and others [97, 98], but the incidence seems to be much lower than in adult oncology [90].

(Experimental) overexpression of *BCL-2* is associated with tumour development, documenting the protective oncogenic effect of apoptosis. Extended cell survival induced by *BCL-2* may increase the opportunity for cells to acquire additional genetic defects of genes promoting growth and proliferation, or of tumour suppressor genes. *BCL-2* is expressed in many cancers and precancerous lesions including high-grade lymphomas and AML [68]. The level of regulated expression of *BCL-2*, but not of *C-MYC*, in different myeloid leukaemia cell lines is associated with their susceptibility to induction of apoptosis by different cytostatic drugs [99]. In the presence of these drugs, *BCL-2*-induced prolonged cell survival can lead to the emergence of drug resistant cell clones, when *C-MYC* is co-expressed [63, 73, 74] and, potentially, to neoplastic transformation [65, 73, 100]. Dysregulation of both *C-MYC* and *BCL-2*, are involved in the pathogenesis of malignant lymphoma. Dysregulated *C-MYC* produces an indolent follicular lymphoma which, upon subsequent acquisition of *BCL-2* t(14;18) translocation to the heavy chain enhancer, eventually develops into a clonal malignant lymphoma [101]. In neuroblastoma, *BCL-2* inhibits apoptosis, promotes drug resistance, and correlates with an unfavourable histology and with *N-MYC* amplification [102]. Inhibition of the susceptibility to apoptosis by deregulated *C-MYC* appears to be one of the pathogenetic mechanisms of acute myeloid and lymphoid leukaemias [99, 103].

CELL MEMBRANE RECEPTORS AND APOPTOSIS

"Activation-induced apoptosis" is triggered by environmental factors, such as antibodies and cytokine withdrawal, and is mediated by cell membrane receptors. One such receptor is TCR/CD3 which, in association with INFτ, triggers apoptosis in cortical thymocytes and peripheral blood lymphocytes [104] during clonal selection for self-reactivity [105, 106]. IL-2 inhibits IFNτ induced apoptosis. Uncoupling of IFNτ and IL-2 gene expression, following TCR/CD3 mobilisation on CD4+ and CD8+ lymphocytes, initiates apoptosis of T cells at different stages during development and activation. The stroma may be instrumental in coupling and uncoupling of IL-2 and IFNτ gene expression [107]. During human embryogenesis, TCR/CD3 transcription is induced by IL-2 [108] on hepatic and thymic CD7+ cCD3+ CD2+ lymphocytes from 9.5 or 10 weeks gestation onwards [109–111]. Abnormal TCR rearrangements, such as TCR α/δ [112], have been reported from highly undifferentiated leukaemias as a result of germ line transcription of TCR genes [113–116]. This raises the question whether abnormal TCR molecules on membranes of leukaemic cells may have failed to mediate induction of apoptosis in the genesis of these neoplasms. NK cells exert antibody-mediated cytotoxicity by apoptosis [117].

B-lymphocytes of germinal centres are programmed for death by apoptosis unless they receive a positive stimulus for rescue, for example by their CD21 receptors. Such stimuli include immune complexes presented by CD23 molecules on dendritic cells [118]. Expression of CD21 is limited to stages of lymphocyte differentiation following the pre-B cell stage. In embryonal ontogeny, CD21 is expressed in liver, bone marrow and spleen from 11 to 14 weeks gestation onwards [119]. Stimulation of IgM receptors which are detectable on B lymphocytes of embryonal liver as early as 12.5 weeks gestation, induces apoptosis by down-regulating *BCL-2* expression [61]. Silenced self-reactive B-lymphocytes are not necessarily committed irreversibly to unresponsiveness and apoptosis. Up to a certain point, this process is reversible and the cells may become reactivated [120]. Germinal centres are formed only after birth in response to antigenic stimulation [121]. *BCL-2* expression in germinal centre cells is restricted to the follicle mantle and to portions of the light zones [9, 67] implicated in selection and maintenance of plasma cells and B memory cells [63, 122]. This suggests that the remainder of germinal centre cells are susceptible to apoptosis.

There are other activation-inducible pathways in monocytes and macrophages which are regulated by interactions of M-CSF and IFNτ. IFNτ sensitises macrophages to apoptosis, and M-CSF increases their resistance [123].

The APO-1/FAS receptor is another membrane antigen that mediates apoptosis, as well as necrotic forms of cell lysis [124–126]. The cDNA for APO-1/FAS encodes a protein which shows homology to the receptors for TNF, nerve growth factor and the CD40 B cell antigen [124, 126]. Like TCR, APO-1 is involved in clonal selection of autoreactive lymphocytes in thymus and blood [127]. APO-1 also mediates apoptosis of CD34+ haematopoietic precursor cells in response to TNF and IFNτ [128]. In fact, FAS antigen and TNF receptor may share the same signalling pathways. *BCL-2* interferes with the apoptotic process mediated by the FAS antigen and TNF receptor [129]. APO-1 is also expressed on neoplastic B cells

[130] and controls cellular growth of some B neoplasms by induction of apoptosis [126]. Nothing is known about expression or functions of this receptor during embryonal development.

CYTOKINES AND APOPTOSIS

Physiological and many types of neoplastic cell systems with rapid turnover rates depend for survival and proliferation on cytokine stimulation, and direct cell to cell contacts with the stroma [131]. Consequently, withdrawal or dysregulation of cytokines may trigger apoptosis. Attempts to understand the role of apoptosis in the genesis of paediatric neoplasms should consider the ontogeny of the microenvironment. This is further emphasized by recent observations that foetal target cells, such as myelopoietic precursor cells of 6–12 weeks gestation, embryonal liver and mononuclear cord blood cells, show responses to TNFα, IFNα and IFN-τ, divergent from bone marrow progenitor cells of adults [132].

Cytokines affect the development of the embryo, even in its pre-implantation stages. Maternal macrophages, lymphocytes and epithelial cells of the reproductive tract provide the fertilised egg with EGF, IGF II, CSF-1, GM-CSF, PDGF, LIF, and other cytokines [133–137]. Totipotent embryonic stem cells produce and/or respond to cytokines EPO, PDGF, LIF, IL-6, CSF, FGF [138–142]. Foetal trophoblasts produce GM-CSF, CSF-1, IL-1, IL-6, TGF, PDGF etc. [143]. This suggests that environmental cytokines control yolk sac haematopoiesis [23], migration of haematopoietic stem cells from the yolk sac and lodgement in the embryonal liver [144], and haematopoiesis in the embryonal liver itself [145]. Although there is no, or very little, myelopoiesis in foetal liver [132, 146–150] GM-CFC are detectable in the liver from 5 to 8 weeks gestation onwards. *In vitro*, these cells respond to GM-CSF and are suppressed by TNF and IFNτ.

The composition of the stroma of the human thymus is already highly complex at the time of its colonisation with CD7+, CD3+ thymic precursor cells at 7–9.5 weeks gestation [151]. It consists of mesenchymal and six types of endocrine epithelial cells, and is derived from the third pharyngeal pouches, brancheal clefts, and pharyngeal arches. The thymic stroma produces a number of cytokines and has been successfully employed in lymphocyte-purged transplantations of patients with SCID [152, 153].

Abnormal cytokine production, including autocrine secretion, plays a major role in neoplastic cell transformation and/or their clonal expansion [154, 155]. For example, GM-CSF upregulates *BCL-2* expression in AML cells [156]. Thus, cytokines protect AML cells from apoptosis. Conversely, withdrawal of cytokines from factor-dependent neoplasms induces apoptosis [157]. Imbalances and functional abnormalities in the cytokine network during embryonal development will result in disturbances of cellular growth and maturation in the foetus and newborn. For example, impaired apoptosis in the embryonal development of metanephrogenic kidney tissues and adrenal medulla will result in the persistence of embryonal rest tissues as primary lesions in the genesis of Wilms' tumour and neuroblastoma, respectively [25, 26].

Our knowledge of the actions and time period of expression during human embryonal development of individual cytokines is very limited (manuscript in preparation). We know, for example, that TGFβ1 is produced by the foetal trophoblast. It has functional activity in mesenchymal cells of the yolk sac, liver and bone marrow, as well as in angiogenesis of human embryos from 8 weeks gestation onwards [158–160]. TGFβ1 has both cell proliferation stimulating and inhibiting properties, and is one of the most potent growth inhibitor polypeptides of mesenchymal, myeloid, lymphoid, epi- and endothelial tissues [161]. TGFβ1 arrests *C-MYC* expression when it interacts with pRb [162], and thus prevents clonogenic cells from entering the mitotic cycle [163]. TGFβ1 induces apoptosis in murine myeloid leukaemic cells [164] and in human hepatic cell cultures [165, 166]. Human AML cells exhibit heterogeneous growth responses to TGFβ1. Some effects of TGFβ1 on myeloid cells occur through apoptosis [167].

EGF is present in the maternal environment of pre-implantation embryos. It stimulates DNA synthesis in various cell systems, and does not appear to promote neoplastic transformation [168]. In the genesis of (rat) metanephrogenic kidney, EGF induces the conversion of mesenchymal into epithelial cells [36].

CONCLUSIONS

Apoptosis is a protective, physiological mechanism of eliminating "unwanted" cells from the body. As a general principle, it is employed in multiple processes of normal embryonal and post-natal development and steady state conditions. Recent studies have shown that failure of apoptotic removal of abnormal cells is one pathogenetic mechanism closely associated with neoplastic cell transformation and clonal expansion. During embryonal/foetal development from the fertilised zygote to the full-term infant, an orderly sequential expression of those genes, cell membrane receptors and cytokines must occur that are operative in apoptosis and in apoptosis prevention, and that also probably determine the clinical behaviour of neoplastic cells.

After we have developed a better understanding of how the failure of apoptosis occurs in the pathogenesis of a particular neoplasm, then the option may arise of increasing apoptotic surveillance of the lymphocyte/macrophage system as a way of preventing—or even treating—neoplastic disease. These measures could operate on three possible levels:

(1) Increasing cellular expression of apoptosis promoting genes, such as *p53*, and down-regulation of apoptosis inhibiting genes, such as *BCL-2*. Such options could be tested in animals using gene knock-out and transfection experiments.
(2) Prevention of the synthesis of abnormal cell membrane receptor molecules which occurs, for example, in some lymphomas as germ line transcription of TCR chains, and increasing the number of normal TCR/CD3 and APO-1 receptors on T-lymphocytes.
(3) Suppression of abnormal growth factor production, for example by monoclonal antibodies in the pathogenesis of factor-dependent leukaemias.

Perhaps some of these techniques could be adapted to post-natal paediatrics, but they will be even more difficult to institute as prophylactic measures during embryonal development when the pathogenetic process of a number of paediatric neoplasms is initiated.

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News

18th Symposium of Clinical Hyperthermia

This symposium will be held on 21–24 May 1995 in Kiev, Ukraine. It is being organised by the International Clinical Hyperthermia Society. For further information contact Prof. Sergej P. Osinski, R.E. Kavetski Institute for Oncology Problems Acad. of Sci. of the UkrSSR, 45 Vasilkovskaja Str, Kiev 22, Ukraine. Tel. 117 044 266 9802; Fax 044 271 7329.

Critical issues in tumour microcirculation, angiogenesis and metastasis: biological significance and clinical relevance

This workshop, a continuing education course of the Harvard Medical School (HMS) and Massachusetts General Hospital (MGH), Boston, Massachusetts, U.S.A., will be held between 5 and 9 June 1995. Topics include tumour angiogenesis, tumour stroma generation, metastasis, tumour blood flow, tumour micro-environment, adhesion molecules, leucocyte–endothelial interactions and delivery of novel and conventional agents. For further information contact Norman Shostak, Department of Continuing Education, 641 Huntington Ave, Boston, Massachusetts 02115, U.S.A. Tel. 617 432 0196; Fax 617 432 1562.

Drug Resistance in Cancer

An international symposium, which will include sessions on clinical, molecular and pharmacological aspects of drug resistance, will be held in Dublin, Ireland between the 20 and 24 September 1995. For more information, please contact Professor Martin Clynes, National Cell & Tissue Culture Centre/Bioresearch Ireland, Dublin City University, Dublin 9, Ireland. Tel. 01 704 5700; Fax: 01 704 5484.