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Minireview

Apoptosis

Biochemical events and relevance to cancer chemotherapy

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Two distinct pathways for cell death exist. Compared to necrotic death, physiological or apoptotic cell death is an active suicidal process that consists of a cascade of well-regulated synthetic events. Participation of specific genes in apoptosis, and its possible molecular regulation, are considered in order to investigate the mechanism of cell death induced by some cancer chemotherapeutic agents.

Anticancer agent; *bcl*-2; Calcium; Cell cycle phase; $p34^{cdc2}$; p53; Programmed cell death; Regulation; Sulphated glycoprotein

1. INTRODUCTION

Apoptosis, or programmed cell death, is a major mechanism of maintenance of homeostasis of several systems of the body under physiological conditions. It is an integral part of embryogenesis, metamorphosis, organ involution, and plays a controlling role in counteracting cell proliferation [1–5]. Compared to necrotic death it is a relatively slower process [6,7], and involves a series of well-regulated synthetic events [8,9]. An apoptotic cell induces a programme so that a cascade of biochemical changes occur in response to the stimulus. The cell actively participates in the suicidal process and destroys itself.

Originally described as 'shrinkage necrosis' [10] apoptosis is characterised by a marked reduction in cell volume and an increase in buoyant density [11,12]. Apoptotic bodies are characterised by their small size, dense chromatin, nuclear fragmentation, randomly assorted organelles in the cytoplasm, loss of characteristic membrane architecture and appearance of blebbing [1,7,13]. Significant alterations in membrane composition take place [2,14].

A wide variety of physical and chemical stimuli induce apoptosis [15]. Though the exact mechanism of selection of the biochemical pathway of cell death is not fully understood it is clear that cells possess effector mechanisms for self-destruction, which may be activated by several 'relatively mild' stimuli. In this review we will briefly consider the participation of specific genes in the process of programmed death in an attempt to elucidate the mechanism of induction of apoptosis, particularly by the cancer chemotherapeutic agents.

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2. BIOCHEMICAL EVENTS

A reversible pre-commitment step for induction of genetic programming through macromolecular synthesis is essential for apoptotic death [16]. Once the cascade of metabolic processes has been initiated within the committed cell it proceeds to completion [17]. Receptors play a major role in perception of the stimulus. A wide variety of messengers and second messengers are released. The process is mostly turned on through synthesis of macromolecules. As a final step the DNA is typically destroyed by the action of endogenous endonuclease [18]. It is becoming evident that several genes participate in the cascade of events.

2.1. *Participation of specific genes*

Identification of the deathless mutants of *Caenorhabditis elegans* initiated the search for the mammalian equivalents of the cell death (*ced*) gene. In this nematode a number of mutations affecting the process of cell death have been identified. In particular the genes *ced-1*, *ced-2* and *nuc-1* affect apoptosis [19,20]. *ced-3* and *ced-4* genes may be involved in determining which particular cells express the fate of apoptosis during development [21] of this nematode.

Specific expression of cell death-associated gene products has been reported. Originally identified as androgen repressed messages [22] in involuting rat ventral

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prostate a group of testosterone-repressed mRNA sequences have subsequently been described [23]. The most abundant of these sequences is the testosterone-repressed prostatic message-2 (TRPM-2), the translation product of which is a 46 kDa protein. The TRPM-2 gene product has been identified in a variety of cell types undergoing apoptosis [24]. A transient but sharp rise of TRPM-2 mRNA has also been reported during early phases of regression of hormone-dependent Schionogi mouse mammary carcinoma [25], rat prostate [26,27], in androgen-independent AT3 prostatic cancer cells undergoing apoptosis following treatment with 5-fluorodeoxyuridine and trifluorothymidine [8], during regression of PC82 human prostate cancer following androgen ablation [28], during induction of apoptosis of L929 tumour cells of C3H mice upon treatment with recombinant human tumour necrosis factor and inhibitors of DNA topoisomerase type II [29], and also during death of estrogen- dependent MCF-7 human breast cancer cells xenografted onto nude mice [30]. Sulfated glycoprotein-2 (SGP-2), a constitutively expressed gene product originally characterised in mammalian Sertoli cells, has gained attention due to its rapid induction in numerous types of mammalian cells undergoing apoptosis. SGP-2 shares extensive sequence homology with TRPM-2, and it has been shown only very recently that both these proteins are encoded by a single gene. Southern blot analysis of *Bgl*II-restricted genomic DNA screened for the presence of restriction fragments homologous to SGP-2 indicated that the human homologue of SGP-2 resides on chromosome 8 [31].

Using the regressing rat ventral prostate gland as a model to study androgen-programmed cell death a series of molecular events that accompany the process was characterised [32]. There is a sequential induction of specific gene transcripts. The first event in the cascade is an induction of the specific gene encoding c-*fos*, the expression of which has been linked to perturbed intracellular Ca^{2+} levels [32]. This is followed by sequential induction of c-*myc* and 70 kDa heat-shock protein mRNA [33–35]. Simultaneous treatment of rats either with verapamil or with nifedipine on castration significantly delayed regression in both cases, indicating that Ca^{2+} flux is an early physiological step involved in the cascade. Furthermore the antagonists suppressed induction of transcripts encoding for both c-*fos* and TRPM-2 [33]; expression of c-H-*ras* and pS2 also decreased [30]. Recently it has been shown that an elevation of Ca^{2+}, 3–6-fold above the base line, induced apoptosis in androgen-independent prostatic cancer cells [36]. It is probable that a sustained increase in Ca^{2+} can play a major controlling role in activating specific genes needed for apoptosis.

Wild-type p53 protein, a product of the tumour suppressor gene, has been reported to induce apoptosis in the murine myeloid leukemic MI line clone, S6 [37]. Isolation and characterisation of a cDNA clone from CEM C7 cells showed homology with the human HL-14 gene encoding a β-galactoside-binding protein. This protein is over-expressed during programmed death induced by glucocorticoids. The mouse homologue acts as a potent cell growth inhibitory factor [38]. Very recently Cheng et al. [39] demonstrated that tumorigenicity of T leukemia Be-13 cells, which lack endogenous p53 protein, can be suppressed when infected with a recombinant retrovirus encoding the wild-type allele of human p53. Expression of p53 reduced growth rate, clonogenic potential and tumorigenicity, indicating the possibility of modulation of the tumorigenic phenotype through high-efficiency infection with retroviruses – at least, in leukemic cells.

The *bcl*-2 protooncogene expression enhances the survival of B cell precursors on withdrawl of growth factors. The finding that *bcl*-2 provides a survival advantage for the cells [40–43] by preventing the onset of apoptosis [44,45] suggests that, in some cells, programmed death may be negatively modulated by other genes. *bcl*-2 has been shown to be involved in prevention of apoptosis in T cells [46], thymocytes [47], germinal center B cells and Burkitt lymphoma cells [48], and in pre-B leukemia lines [49]. Recent evidence suggests that high levels of *bcl*-2 enhances cell survival under conditions of repressed c-*myc* expression, probably by mobilisation of Ca^{2+} from mitochondria to cytoplasm. This in turn can activate protein kinase C and help in survival [49,50]. Epstein–Barr virus in circulating B cells may influence suppression of apoptosis by the expression of Epstein–Barr virus latent genes [51]. DNA transfection into human B cells demonstrated that expression of the viral membrane protein 1 suppresses apoptosis by up-regulating expression of the oncogene, *bcl*-2. The mouse anti-*Fas* monoclonal antibody binds to a 36 kDa transmembrane receptor which has significant homology with human tumour necrosis factor receptor, human nerve growth factor receptor and human B cell antigen, CD40. The antibody, when recognised by the appropriate cell surface antigen, can induce apoptosis either by preventing the activity of a factor necessary for survival or by serving as a positive death-inducing signal [52].

Methylation is inhibited by the drug, 5-azacytidine. Murine thymocyte sub-line, SAK8, reported to possess functional glucocorticoid receptors but resistant to hormone-induced death [53], becomes sensitive to the hormone treatment when treated with 5-azacytidine. The DNA remains in a demethylated state following the drug treatment, and the resistance of the cell correlates well with the level of methylation of DNA [54], indicating thereby that certain genes must remain in a transcriptionally activated demethylated state for glucocorticoids to be effective.

2.2. *Molecular regulation*

Little is known about molecular regulation and the

factors influencing the onset of apoptosis. Chinese hamster V79 fibroblast cells, when subjected to cold shock, undergo apoptotic death. Cells at the transition from exponential to stationary growth are the most sensitive [55]. However, epidermal cells in their synthetic phase are reported to be more prone to damage [56]. One fundamental question that remains to be answered is whether apoptotic death can be induced at a specific point of the cell cycle progression. Unfortunately only scant information is available on this topic. While it is known that transcriptionally active chromatin is preferentially cleaved during programmed death [57] Kung et al. [58] reported an absence of correlation between cell cycle phase at the time of anticancer drug addition and subsequent morphology of cell death.

L1210 cells, when treated with cisplatin [59], and murine BW514 thymoma cells, when treated with dexamethasone or irradiated with γ-rays [60], showed inhibition of DNA synthesis that correlated with arrest of the cells in the G_2 phase of the cell cycle. Inhibition of total RNA synthesis was observed initially. This was followed by a recovery that corresponded well with the passage of the cells through the G_2 phase [59]. DNA double-strand breaks appeared in cells destined to die [59]. An increase in poly(ADP-ribosyl)ation is commonly associated with DNA breaks [61], causing a decrease in the NAD pool and in ATP levels. However, Sorenson et al. [59] clearly demonstrated that poly(ADP-ribosyl)ation occurs after DNA degradation as an effect of the damage. Their studies showed that initial suppression of DNA synthesis during S phase was followed by a recovery, reflecting a passage of the cells into G_2 phase. Cells were subsequently blocked at the G_2 phase. This arrest could not be attributed to detectable changes in transcription.

A probable explanation may be given from the *RAD 9* gene function [62]. The product of this gene is essential for arrest of cell division following DNA damage in *Saccharomyces cerevisiae*. As postulated previously by Tobey [63] RAD 9, or its quivalent gene product, may be involved in the surveillance mechanism. Cells may be lethally damaged yet continue to progress in the cell

Table I

Examples of anticancer agents reported to induce apoptosis in cultured cells

Drug	Cell lines tested	Concentration (μM)	Reference
Amsacrine	Thymocytes	10	[72]
Aphidicolin	CHO strain AA8	1–10	[58]
	CHO strains AA8, UV41	0.2	[85]
1-β-D-Arabinofuranosylcystosine	HL60, KG1A	3	[71]
BCNU	CCRF/CEM C7, F89, Molt-4-F, EB1, EB2-3945	219	[86]
Camptothecin	HL60, KG1A	0.1	[71]
Cisplatin	CHO strains AA8, UV41	0.025–40	[85]
	HL60, KG1A	20	[71]
	L1210/0	2–27	[59]
Etoposide	Thymocytes	50	[72]
	CHO strains AA8, UV41	40	[85]
	HL60, KG1A	17	[71]
	CHO	25	[65]
	Chronic lymphocytic leukemia	40	[87]
5-Fluorodeoxyuridine	CHO strains AA8, UV41	1	[85]
5-Fluorouracil	CHO strains AA8 UV41	10	[85]
	CCRF/CEM C7, F89, Molt-4-F, EB1, EB2-3945	77	[86]
Methotrexate	CHO strains AA8, UV41	0.5–128	[85]
	HL60, KG1A	1	[71]
Melphalan	CCRF/CEM C7, F89, Molt-4-F, FB1, EB2-3945	65	[86]
Teniposide	Thymocytes	5	[72]
Vincristine	CHO strain AA8	0.05-0.1	[58]
	F89, Molt-4-F, EB1, EB2-3945	2.2	[86]

cycle and eventually destroy themselves by apoptosis at the G_2M phase transition [58]. Poisons of DNA topoisomerase type-II may alter the activity of specific proteins [64,65] and cause a block of cells at the G_2 phase. Clear dissociation between drug-induced DNA topoisomerase II complexes and cytotoxicity has been reported [65–69]. Furthermore toxicity of mAMSA and etoposide was reduced by concomittant treatment with cycloheximide; but the drug–DNA topoisomerase II complexes were not decreased [67,70]. Fragmentation of internucleosomal DNA, so typical of apoptosis, has been reported after administration of etoposide [71], teniposide and amsacrine [72]. Here DNA cleavage had two distinct patterns: the first cleavage was due to drug–topoisomerase II interactions, whereas the second cleavage was due to entry of the drug-treated cells into a phase of programmed death [72]. It is probable that biochemical events that routinely occur at the G_2/M phase transition may be involved in programmed death of a cell.

The activity of $p34^{cdc2}$ kinase remains inhibited during the onset of apoptotic cascade following administration of etoposide or γ-irradiation [65], and correlates well with the G_2 arrest of the cells. This arrest of cells at the G_2 phase following etoposide treatment, however, is transient. Abnormally elevated $p34^{cdc2}$ kinase activity was detectable during mitosis, and was related to cell death [73]. A prolonged mitosis, during which activities of specific cell cycle-regulated proteins/regulatory proteins are altered, may result in DNA damage and cell death. The *cdc*2 gene is activated and inactivated by specific phosphorylations and by a variety of associated proteins [74–76]. Regulation of the gene is defined well in yeast: while *cdc*25 activates the kinase [77] *wee*1 suppresses it. Cells can undergo a catastrophe when mutation causes imbalance in these genes [78].

DNA damage alone may not be a prerequisite for cell death. In human cells the $p34^{cdc2}$ kinase complex interacts directly with p13 [74]. Microinjection of p13 or antibodies to p13 to rat fibroblasts causes micronucleation and death [76]. Kung and co-workers [58] suggested that cytotoxicity does not derive directly from the specific biochemical action of the drug per se, but results from the disparate inhibition of certain cell cycle processes or from dissociation of normally integrated and linked cell cycle events. In support of their hypothesis the authors provided examples of apoptotic death of Chinese hamster ovary cells treated with aphidicolin or vincristine. Further support of the hypothesis comes from the studies reporting potentiation of cytotoxicity of cells treated with caffeine to uncouple cell cycle events leading to mitosis [79–81], or by uncoupling protein synthesis from DNA synthesis [82,83]. Enoch and Nurse [84] provided additional support. Their studies demonstrated that in *Schizosaccharomyces pombe cdc*25 mutants, which are defective in the control of mitosis, are highly susceptible to the lethal effect of DNA synthesis inhibition. Presence of an additional mutation at the *cdc*10 gene (preventing cells from entering start) killed only those cells that entered S phase prior to DNA synthesis inhibition. This result supports the concept that an ordered progression of cell cycle events is an essential prerequisite for cellular viability.

3. HYPOTHETICAL RELEVANCE TO CANCER CHEMOTHERAPY

As shown in Table I several anticancer drugs induce apoptosis in different cancer cells and in thymocytes. This phenomenon has never been reported in cells taken from tumour biopsies of tumour-bearing animals or from cancer patients after drug treatment. However, the lack of in vivo data does not reduce the potential relevance of apoptosis for the anti-tumoral activity of the drug, since the cells undergoing apoptosis are probably rapidly destroyed by phagocytic cells, and it is their very short 'half-life' that makes it difficult to detect them.

It is evident that apoptosis can be induced by a variety of drugs with diverse chemical structure and different mechanism of action. Among the long list of drugs reported to induce apoptosis are (i) the DNA-damaging agents, such as BCNU, melphalan and *cis*-platinum, known to cause several types of DNA damage, such as DNA interstrand and intrastrand crosslinks and DNA–protein crosslinks [88], (ii) drugs like camptothecin that cause protein-associated DNA strand breaks mediated by the enzyme DNA topoisomerase I, (iii) the epipodophyllotoxins and intercalating agents that are poisons of DNA topoisomerase II, (iv) inhibitors of mitotic spindle apparatus, such as the vinca alkaloids, (v) inhibitors of DNA synthesis (aphidicolin), and (vi) several antimetabolites.

The fact that apoptosis can be induced by perturbing cell biochemistry with a variety of antineoplastic drugs suggests that the phenomenon can be triggered by multiple mechanisms, or that it occurs via a common mechanism which is activated by all these drugs. All these drugs cause a block of macromolecule synthesis and of the progression of the cell cycle, which is reported to occur before the cytotoxicity is evident. The various drugs listed in Table I can block the cells at different points of the cell cycle: while some block the cells at the beginning of the S phase, others block in G_2, while some other drugs induce a block in mitosis [63]. It seems unlikely, therefore, that the activation of apoptosis is mediated by a specific block in the cell cycle. More probably it appears to be related to a general metabolic stress and/or damage of cell structure(s). Unfortunately the steps leading to endonuclease activation following perception of the death signal are still very poorly elucidated.

As previously indicated a number of genes involved in the apoptotic process have already been identified.

Biochemical characterisation of the function of the gene products will allow the investigation of the mechanism of drug-induced apoptosis. It appears that the majority of cells, neoplastic or otherwise, in which the phenomenon of drug-induced apoptosis has been described, are derived from the haemopoietic system. It is unclear whether the lack of data on drug-induced apoptosis in cells derived from solid tumours is due to paucity of studies or to the fact that negative data are generally too difficult to publish. It seems possible that the phenomenon of apoptosis is phenotypically determined and can be induced only in some cell types. It may be that the drugs can induce or enhance the biochemical pathways leading to apoptotic death only in the cells where the phenomenon can occur naturally under certain physiological conditions. It seems tempting to speculate that tumours that respond to chemotherapy (e.g. some leukemia, lymphoma and embryonal carcinomas) are those derived from tissues already 'predisposed' to apoptotic death. Here the drugs probably work by simulating a stimulus which normally activates the suicidal programme. Experimental studies aimed at comparison of induction of apoptosis by cancer chemotherapeutic agents in neoplastic and normal cells of different origin are warranted to elucidate this point.

4. CONCLUSIONS

In this paper we have critically reviewed the most relevant available literature on the biochemical events and molecular regulation of apoptosis. We have also discussed the possibility that this phenomenon could be relevant for the susceptibility of the tumours to chemotherapeutic agents. This hypothesis is still speculative at the moment, but the availability of a better knowledge of the molecular events involved in the apoptotic process makes it realistic to investigate the mechanisms involved in the induction of programmed death by anticancer drugs.

It is still unclear if all cells can undergo apoptosis under certain circumstances or whether it is a characteristic of only some tissues which are under the regulation of specific hormone or growth factors. An important issue, which has not been discussed here, concerns the choice of the most suitable method for the evaluation of the phenomenon. This issue is of potential relevance when the process of apoptosis is evaluated after exposing the cells to chemicals that can cause necrosis or apoptosis, depending on the intensity or severity of the stimulus. Only a very fine line separates the two intensities producing apoptosis and necrosis. Therefore a reliable quantitative measure of the two types of death over time is needed.

As indicated in this review there are reasons to believe that the mechanism of programmed cell death is regulated by a definite action of genes. It may be possible that the modulation of the gene in a coordinated fashion will result, advantageously, in the selection of cell killing by antineoplastic agents specifically against tumoral cells [89]. Although this hypothesis is still entirely speculative it indicates a radically novel approach for the identification of new effective anticancer therapies.

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