Programmed cell death: beyond the frontiers of science

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REPORTE

Programmed Cell Death (PCD) is an emerging topic contributing actively to basic biology, and in the near future, we could expect practical applications improving human health and the productivity of our crops. Current results relate this complex and paradoxical process with the physiological development, stress response and diseases of plants and animals. With the aim of improving the exchange as a starting point to future cooperation in the field of PCD, the International Cell Death Society (ICDS) and the European Cell Death Organization (ECDO) organized in Havana on February 24th the International Seminar Programmed Cell Death in Plants: *a challenge to the new millennium*, sponsored by the Tobacco Research Institute of Havana. To this historical meeting, the first co-organized by the two most important organizations on PCD, were invited recognized scientists from Italy, USA, France, Belgium, Switzerland, Czech Republic and Cuba. Although the seminar was organized to deal with the basic aspects of cell death in plants, speeches referred frequently to animal models.

Session chairs, Mauro Piacentini and Zahra Zakeri, presidents of ECDO and ICDS, respectively, guided thirty minute speeches of ten speakers. The opening words of Vladimir Andino, head of the Tobacco Research Institute of Havana, and Mauro Piacentini, were followed by the speech of Richard A Lockshin from the Department of Biological Science of St. John's University, USA. He dealt with the historical origins of PCD research [1]. According with his speech, cell death as a normal, physiological process was recognized in the 19th Century. One hundred years later, many of these deaths (in animals) were described as programmed, deriving from the recognition that, in embryonic development and metamorphosis, cells died at predictable times and places. Thus the assumption was that cell death was genetic in origin, and not a random loss of control. Later, Kerr, Wyllie, and Currie [2] called attention to a common morphology of many cell deaths and coined the term "apoptosis" in order to assert its importance in homeostasis as opposite and equal to mitosis (Figure 1). Shortly thereafter, a group of researchers ultimately led by Horvitz [3] proved the existence of genes that controlled all cell deaths in the embryo of the nematode *Caenorhabditis elegans.* Their research led rapidly to the identification of these genes. These included genes that could turn on or off the activation of death, genes that produced products that could kill cells, inhibitors of those products-activation of death often consisted of release from inhibition of death-and genes involved in the scavenging of the remnants of the dead cells. Two profound arguments developed from these discoveries: First, that all cells carried within themselves the capacity to self-destruct, with this capacity typically held in abeyance until the inhibition was released; and, second, the realization that the primary killer gene was often a sequence-specific protease now termed a caspase. Richard A Lockshin was one of the first researchers defining the cell death as a programmed process and is the co-editor of the most successful review on PCD: *When Cells Die* I and II [4, 5] published by John Wiley and Sons.

Peter Vandenabeele, from Molecular Signalling and Cell Death Unit, Department of Molecular Biomedical Research, Ghent University, Belgium, described the distinct types of cell death. Morphologically, three distinct types of cell death have been described. Type I apoptotic cell death is characterized by cell shrinkage and extensive chromatin condensation; type II autophagic cell death is associated with the formation of autophagic vacuoles inside the dying cell; whereas type III necrotic cell death is distinguished bya rapid loss of plasma membrane integrity and spillage of the intracellular content. Biochemically, cell death programs are subdivided into initiation, amplification and executioner phases (Figure 2). Depending on the stimulus and the cellular context, one distinct cell death program will become apparent, most probably because every cell death program is a result of self propagating signals and signals that suppress the other cell death programs. He mainly considered the signaling pathways initiated by death receptors, a family of receptors that bind ligands of the tumor necrosis factor (TNF) superfamily, that contain a characteristic death domain (DD) and that are implicated in cell death during homeostasis and pathology [6]. In type I apoptotic cell death the pivotal role of caspases, pro-and antiapoptotic Bcl-2 members and the release of intermembrane space mitochondrial proteins have been extensively documented. A wide variety of apoptotic stimuli converge on the activation of caspases and/or the translocation of proapoptotic Bcl-2 family members to the mitochondria causing the release of intermembrane space proteins. The final apoptotic morphological features and dismantling of the cell are executed by caspase-dependent specific proteolysis of a wide number of caspase substrates. Autophagy is part of an evolutionary conserved subcellular homeostatic mechanism that eliminates damaged subcellular structures and may function in remodeling and catabolic recovery. However, autophagy is also the principal feature of type II autophagic cell death that occurs in development, homeostasis, senescence and pathologies and is characterized by the occurrence of autophagosomes [7]. Recently, a connection between

1. Lockshin RA, Zakeri Z. (2001) Programmed cell death and apoptosis: origins of the theory. *Nat. Rev. Mol. Cell. Biol.* 2 (7):545-50.

2. Wyllie AH, Kerr JF, Currie AR (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251-306.

3. Horvitz H, Shaham S, Hengartner M (1994). The genetics of programmed cell-death in the nematode *Caenorhabditis elegans. Cold Spring Harbor Symp. Quant. Biol.* 59:377-85.

4. Lockshin RA, Zakeri Z, Tilly JL (1998). *When Cells Die I: A Comprehensive Evaluation of Apoptosis and Programmed Cell Death*, Wiley-Liss, New York;680 pp.

5. Lockshin RA, Zakeri Z (2004). *When Cell Die II: A Comprehensive Evaluation of Apoptosis and Programmed Cell Death*, Wiley-Liss, New York;548 pp.

6. Jupp OJ, Vandenabeele P, MacEwan DJ (2003). Distinct regulation of cytosolic phospholipase A2 phosphorylation, translocation, proteolysis and activation by tumour necrosis factor-receptor subtypes. *Biochem. J.* 374 (Pt 2):453-61.

7. Lockshin RA, Zakeri Z (2002). Caspase-independent cell deaths. *Curr. Opin. Cell. Biol.* 14(6):727-33.

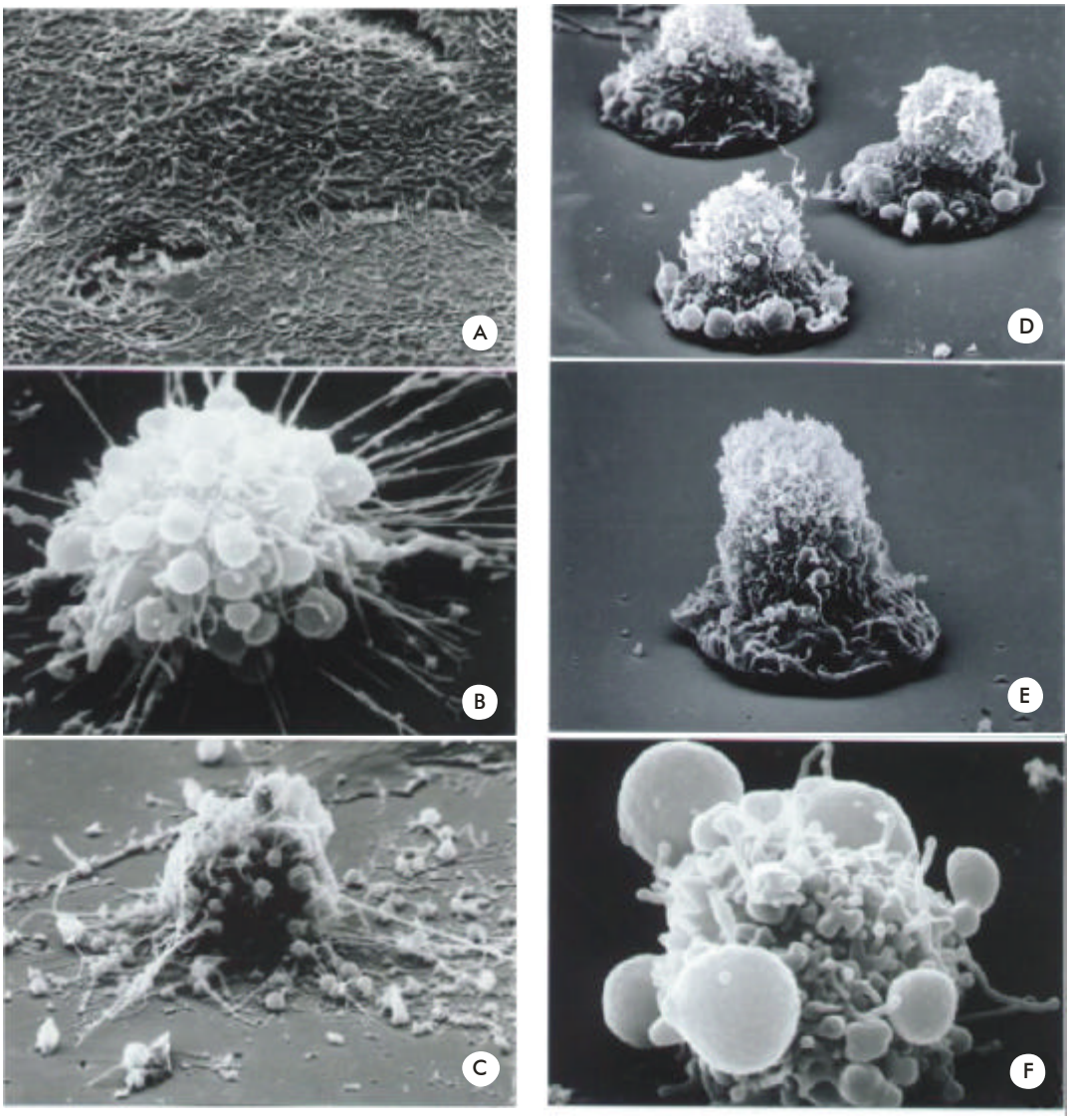

Figure 1. Scanning electron microscopy. Different apoptotic stages in epithelial cells. Flat cells (A) undergo different forms of rounding, surface blebbing and cell retraction (B-E) preceding the tipical apoptotic figures shown in (F). From Walter Malorni, with permission.

death receptors and autophagic cell death has been proposed. Type III necrotic cell death has often been defined as passive cell death due to physicochemical insults. However, recent results support the view that necrotic cell death can also be initiated by death receptors and has its own amplification and execution pathways. His overview stressed the existence of different cell death programs that may be differentially implicated in pathologies. The overwhelming number of reports on apoptotic cell death should not bias our attention and critical examination that other cell death programs may be associated with cell death occurring in different pathologies and stages of pathologies.

The research director of the Institute of Gustave Roussy, France, Guido Kroemer, presented the implication of mitochondria in cell death. A profound alteration in mitochondrial function constitutes an obligatory early event of the apoptotic process of mammalian cells. The molecular mechanism accounting for this alteration is mitochondrial membrane permeabilization (MMP), which is regulated by proteins from the Bcl-2 family. Anti-apoptotic Bcl-2-like proteins inhibit MMP, while pro-apoptotic proteins from the Bcl-2 family induce MMP. MMP is both sufficient and (mostly) necessary for apoptosis to occur. MMP results in mitochondrial failure as well as in the release of catabolic hydrolases and their activators from mitochondria and thus initiates the degradation phase of apoptosis [8] (Figure 3). MMP and the activation of caspases constitute two intertwined phenomena, meaning that induction of MMP can trigger the activation of caspases while activated caspases induce MMP [9]. The concept that MMP

8. Kroemer G, Reed JC (2000). Mitochondrial control of cell death. *Nat. Med.* 6:513-19.

9. Kroemer G, Dallaporta B & Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu. Rev. Physiol.* 60:619-42 (1998).

constitutes a central event of the apoptotic process has profound implications for the design of pharmacological strategies for apoptosis inhibition. It can be spectulated that mitochondria also play an important role in plant cell death.

Tissue transglutaminase (TGase2/tTGase) is a protein-crosslinking enzyme known to be associated with the in vivo apoptosis programs. Laszlo Fesus, head of the Department of Medical and Health Science Center, University of Debrecen, Hungary, reviewed recent studies concerning the expression and the possible role of this enigmatic enzyme in apoptotic cells during development as well as in those undergoing apoptosis in various patho-physiological and experimental settings [10]. In TGase2-/-mice, the lack of TGase2 prevented the production of active transforming growth factor-beta 1 in macrophages exposed to apoptotic cells, which is required for the up-regulation of TGase2 in the thymus in vivo, for accelerating deletion of CD4+CD8+ cells and for efficient phagocytosis of apoptotic bodies. The deficiency is associated with the development of splenomegaly, autoantibodies, and immune complex glomerulonephritis in TGase2-/-mice. These findings have broad implications not only for diseases linked to inflammation and autoimmunity but also for understanding the interrelationship between the apoptosis and phagocytosis processes [11]. Mauro Piacentini has also worked in this enigmatic field "tissue trans-glutaminase" in cell death and jointly with Lazlo Fesus has published many papers.

The president of the International Cell death Society, Zahra Zakeri, gave an instructive overview on the approaches in cell death studies. She said that to study cell death leading to the understanding of underlying regulatory mechanisms. There are many means by which cells reach physiological death, and it is important to be prepared to assess cell death by several different approaches [12]. These approaches can use both *in vivo* and *in vitro* model systems and include studies of the morphology and structure of the cell such as nuclear fragmentation, measurement of fragmentation of DNA into specific sizes and, in animals, assessment of the permeability of cell membranes by measuring the entry of vital dyes or the active exteriorization of phosphatidylserine to the outer leaflet of the cell membrane. She and her group also study organelle function, *i.e.*, mitochondria and endoplasmic reticulum, measurement of the activity of second messengers and the destructive pathways of the cells such as caspases, calpains and lysosomal enzymes. Enzyme activation can be determined in some instances by Western blot or immunohistochemistry, using antibodies specific for the activated forms of the enzymes, or by assessing proteolytic activation leading to decrease in size of enzymes. In other instances, enzymatic activity can be assessed using fluorogenic substrates or by cleavage of protein substrates into fragments of defined sizes (indicating cleavage at specific sites). Since not all forms of death are apoptosis, it is important to recognize that apoptosis-specific markers may not detect these, likewise important, instances of cell death. In specific instances, the presence and activity of phagocytes can also be evaluated. She and her group have used cell death during

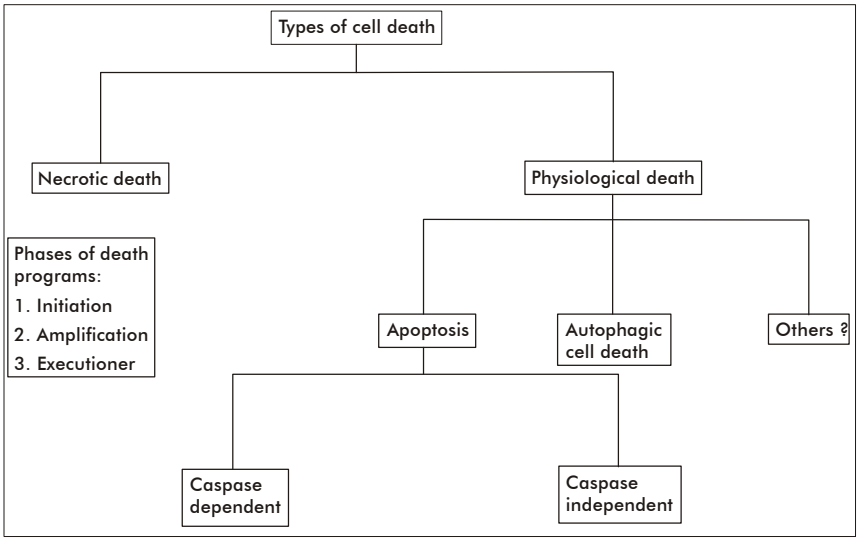

Figure 2. Morphological types of cell death and phases of death programs.

mouse embryonic development to study the mode of cell death as well as how it is regulated by the different pathways involved. They have found that specific enzymatic activities are activated during cell death, such as cell cycle dependent kinase 5. This enzyme is also differentially regulated in cell death ocurring in a number of different situations, both normal and pathological. In different systems they have analyzed the mode of cell death in insects such as the tobacco hornworm, *Manduca sexta*, and Drosophila. In the hornworm they find that the first sign of impending death is activation of lysosomes, and that most of the cytoplasm is destroyed before any signs of apoptosis appear, such as DNA cleavage, morphological change in nuclei, mitochondrial depolarization, or exteriorization of phosphatidylserine. Thus evaluation of cell death involves many features.

Walter Malorni from the Department of Ultrastructures, Instituto Superiore della Sanitá, Italy, charmed us with his excellent pictures on cell polarization and apoptosis. One of them magnificently illustrates this report (Figure 1). Cell polarization is a general feature of cells. It essentially depends on cytoskeleton (mainly on the microfilament system and ezrin-radixin-moesin, ERM proteins) and is of great importance in all cell types, neuronal epithelial or lymphoid cells. In particular, the functional state and fate of leukocytes depend on the acquisition of a polarity predisposing lymphocytes either to migration, activation or apoptosis. They have in fact two poles: the *leading edge*, which deserves as attachment-privileged site where the cell/substrate attachment takes place and the direction of cell movements is established, and, on the opposite side, the *uropod*, which actually "communicates" in a variety of immune cell activities including activation and apoptosis. Thus, an important requirement for a proper immune response is a transient contact with different substrates, e.g. the extracellular matrix, via the leading edge, as well as a "private" contact both among the immune cells

10. Melino G, Piacentini M (1998). «Tissue» transglutaminase in cell death: a downstream or a multifunctional upstream effector? *FEBS Letters*. 430:59-63.

11. Fesus L, Piacentini M (2002). Transglutaminase 2: An enigmatic enzyme with diverse functions. *Trends. Biochem. Sci.* 27 (10):534-9.

12. Zakeri Z, Lockshin RA (2002). Cell death during development. *J Immunol Methods*. 265(1-2):3-20.

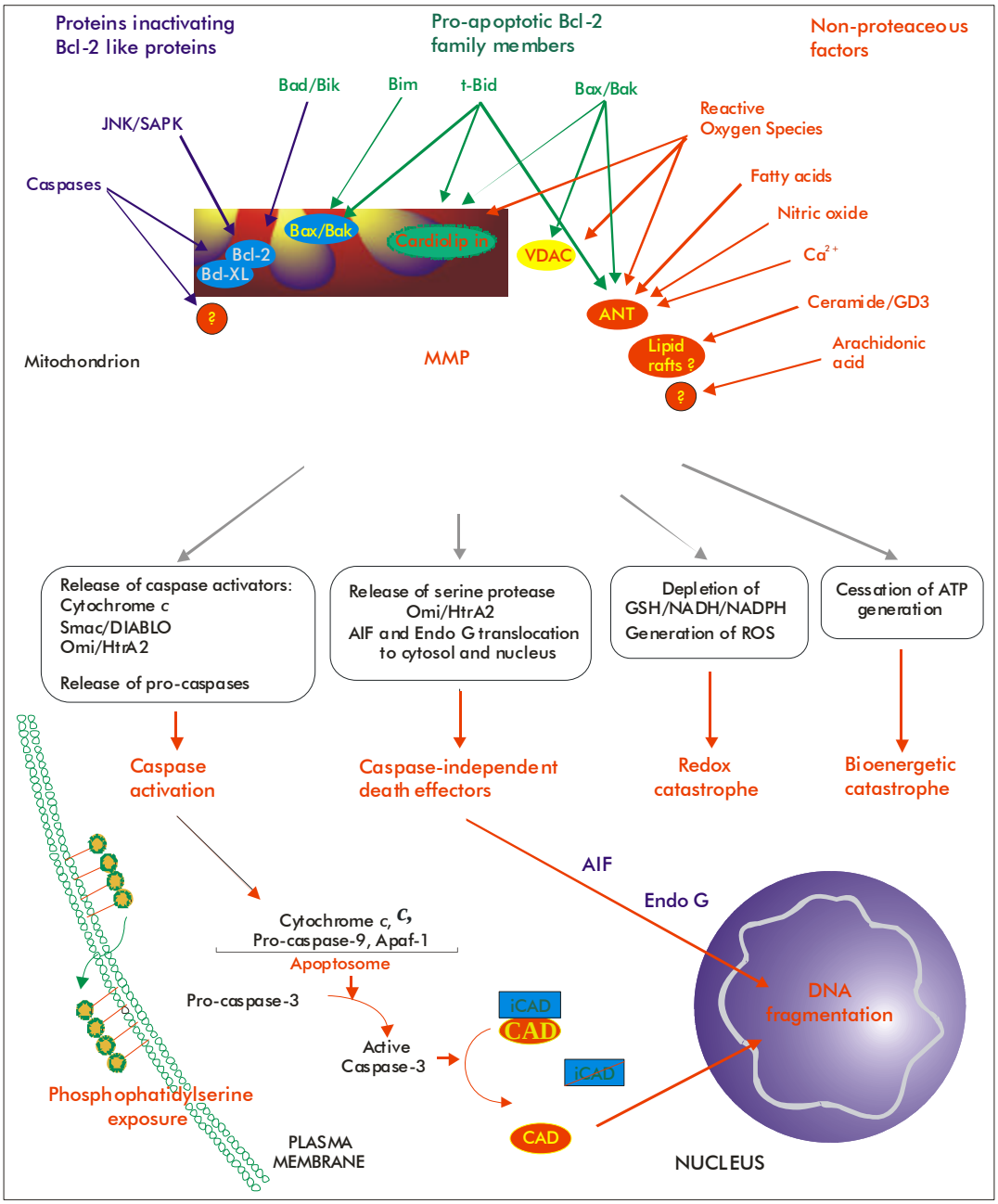

Figure 3. Phases of the apoptotic process with respect to mitochondrial membrane permeabilization (MMP). In the upper part of the figure, pro-apoptotic pathways converging on mitochondria are shown. Different apoptogenic molecules act on a variety of tentatively identified mitochondrial receptors (arrows), which in turn regulate MMP. The degradation pathways triggered by MMP are depicted in the lower part of the figure. The activation of caspases, as well as caspase-independent death effectors then trigger different manifestations of apoptosis including the exposure of phosphatidylserine on the plasma membrane surface and chromatinolysis. The complexity of apoptosis regulation is also visible at the level of DNA fragmentation. Thus, activation of CAD (by the caspase-3-mediated degradation of its inhibitor ICAD) leads to DNA degradation. DNA degradation can also be triggered by two mitochondrial proteins (AIF and Endo-G) that translocate to the nucleus. Note that a massive redox and bioenergetic catastrophe may cause accelerated killing of cells (necrosis) without which caspases would not come into action. Note also that caspase can act both upstream and downstream of MMP. AIF, apoptosis inducing factor; ANT, adenine nucleotide translocase; ATP, adenine nucleotide triphosphate; CAD, caspase-activated DNAse; Endo-G, endonuclease G; GD3, ganglioside GD3; GSH, glutathione; iCAD, inhibitor of CAD; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; VDAC, voltage-dependent anion channel. From Guido Kroemer, with permission.

and between immune cells and their targets. Before and during this complex cross-talk between cells and extracellular matrix components, a number of receptors and counter receptors crowd in the contact sites in order to allow efficient cell-to-cell or cell-substrate interaction. The membrane/cytoskeleton interaction plays a crucial role in tuning these activities and in "redisposing" the immune cells to

their function through the acquisition of a polarized phenotype. In particular, on the basis of the literature data, including their results, he suggested that cell protrusion called uropod, actively bobs up and down in the microenvironment as a sensory end, instead of crawling as a "passive" tail of leukocytes [13]. Thus, he proposed a role of "antenna-like" ("keraiosome") for this cell projection, and hypothesized that the keraiosome could be involved in determining the survival or death of leukocytes.

Many pathologies are associated with increased cell death such as autoimmune diseases, neurodegenerative diseases, vascular diseases and ischemia-reperfusion, while others such as cancer exhibit reduced cell death sensitivity. Boris Zhivotovsky, from the Division of Toxicology, Institute of Environmental Medicine, Karolinska Institute, Stockholm, Sweden, spoke about the ability of malignant cells to evade apoptosis as a hallmark of cancer; their resistance to apoptosis constitutes an important clinical problem. Cell death pathways in tumor cells appear to be much more complicated than was originally anticipated. Resistance of many tumors to chemotherapy is associated with either defects or dysregulation of death machinery. None of the components of this machinery operates in isolation, and the activation of a single caspase-dependent route for death is not sufficient to kill all types of cells. It is clear that exploiting a combination of caspase dependent and independent pathways may be more effective in killing tumor cells. Therefore, Zhivotovsky argued that it might ultimately be more useful to understand the interplay of various pathways rather than evaluating the isolated components. This approach might identify new targets for chemotherapy or new combinations for therapeutic interventions [14, 15].

Plants also manifest many dramatic forms of programmed cell death, including seasonal leaf drop (the etymological origin of the term "apoptosis") senescence of various organs and entire plants. Plant physiologists recognized and turned to the study of these deaths at approximately the time that animal biologists did, as Richard Lockshin said.

In plants, argued Zdenek Opatrny from the Department of Plant Physiology, Charles University Prague, Czech Republic, cell death comes up during both normal ontogenesis of the organized plant body and as a part of the plant defense mechanism against biotic or abiotic stress factors. For studies on cellular and molecular levels, cell lines can be used as an alternative to the complex organism. Tens of plant cell suspension cultures have been derived and employed during the last fifty years. However, only a few of them have been applied successfully to the detailed molecular, cytological and biochemical studies. Only tobacco cell lines VBI-0 [16] (originated 1967) and BY-2 [17] (originated 1974) seem to be the convenient bioassay systems to study the effects on plant cells of both internal factors (such as phytohormones) or external factors (stress, xenobiotics, growth regulators). In relation to this, the modifications of processes of cell division, growth, differentiation, polarity, and/or patterning have been investigated in detail using these lines. Responses of particular intracellular structures (such as the nucleus, endoplasmic reticulum, tonoplast and, in particular, both microfilamentous and microtubular cytoskeleton) to the application of abiotic stress factors have been characterized. For this purpose, special transgenic "GFP labeled" cell sublines have been derived especially from the BY-2 parental line, which enable the investigation of the reaction(s) of the cells "*in vivo and in situ*". Depending on both the nature and dosage of these factors, various responses of treated cells, from short temporary growth changes or cell cycle inhibition to accelerated senescence and/or cell death, are induced. Tobacco cell lines also have been used for the detailed characterization of progressive symptoms of cell death and for specification of its programmed character. Molecular (DNA destruction), cytological (nuclear fragmentation, destruction of membrane systems) and biochemical (e.g. levels of polyamines and other modifications of metabolism of phenolic compounds) aspects of cell development have been described after the application of abiotic stress factors, such as heavy metals or extreme temperature. The role of the cytoskeleton (active or passive) has been considered. Recently the mechanisms of action of other potential programmed-cell-death-inducing factors (such as plant pathogen toxins, or various cytostatics) have been investigated thoroughly.

The last expositor, another friendly woman, Donatella Seraffini-Fracassini, from Dipartimento di Biologia Evoluzionistica Sperimentale, Universita di Bologna, Italy, spoke about senescence and programmed cell death in tobacco. Several morphological and biochemical characteristics of developing and senescent petals of the tobacco flowers were studied: membrane integrity, cell wall modification, decline in protein, water, chloroplast, anthocyanin, increase of the protease activity, nuclear blebbing, DNA laddering. The supply of spermine (SM) to excised flowers delayed senescence and PCD, and caused an increase in PA levels in the corolla. SM was the most efficient polyamine in delaying PCD. The pigmentation of the corolla of treated flowers remained more intense for longer periods than in the controls and the appearance of a brown ring in the future abscission zone was also delayed. It is hypothesized that the mechanism by which PA exert this protective effect could be mediated by the enzyme transglutaminase (TGase). Post-translational modification of protein by conjugation of polyamines is catalyzed by TGase, that by conjugating polyamines to endoglutamine residues of proteins, produced bis- and mono-glutamyl putrescine (PU) and bis-glutamyl spermidine (SD). TGase activity was studied in the corolla at different stages of senescence. Bis-PU was the most abundant derivative before the DNA laddering stage; thereafter, bis-PU decreased and mono-PU became the most abundant derivative. When His_6-Xpress-GFP, a histidine-tagged green fluorescent protein utilized as a substrate for TGase of animal cells, was added to flower slices, a green fluorescence was also detected in senescing corolla cells. Using the same substrate, TGase activity was monitored by fluorescence and radioactivity detection on SDS-PAGE showing a change of the GFP molecular mass by labeled SM conjugation. Flower TGase was immunodetected; it caused the same modifications of GFP migration on the gel, as well as the appearance of high molecular mass products not resolved in the gel. By adding labeled SM to the cell free

13. Fais S, Malorni W (2003). Leukocyte uropod formation and membr e. 2 (1): 31-3.

15. Zhivotovsky B. (2003) Defects in the apoptotic machinery of cancer cells: role in drug resistance. *Semin Cancer Biol*. 13 (2):125-34.

16. Opatrný Z, Opatrná J (1976). The specifity of the effect of 2,4-D and NAA on the growth, micromorphology and occurence of starch in long-term *Nicotiana tabacum* L. cell strains. *Biol. Plant*. 18:359-65.

17. Nagata T, NemotoY, Hasezawa S (1992). Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. *Int. Rev. Cytol*.132:1-30.

extract of the corolla, mainly products of high mass in senescence phases and some bands of about 60 kDa were detected by gel autoradiography. In the late stages, the label was also detected on the front of the gel, possibly due to degradation products. The separation of glutamyl-PAs, produced by endogenous TGase, showed that during senescence mainly mono, bis-PU and bis-SD increased when compared to flowers of earlier stages. Mono-SM gradually increased during the flower's life-span. This data suggests that the effect of polyamines in the delay of senescence and PCD could be mediated by TGase [18].

After more than six hours of exchange and discussions, the concluding remarks were given by Zahra Zakeri. Beside the beautiful and grateful words of the president of the International Cell Death Society, the speakers offered the start of the collaboration between Cuban science and the international scientific community working on cell death. Havana University and the Tobacco Research Institute received books and reference materials related with PCD. An entire collection of the specialized publication Cell Death and Differentiation (CDD), the most important and quoted pubblication in this field, was given to the National Library of Science. Also, the ICDS and the ECDO will support the participation of three Cuban researchers in the next international meeting of these organizations in Ireland, June 2004, and Brazil, 2006. The very brief and historical meeting celebrated in Havana last February not only give a complete overview of the PCD outside of traditional and expensive frames. This meeting also fed the hope of young researchers. As said by Dr Zakeri "science is, like a monarch butterfly. It goes from country to country, tasting the sweetness of each land, and sharing its beauty will all".

18. Seraffini-Fracassini D, Del Duca S, Monti F, Poli F, Sacchetti G, Bregoli AM, Biondi S, Della Mea M (2002). Transglutaminase activity during senescence and programmed cell death in the corolla of tobacco (*Nicotian atabacum*) flowers. *Cell Death and Diff.* 9(3):309-21.

Mitochondria as cell death regulators

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A profound alteration in mitochondrial function constitutes an obligatory early event of the apoptotic process of mammalian cells. The molecular mechanism accounting for this alteration is mitochondrial membrane permeabilization (MMP), which is regulated by proteins from the Bcl-2 family. Anti-apoptotic Bcl-2-like proteins inhibit MMP, while pro-apoptotic proteins from the Bcl-2 family induce MMP. MMP is both sufficient and (mostly) necessary for apoptosis to occur. MMP results in mitochondrial failure as well as in the release of catabolic hydrolases and their activators from mitochondria and thus initiates the degradation phase of apoptosis. MMP and the activation of caspases constitute two intertwined phenomena, meaning that the induction of MMP can trigger the activation of caspases while activated caspases induce MMP. The concept that MMP constitutes a central event of the apoptotic process has profound implications for the design of pharmacological strategies for apoptosis inhibition. It can be spectulated that mitochondria also play an important role in plant cell death.

Cell deaths: Origin of our understanding and the range of possibilities

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Cell death as a normal, physiological process was recognized in the 19th Century. One hundred years later, many of these deaths (in animals) were described as programmed, deriving from the recognition that, in embryonic development and metamorphosis, cells died at predictable times and places. Thus the assumption was that cell death was genetic in origin, and not a random loss of control. Later, Kerr, Wyllie, and Currie called attention to a common morphology of many cell deaths and coined the term «apoptosis» in order to assert its importance in homeostasis as opposite and equal to mitosis. Shortly thereafter, a group of researchers ultimately led by Horvitz proved the existence of genes that controlled all cell deaths in the embryo of the nematode *Caenorhabditis elegans*. Their research led rapidly to the identification of these genes. These included genes that could turn on or off the activation of death, genes that produced products that could kill cells, inhibitors of those products—activation of death often consisted of the release from inhibition of death—and genes involved in the scavenging of the remnants of the dead cells. Two profound arguments developed from these discoveries: First, that all cells carried within themselves the capacity of self-destruction, with this capacity typically held in abeyance until the inhibition was released; and, second, the realization that the primary killer gene was often a sequence-specific protease now termed a caspase. Today we understand that this form of cell death, apoptosis, the most common form of cell death in animals, is dominated by these caspases. More than one caspase is involved, typically in a sequence in which an initiator caspase is activated and then this proteolytically activates an effector caspase, which attacks several structural and other essential proteins in the cell. The initiator caspase is activated either following the interaction of a cell surface receptor with a ligand or by interacting with cytochrome c and other materials released from mitochondria. The relative sensitivity of mitochondria to depolarization and the release of cytochromec is adjusted by pro- and anti-apoptotic proteins that bind to the mitochondria. The digestion of the proteins targeted by these caspases, including proteins of the cytoskeleton and chromatin as well as DNA repair enzymes, accounts for much of the morphology of apoptosis as well as the activation of DNases that cut the DNA in a characteristic, inter-nucleosomal, fashion.

Plants also manifest many dramatic forms of programmed cell death, including seasonal leaf drop (the etymological origin of the term «apoptosis,» senescence of various organs and entire plants, and defense mechanisms such as hypersensitive response. Plant physiologists recognized and turned to the study of these deaths at approximately the time that animal biologists did. Although these deaths are biologically extremely important, they have not attracted much attention among those studying animal cells because plants lack clearly identifiable caspases; the morphology of death does not resemble apoptosis; and the characteristic oligonucleosome-sized fragments of DNA are not observed. However, there are many other forms of cell death in animals as well as plants. These forms of cell death involve proteases other than caspases. The biological reasons for the deaths are similar to those of animals, and the control mechanisms are similar.

Cell polarization and apotosis

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Cell polarization is a general feature of cells. It essentially depends on the cytoskeleton (mainly on microfilament system and ezrin-radixin-moesin, ERM proteins) and is of great importance in all cell types, neuronal epithelial or lymphoid cells. In particular, functional state and fate of leukocytes depend on the acquisition of a polarity predisposing lymphocytes either to migration, activation or apoptosis. They have in fact two poles: the *leading edge*, which deserves as attachment-privileged site where the cell/substrate attachment takes place and the direction of cell movements is established, and, on the opposite side, the *uropod*, which actually «communicates» in a variety of immune cell activities including activation and apoptosis. Thus, an important requirement for a proper immune response is a transient contact with different substrates, e.g. the extracellular matrix, via the leading edge, as well as a «private» contact both among the immune cells and between immune cells and their targets. Before and during this complex cross-talk between cells and extracellular matrix components, a number of receptors and counterreceptors crowd in the contact sites in order to allow efficient cell-to-cell or cell-substrate interaction. The membrane/cytoskeleton interaction plays a crucial role in tuning these activities and in «predisposing» the immune cells to their function through the acquisition of a polarized phenotype. In particular, on the basis of the literature data, including our results, we suggest that the cell protrusion called uropod, actively bob up and down in the microenvironment as a sensory end, instead of crawling as a «passive» tail of leukocytes. Thus, we propose a role of «antenna-like» («keraiosome») for this cell projection, and we hypothesize that the keraiosome could be involved in determining the survival or death of leukocytes.

Tobacco cell lines as experimental models for studies of stress effects and programmed cell death in plants

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The Self-destructive behaviour of cells and/or cell complexes represents an indispensable part of the life of any multicellular organism. In plants, it comes up during both normal ontogenesis of an organized plant body and as a part of the plant defence mechanism against biotic or abiotic stress factors. For studies on cellular and molecular levels, cell lines can be used as an alternative to the complex organism. Tens of plant cell suspension cultures have been derived and employed during last half a century. However, only a few of them have been applied successfully for the detailed molecular, cytological and biochemical studies. Only tobacco cell lines VBI-0 (originated 1967, see *Opatrný and Opatrná 1976)* and BY-2 (originated 1974, see *Nagata et al. 1992*) seem to be the convenient bioassay systems to study the effects on plant cells of both internal (such as phytohormones) or external (stress, xenobiotics, growth regulators) factors. In relation to this, the modifications of processes of cell division, growth, differentiation, polarity, and/or patterning have been investigated in detail using these lines (*Domažlická and Opatrný 1989, Petrášek et al. 1998, 2002, 2003, Zažímalová et al. 1995,1996, Pokorná et al 2003 , Boøíková et al. 2003, Campanoni et al. 2003).* Responses of particular intracellular structures (such as nucleus, endoplasmic reticulum, tonoplast and, in particular, both microfilamentar and microtubular cytoskeleton) to application of abiotic stress factors have been characterized. For this purpose special transgenic „GFP labeled» cell sublines have been derived especially from the BY-2 parental line, to investigate the reaction(s) of the cells *„in vivo and in situ»* .

Depending on both nature and dosage of these factors, various responses of treated cells, from short temporary growth change or cell cycle inhibition to accelerated senescence and/or cell death, are induced. Tobacco cell lines have been used also for a detailed characterisation of progressive symptoms of cell death and for the specification of its programmed character. Molecular (DNA destruction), cytological (nuclear fragmentation, destruction of membrane systems) and biochemical (e.g. levels of polyamines and other modifications of metabolism of phenolic compounds) aspects of cell development have been described after the application of abiotic stress factors, such as heavy metals or extreme temperature (*Kuthanová et al. 2003*). The role of the cytoskeleton (active or passive) has been considered. Recently the mechanisms of action of other potential programmed-cell-death-inducing factors (such as plant pathogen toxins, or various cytostatics) have been investigated thoroughly.

Senescence and programmed cell death in tobacco petals

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Several morphological and biochemical characteristics of developing and senescent petals of tobacco flowers were studied: membrane integrity, cell wall modification, decline in protein, water, chloroplast, anthocyanin, increase of the protease activity, nuclear blebbing, DNA laddering (Serafini-Fracassini *et al*, 2002).

The supply of spermine (SM) to excised flowers delayed senescence and PCD, and caused an increase in PA levels in the corolla. SM was the most efficient polyamine in delaying PCD.

The pigmentation of the corolla of treated flowers remained more intense for longer periods than in the controls and the appearance of a brown ring in the future abscission zone was also delayed.

It is hypothesised that the mechanism by which PA exert this protective effect could be mediated by the enzyme transglutaminase (TGase). Post-translational modification of the protein by conjugation of polyamines is catalysed by TGase, which, conjugating polyamines to endoglutamine residues of proteins, produced bis- and mono-glutamyl putrescine (PU) and bis-glutamyl spermidine (SD). TGase activity was studied in the corolla at different stages of senescence.Bis-PU was the most abundant derivative before the DNA laddering stage; thereafter, bis-PU decreased and mono-PU became the most abundant derivative.

When His_6-Xpress-GFP, a histidine-tagged green fluorescent protein utilised as a substrate for TGase of animal cells, was added to flower slices, a green fluorescence was also detected in senescing cells of corolla. Using the same substrate, TGase activity was monitored by fluorescence and radioactivity detection on SDS-PAGE showing a change of the GFP molecular mass by labelled SM conjugation.

Flower TGase was immunodetected; it caused the same modifications of GFP migration on the gel, as well as the appearance of high molecular mass products not resolved in the gel. By adding labelled SM to the cell free extract of corolla, mainly products of high mass in senescence phases and some bands of about 60 kDa were detected by gel autoradiography. In the late stages, the label was also detected on the front of the gel, possibly due to degradation products. The separation of glutamyl-PAs, produced by endogenous TGase, showed that during senescence mainly mono-, bis-PU and bis-SD increased when compared to flowers of earlier stages. Mono-SM gradually increased during the flower's life-span. This data suggests that the effect of polyamines in the delay of senescence and PCD could be mediated by TGase.

More than one way to die, molecular mechanisms of cell death

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Many pathologies are associated with increased cell death such as autoimmune diseases, neurodegenerative diseases, vascular diseases and ischemia-reperfusion, while others such as cancer exhibit reduced cell death sensitivity. Morphologically, three distinct types of cell death have been described. Type I apoptotic cell death is characterised by cell shrinkage and extensive chromatin condensation; type II autophagic cell death is associated with the formation of autophagic vacuoles inside the dying cell; whereas type III necrotic cell death is distinguished by a rapid loss of plasma membrane integrity and spillage of the intracellular content. Biochemically, cell death programs are subdivided in an initiation, amplification and executioner phase. Depending on the stimulus and the cellular context one distinct cell death program will become apparent, most probably because every cell death program is a result of self propagating signals and signals that suppress the other cell death programs. In this overview we will mainly consider the signalling pathways initiated by death receptors, a family of receptors that bind ligands of the Tumor Necrosis Factor (TNF) superfamily, that contain a characteristic death domain (DD) and that are implicated in cell death during homeostasis and pathology.

In type I apoptotic cell death the pivotal role of caspases, pro- and antiapoptotic Bcl-2 members and the release of intermembrane space mitochondrial proteins have been extensively documented. A wide variety of apoptotic stimuli converge on the activation of caspases and/or the translocation of proapoptotic Bcl-2 family members to the mitochondria causing the release of intermembrane space proteins. The final apoptotic morphological features and dismantling of the cell are executed by caspase-dependent specific proteolysis of a wide number of caspase substrates. Autophagy is part of an evolutionary conserved subcellular homeostatic mechanism that eliminates damaged subcellular structures and may function in remodelling and catabolic recovery. However, autophagy is also the principal feature of type II autophagic cell death that occurs in development, homeostasis, senescence and pathologies and is characterised by the occurrence of autophagosomes. Recently a connection between death receptors and autophagic cell death has been proposed. Type III necrotic cell death has often been defined as passive cell death due to physicochemical insults, however recent results support the view that necrotic cell death can also be initiated by death receptors and has its own amplification and execution pathways.

Our overview aims to stress the existence of different cell death programs that may be differentially implicated in pathologies. The overwhelming number of reports on apoptotic cell death should not bias our attention and critical examination that other cell death programs may be associated with cell death occurring in different pathologies and stages of pathologies.

Models and approaches to study cell death

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Cell death is an important and controlled process in many situations, including normal development, physiological turnover of cells, and response of the organism to disease. Physiological cell death, under the control of the organism, is different from necrosis. In the latter situation, cells lose control of their ionic pumps, imbibe water, and rupture, disseminating their contents throughout the organism in an uncontrolled manner. In animals, this type of death often leads to inflammation. In physiological cell death, the mode of death is closely controlled by the organism and the cell, resulting in a regulated destruction or release of biological materials. We have used a number of model systems to study cell death and the underlying regulatory mechanisms. There are many means by which cells achieve physiological cell death, and it is important to be prepared to assess cell death by several different approaches. These approaches can use both *in vivo* and *in vitro* model systems and include studies of the morphology and structure of the cell such as nuclear fragmentation, measurement of fragmentation of DNA into specific sizes and, in animals, assessment of the permeability of cell membranes by measuring the entry of vital dyes or the active exteriorization of phosphatidylserine to the outer leaflet of the cell membrane. They also study organelle function, *i.e.*, mitochondria and endoplasmic reticulum, measurement of the activity of second messengers and the destructive pathways of the cells such as caspases, calpains and lysosomal enzymes. Enzyme activation can be determined in some instances by Western blot or immunohistochemistry, using antibodies specific for the activated forms of the enzymes, or by assessing proteolytic activation leading to decrease in size of enzymes. In other instances, enzymatic activity can be assessed using fluorogenic substrates or by cleavage of protein substrates into fragments of defined sizes (indicating cleavage at specific sites). Since not all forms of death are apoptosis, it is important to recognize that apoptosis-specific markers may not detect these, likewise important, instances of cell death. In specific instances, we can also evaluate the presence and activity of phagocytes. We have used cell death during mouse embryonic development to study the mode of cell death as well as how it is regulated by the different pathways. We have found that specific genes are activated and differentially regulated during cell death, such as cell cycle dependent kinase 5. This gene is also differentially regulated in cell death occurring in a number of different situations, both normal and pathological. In different systems we have analyzed the mode of cell death in insects such as the tobacco hornworm, *Manduca sexta*, and Drosophila. In the hornworm we find that the first sign of impending death is activation of lysosomes, and that most of the cytoplasm is destroyed before any signs of apoptosis appear, such as DNA cleavage, morphological change in nuclei, mitochondrial depolarization, or exteriorization of phosphatidylserine. Thus evaluation of cell death involves many features that must be evaluated.

Defects in the apoptotic machinery of cancer cells: Role in drug resistance

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The ability of malignant cells to evade apoptosis is a hallmark of cancer, and their resistance to apoptosis constitutes an important clinical problem. Cell death pathways in tumor cells appear to be much more complicated than was originally anticipated. Resistance of many tumors to chemotherapy is associated with either defects or dysregulation of death machinery. None of the components of this machinery operate in isolation, and the activation of a single caspase-dependent route for death is not sufficient to kill all types of cells. It is clear that exploiting a combination of caspase-dependent and -independent pathways may be more effective in killing tumor cells. Therefore, it might ultimately be more useful to understand the interplay of various pathways rather than evaluating the isolated components. This approach might identify new targets for chemotherapy or new combinations for therapeutic interventions. Many chemotherapeutic drugs do not interact directly with mitochondria. On the other hand, several Bcl-2 family members operate at the mitochondria level and are involved in the protection of tumor cells. Thus, agents that induce damage to mitochondria may constitute a novel strategy for overcoming resistance to cell death. These agents might induce both caspase-dependent and -independent types of cell death. Mutations or hypermethylation of genes encoding several pro-apoptotic proteins were described. Restoration of activity of these genes/proteins may be achieved by using specific peptides. Finally, disturbances in the delivery of drugs often result in resistance to treatment. In this case, activation of transporter expression in combination with activation of cell death machinery might lead to successful results. We believe that improved understanding of the signaling pathways and the disturbances in their function in tumor cells is required to develop novel compounds that regulate death of tumor cells.