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Cell Death: Current Difficulties in Discriminating Apoptosis From Necrosis in the Context of Pathological Processes In Vivo

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Abstract The term apoptosis was proposed to define a type of cell death morphologically, biochemically, and molecularly distinct from necrosis, which plays a fundamental regulatory function in the control of the overall size of cell populations, being complementary but opposite to cell proliferation [Kerr et al. (1972): Br J Cancer 26:239–257]. This view has led to the appreciation that apoptosis is an integral part of normal biological processes and may impact on disease states. Introduction of the concept of apoptosis has raised great interest and many studies have been aimed to the identification of genes responsible for the induction of cell death. Indeed, over the past few years, many genes whose expression is associated with cell death have been described, and the molecular mechanisms underlying cell death have been, in some circumstances, clearly established. However, it is now evident that extension of the conclusions achieved by studies performed with highly selected in vitro systems (simple systems), to in vivo conditions (complex systems), has generated a certain degree of confusion. This is in part due to the indiscriminate use of the term apoptosis and to the uncertainty whether apoptosis is always different from necrosis, and, if this is the case, to the lack of well established criteria to discriminate the two processes; in addition, it still remains to be established whether both types of cell death, although different, could be induced simultaneously by the same agent, depending on the cell type and the experimental condition used. The distinction between apoptosis and necrosis, is not simply a problem of terminology; if necrosis and apoptosis are different from a mechanistic point of view, and if necrosis is merely the passive result of cellular injury (still to be shown), it becomes critical to discriminate between the two processes, in order to understand how to modulate apoptosis in view of its potential therapeutic use. This review will summarize existing informations and discuss some of the conflicting issues related to cell death in the liver.

Key words: apoptosis, necrosis, liver, programmed cell death, carcinogenic processes

The term apoptosis, often unappropriately used as synonimous of programmed cell death, defines a type of cell death distinct from necrosis, on the basis of the following morphological features: a progressive condensation of the chromatin to the inner face of the nuclear membrane; cell shrinkage with consequent loss of membrane contact with neighbouring cells; fragmentation of the cell with formation of membrane-bound acidophilic globules (apoptotic bodies), often containing nuclear material and frequently found within the cytoplasm of intact cells, indicating their phagocytosis by adiacent cells. In addition, apoptosis is not commonly associated with the inflammatory response that accompanies necrosis (1).

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At the molecular level, these changes are thought to be accompanied by activation of a Ca^{2+}, Mg^{2+}-dependent endonuclease that results in the formation of DNA fragments that are multiple of approximately 180 bp. These fragments can readily be seen after agarose gel electrophoresis, wherein a characteristic "ladder" develops. In necrosis, in contrast, the progressive disappearance of chromatin seen morphologically at late stages of degeneration is accompanied by random DNA breakdown, a diffuse smear appearing in gels [2].

Another parameter often used to discriminate apoptosis from necrosis stems from the concept that the former, is an active mode of cell death, requiring active RNA and protein synthesis [3,4]. The concept of programmed, active cell death appears to be supported also by the existence of pro- and anti-apoptotic genes. A great deal of studies aimed to determine the role of such

genes have been performed in the last few years, especially following the discovery that an abnormal expression of a gene, bcl-2, plays a fundamental role in suppressing cell death and favouring tumor formation [4,5].

Most of the presumptive differences between apoptosis and necrosis have been derived from studies based on cell death occurring in thymocytes challenged with glucocorticoids [2]. Under these conditions, a fairly reproducible series of biochemical and morphological events has been established. However, the distinction between apoptosis and necrosis, in other cell types and under other experimental conditions, is far from clear. In particular, it is now evident that despite the many efforts, the fundamental biochemistry of cell death is still obscure and this lack of knowledge makes it extremely complicated to understand why a cell dies by apoptosis or by necrosis or even worse whether the cell dies by apoptosis or necrosis. The absence of a clear understanding of the mechanisms responsible for cell death makes interpretations particularly complicated, especially if one wants to reconcile the merely descriptive term of apoptosis with that, much more demanding, of "programmed cell death," in conditions where cell death is induced by agents or conditions that are clearly pathological. In the existing literature, the term "programmed cell death" is indifferently applied to at least three quite different conditions: (1) cell death occurring at predictable times during normal development and/or metamorphosis. This type of cell death is clearly regulated by a specific gene program, and pathological conditions cannot be invoked for triggering the death of the cell; (2) cell death occurring in adult organisms during regression of hyperplastic organs or tissues, resulting from increased cellular proliferation induced by endogenous or exogenous factors. This form of cell death, unlike the previous one, may not necessarily occur during the life of the organism, but some program exists in the cell such that death is triggered anytime an excess of cell is generated. In this case also, cell death appears to be a physiological response; (3) cell death occurring after application of pathologic stimuli of a chemical, physical, and biological nature. Cell death induced under these conditions is clearly not a physiological type of cell death, and to define it as programmed cell death appears a distortion, as it clearly represents the response (active or passive is still to be established) to the toxicity of the causative agent. Although there is evidence that this type of cell death shares several morphological and biochemical features with the previous two types (see below), no information exists on when and how the same agent may trigger a program of events leading to a type of cell death morphologically similar to apoptosis, or to necrosis. In an attempt to rationalize the use of the term "programmed" and limit the confusion arising from the indiscriminate use of these terms in different contexts, Farber [6] has recently proposed that cell death should be defined as (1) developmentally programmed, (2) physiologically programmed, and (3) biochemically programmed cell death, with reference to the above-mentioned conditions. Although this distinction may be viewed as an unnecessary attempt to introduce a further classification, and may not be fully representative of the differences existing in the various conditions, it may nevertheless be helpful in defining the system that one is using to study cell death.

Here, we will review some of the current knowledge on cell death in a system, rat liver, wherein, based mainly on morphological criteria, the occurrence of both apoptosis and necrosis is often observed. Physiologically programmed cell death (apoptosis occurring during regression of hyperplastic liver) in carcinogenic processes will be first discussed in view of its potential significance in chemoterapeutic approaches. Secondly, we will examine the far more complex area of cell death induced by hepatotoxins. The consistency of parameters used to discriminate apoptosis from necrosis, such as morphology, DNA fragmentation, and requirement for a specific gene program, and the controversial issue of whether protein or DNA synthesis is a necessary requirement for apoptosis, will also be discussed.

APOPTOSIS (DEVELOPMENTALLY AND PHYSIOLOGICALLY PROGRAMMED CELL DEATH) IN TISSUE HOMEOSTASIS

It is well established that apoptosis is an important form of cell death during embryonic and fetal development. Examples of developmentally programmed cell death are the regression of Mullerian duct in male embryos, the removal of interdigital webs, and amphibian tail regression during metamorphosis [7]. In adult tissues, physiologically programmed cell death plays an opposite role to mitosis in the maintenance of cell populations. Thus, cell deletion occurs at a

high rate in rapidly dividing tissues such as intestinal crypts, in contrast to resting tissues such as the liver [8–11]. Apoptosis is also responsible for cellular loss in the premenstrual endometrium [12] and human breast towards the end of the menstrual cycle [13]. An interesting and well-studied model of the reciprocal roles played by apoptosis and cell proliferation in the maintenance of the size of a cell population, is the regression of liver hyperplasia. It is known that many chemicals of various nature (primary mitogens) can induce a mitotic response of liver cells. The proliferative event gives rise to an excess of cells that is subsequently removed by apoptosis, leading to regression of the original hyperplasia [9,10]. Under these conditions, cell death takes place only after the proliferative event, suggesting that it is not the result of toxicity of the mitogen, and is completed once the hepatic DNA content has returned to original values. Interestingly, despite a loss of almost 50% hepatic DNA, no inflammatory response occurs, nor is there any increase in the level of serum transaminases [10]. This set of events is not unique to hepatocytes, since similar findings have been made during involution of biliary duct hyperplasia, regression of renal hyperplasia, and regression of the pancreas after discontinuation of a diet containing trypsin inhibitor [14–16]. In these circumstances, it appears that apoptosis is a physiological type of cell death, being part of a homeostatic mechanism triggered to restore the original number of cells. How the cells dye, and which cells are deleted from the liver is not known. As to the first question, occurrence of apoptosis during regression of the liver hyperplasia induced by lead nitrate (LN) is associated with an increased activity in the liver of tissue transglutaminase (tTG), an enzyme considered to be a specific marker for this type of cell death [17]. This enzyme crosslinks lysine and glutamine residues, tightening the membrane of apoptotic bodies, thus preventing lysis before phagocytosis and intracellular degradation [18]. On the other hand, no increase in the transcriptional activity of other genes thought to play a role in aptoptosis, such as TRPM-2 or p53, was observed under the same condition [19,20]. Recent interest has focused on transforming growth factor-β1 (TGF-β1). TGF-β1 induces apoptosis of uterine epithelial cells in culture [21], and TGF-β1 mRNA expression increases during regression of the prostate after castration [22]. In the liver, TGF-β1 has been shown to increase the apoptotic index in regressing liver, and to induce apoptosis in primary cultures of hepatocytes [23,24]. Recently, a member of the TGF-β1 superfamily, activin A, was shown to induce a significant degree of apoptosis in normal rodent liver, and in primary hepatocyte cultures [25]. These results suggest that apoptosis occurring during regression of the liver may be under the control of growth-regulatory signals. Nevertheless, the fact that in spite of a striking increase in TGF-β1 mRNA expression during liver rigeneration after surgical partial hepatectomy [26] no apoptosis takes place, suggests a more complex network in the regulation of apoptosis.

As to the second question, no clear evidence exists on whether apoptosis during liver regression is a random or a specific phenomenon. While Bursch et al. reported a preferential removal of old, non-proliferating hepatocytes [27], we have found that apoptosis occurring after lead nitrate (LN)-induced hyperplasia affects both non-dividing as well as dividing hepatocytes [28].

PHYSIOLOGICALLY PROGRAMMED CELL DEATH AND LIVER CARCINOGENESIS

Apoptosis is frequently observed in human hepatocellular carcinomas, as it is in a variety of other malignant neoplasms [reviewed in ref. 7]. However, the significance and contribution of apoptosis to the clonal expansion of preneoplastic cell populations induced by carcinogens are difficult to evaluate; the high incidence of apoptosis could be a consequence of sustained proliferative activity, or the result of a homeostatic response triggered to limit nodular growth. In the former case, it is possible that cell death occurs because of the accumulation of genomic hits in a cell population already altered by the carcinogen; triggering of cell death, may in turn generate a highly localized compensatory proliferation, thus providing an endogenous proliferative stimulus. In this case, apoptosis may favor tumor progression by eliminating "weak" cells and selecting more aggressive cell populations. On the contrary, according to the latter case, the elimination of preneoplastic cells due to a homeostatic mechanism may not imply a compensatory regeneration of neighbouring cells, and it may act as a limiting factor for the progression of the process. In the last few years several studies have addressed the role and significance of cell death in the carcinogenic process of rodent's liver. It is now widely accepted that cell

proliferation plays an important role in the multistep process of carcinogenesis. It is needed at the initiation step, for generation of a population of irreversibly altered cells, through fixation of carcinogen-induced damage in the newly made DNA [29]. It is also important for the expansion of sequential clones of initiated cells, to preneoplastic and neoplastic stages [30]. There is now evidence that together with cell proliferation, apoptosis also plays an important role in carcinogenesis. For example, it has been shown [31] that administration of low doses of carcinogens, coupled with liver cell proliferation induced by primary mitogens, fails to induce the appearance of initiated cells, monitored as preneoplastic foci. On the contrary, a similar dose of carcinogen given during liver regeneration compensatory to CCl_4-induced necrosis resulted in a large number of initiated cells. These findings seem to suggest that physiologically programmed cell death, occurring during regression of liver hyperplasia, eliminates a vast majority of carcinogen-initiated cells, thus questioning the irreversibility of the initiation process. Moreover, initiated cells seem to maintain their sensitivity to apoptosis for some time. In fact, a single round of proliferation followed by apoptosis 2 weeks after initiation led to a loss of approximately 50% of enzyme-altered preneoplastic foci positive for the placental form of Glutathione S-transferase (GST-P) [28]. On the other hand, necrosis followed by regeneration exerted a promoting effect on the growth of GST-P positive foci, indicating that while initiated cells possess a special biochemical phenotype that makes them resistant to necrosis, they are not resistant to physiologically programmed cell death triggered by a homeostatic mechanism. A similar phenomenon might explain the loss of a majority of the preneoplastic nodules, following exposure of carcinogen-initiated rats to a clofibrate-supplemented diet prior to the promoting regimen [32]. A high susceptibility of initiated cells to apoptosis is also suggested by mathematical and biological studies, indicating that a large fraction of carcinogen-induced GST-P positive hepatocytes are lost in the first few weeks after initiation [33,34]. Elimination of preneoplastic hepatocytes by apoptosis seems to occur also at later stages. An increased incidence of apoptosis has been in fact observed during the promotion stage in at least 4 different models of liver carcinogenesis [9,35]. Interestingly, the number of apoptotic bodies was found to be higher in preneoplastic lesions, than in surrounding liver, suggesting a shorter life cycle of these cells. Furthermore, apoptosis can be induced in preneoplastic hepatocytes by removal of the promoting agent, suggesting that an important property of tumor promoters is their capacity to inhibit death of preneoplastic cells, and not necessarily their stimulatory effect on cell replication [9]. That death of preneoplastic cells is a modulable process is also shown by the finding that loss of preneoplastic foci induced by chronic administration of S-adenosyl-L-methionine is associated with an increased apoptotic incidence [36]. Taken together, these results suggest that apoptosis induced in the liver under certain well-defined conditions may be very effective in favoring the deletion of carcinogen-altered cells. Whether artificially-induced apoptosis is effective also during the progression stage or whether fully autonomous preneoplastic cells lose their sensitivity to apoptosis remains to be established.

INDUCTION OF LIVER CELL DEATH BY TOXINS

The liver, being the main organ for the detoxification of xenobiotics as well as of endogenous compounds, is subjected to the toxic action of many chemicals. It is therefore not surprising to find that despite a very efficient defensive biochemical machinery, hepatocyte injury occurs frequently following administration of a variety of agents. For a long time, studies of the mechanisms leading to hepatocyte death have dealt with a type of cell death commonly defined as lytic necrosis. This type of cell death is morphologically characterized by extensive cell loss, most often but not always around the terminal hepatic vein, and resulting in the destruction of the hepatic architecture and recruitment of inflammatory cells. However, the finding in the liver, as well as in many other tissues, of so-called "single cell necrosis," morphologically indistinguishable from apoptosis, together with extensive necrosis, following damage induced by chemicals, microorganisms, or ischemia, has been repeatedly reported. The significance of the presence of two morphological types of cell death under these conditions is still not understood. Several laboratories, have attempted to elucidate the significance of the concomitant occurrence of apoptosis and necrosis following injury. Induction of apoptosis by toxic agents is sometimes explained as a function of the concen-

tration of the stimulus leading to cell death. HL-60 cells, for example, can be induced to undergo apoptosis by moderate concentrations of various drugs, or low irradiation, and to necrosis by higher concentrations; murine mastocytoma cells have been reported to undergo apoptosis after moderate heat shock, whereas the same cells die via necrosis when exposed to a higher load. Similar conclusions can be drawn from several other reports [for a review see ref. 37]. However, this may not be true in all occasions. In the liver, for example, administration of both relatively low (25 mg/kg) and high doses (200 mg/kg) of thioacetamide (TH) induce, soon after treatment (3 to 6 h), a type of cell death that exhibits all the morphological features characteristic of apoptosis (chromatin condensation, cellular shrinkage, formation of the globules, absence of inflammatory response, no change in serum, transaminases) in the absence of signs of lytic necrosis [38]. Apoptosis is then followed by necrosis associated with a strong inflammatory response. The severity of necrosis, rather than the extent of apoptosis, seemed to be a function of the dose of TH. In addition, the occurrence of apoptosis, as the only initial form of cell death, was observed by us 2 to 3 h after a high dose (2 ml/kg) of carbon tetrachloride (by definition a classical necrogenic agent) that will, at later times, kill up to 50% of hepatocytes via necrosis. From these studies and those of others, there is now accumulating evidence that using morphological criteria, (1) most of the agents that are capable to induce death of liver cells, induce both apoptosis and necrosis, and (2) apoptosis most often precedes necrosis. On the contrary, very little evidence exists, in the liver, of the occurrence of necrosis in the absence of preceding or concomitant apoptosis. This raises the question of whether the mechanisms leading to hepatic cell death following cytotoxic drugs, are really different between apoptosis and necrosis, or whether apoptosis is simply the initial step of a process that ultimately will lead to necrosis. The possibility that the morphological differences observed between these two types of cell death are the expression of biochemical events occurring post-mortem, rather than the result of different signals involved in the death of the cell, as recently argued by Farber [6], should also be considered. In the absence of such a knowledge, the results of studies aimed to determine possible differences in the molecular and biochemical mechanisms responsible for these two types of cell death after liver injury, are, at best, difficult to interpret.

DNA FRAGMENTATION

A widely used biochemical parameter for discriminating apoptosis cell death from necrosis is the presence of DNA fragmentation resulting in the ladder effect [2]. The fact that DNA fragmentation is a common finding in several experimental conditions associated with cell death, does not demonstrate that DNA fragmentation is a prerequisite for apoptosis, or that it is exclusive of apoptosis. In fact, if on the one hand many studies have shown an association between the presence of the ladder and occurrence of apoptosis, a few others have indicated that this finding may represent the result rather than the cause of cell death [39], or that apoptosis may occur in the absence of DNA fragmentation [24]. In the liver, in particular, it is extremely difficult to obtain a definitive answer. DNA fragmentation has been shown in primary cultures of hepatocytes treated with microtubule antagonists (Vinblastin and Colchicine) [40] and in cell death occurring in neonatal hepatocytes [41]. In contrast, in vitro hepatocyte death induced by TGF-β1 was shown to occur in the absence of DNA fragmentation [24]. In vivo, while some studies have observed the occurrence of oligosomal fragmentation of DNA in apoptotic bodies isolated during involution of lead nitrate-induced hyperplasia [41], others have found that liver apoptosis triggered by TGF-β1 is not accompanied by DNA fragmentation [23]. One possible explanation for these contrasting results is that a minimum number of cells undergoing apoptosis is required to detect the ladder pattern. Support for this interpretation comes from the very recent study by Hully et al. [25]. These authors, have shown that infusion with a member of the TGF-β1 family, Activin A, caused an impressive loss of liver mass, accompanied by several morphological signs of apoptosis. In this study, evidence for DNA fragmentation was provided by means of in situ nick-end labelling of DNA and presence of the typical oligosomal ladder. Thus, at present, it appears that DNA fragmentation in the liver is a rather inconstant finding and it can be hardly considered a reliable marker for apoptosis. To add to the confusion, there are studies showing that liver of rats subjected to ischemia or poisoned with carbon tetrachloride, exhibit oligosomal fragmentation of DNA, in spite of the fact that histological exami-

nation of the liver in both cases demonstrated a clear morphology of classical necrosis [42]. Whether this is a unique example of the presence of the ladder in necrotic cells or whether a similar finding can be extended to other necrotic conditions, is an open question that needs to be investigated.

DEPENDENCE UPON MACROMOLECULAR DNA SYNTHESIS

It is generally accepted that apoptosis is an active type of cell death, requiring protein and RNA synthesis [2,3]. Indeed, apoptosis induced by a variety of agents/conditions can be inhibited by inhibitors of either protein or RNA synthesis. However, in just as many cases inhibitors of macromolecular synthesis do not protect cells [for a review see ref. 43]. In addition, a series of in vitro studies have shown that apoptosis can be induced by protein or RNA synthesis inhibitors [37]. Recently, we have shown that a dose of cycloheximide (CHX), that inhibits 80–85% of hepatic protein synthesis, is per se able to induce hepatocyte death, morphologically similar to apoptosis, in the rat [44]. CHX-induced apoptosis appears very early after treatment (2 h), and is not associated to changes in the levels of serum transaminases. The appearance of CHX-induced apoptosis, unlike physiologically programmed cell death seen in the regressing liver after LN, is associated with an increase in the levels of TRPM-2 and p53 mRNA. Interestingly, unlike treatment with TH or CCl_4, apoptosis induced by this dosage of CHX is not followed by lytic necrosis and inflammation. Although the reason why CHX induces apoptosis is not known, it is possible that CHX treatment may inhibit anti-apoptotic gene products, normally present in liver tissue. Induction of apoptosis can also be achieved by inhibitors of RNA synthesis. In fact, Actinomycin D was also able to induce rat liver apoptosis early after treatment. It appears therefore that new protein or RNA synthesis may not be a universal requirement for apoptosis to occur, but it may depend upon cell type, the state of the cell, and the nature of the inducing stimulus.

Moreover, it should also be stressed that pretreatment with inhibitors of protein synthesis can be very effective in preventing hepatic necrosis induced by carbon tetrachloride or other hepatotoxicants [45]. Although inhibition of CCl_4 induced necrosis by pretreatment with CHX can be due an inhibitory effect on CCl_4 biotransformation, and, consequently the generation of its toxic metabolites, the question still remains whether the protein synthesis requirement is a unique feature of apoptosis, or also of necrosis.

GENE EXPRESSION AND CELL DEATH

An important area of future research is identification of the genes that are involved in the apoptotic program of cell death. In fact, the finding that cell death occurs at a certain time and at the right location during precise stages of normal development or metamorphosis implies that there are genes responsible for the occurrence of cell death. This area is still in its initial phase, but a few interesting genes are currently under study. The best studied example of a cell death-associated gene is probably bcl-2 gene, a gene first isolated from the breakpoint in the translocation between human chromosomes 14 and 18 in follicular B-cell lymphomas. Deregulated bcl-2 expression allows prolonged survival, and blocks apoptosis of hematopoietic cell lines following growth factor deprivation [5]. Altered expression of this gene has also been claimed to interfere with the normal cell death program in several other cell types. Interestingly, bcl-2 prevents programmed cell death in *Caenorhabditis elegans* and may be the functional equivalent of the ced-9 gene in the nematode [46]. Other genes (p53, c-myc, TRPM-2, Fas, and tissue transglutaminase gene), have been described to play a role in the apoptotic process [47]. However, whether these genes are important in modulating cell death, or simply represent an epiphenomenon remains to be established in most cases. Once again, if we look at the liver, evidence of a gene or set of genes involved in apoptosis is at the best scanty. Recently, injection of antibodies against FAS/APO 1 antigen was reported to induce apoptosis in mice liver [48]. Apoptosis was identified by electron microscopy on the basis of the presence of typical apoptotic bodies. However, a 1,000-fold increase in the serum level of enzymes commonly used as markers of necrosis was also reported. In addition, a massive inflammatory reaction was observed in the liver. Thus, an involvement of FAS in apoptosis, under the conditions described by the authors, is questionable. Bcl-2, as already mentioned is another gene involved in the apoptotic process. However, there are no data available as yet about an anti-apoptotic role of this gene in the liver. Very recent studies of normal human liver have shown the presence of BCL-2

protein in normal ducts, but not in the hepatocytes, which are characterized by a very long life span. Furthermore, immunolocalization of the BCL-2 protein was shown to be positive in cholangiocarcinomas, but not in hepatocellular carcinomas [49]. Evidence for an association between alteration in the expression of the TRPM-2 gene, and apoptosis in the liver, is also lacking. The TRPM-2 gene was originally isolated from regressing prostate after androgen ablation and was suggested to be an apoptotic gene in this organ. However, no change in the expression of TRPM-2 was found by in situ hybridization or Northern blot analysis during apoptosis occurring in regressing liver following hyperplasia induced by several liver mitogens [19,20]. If at all, an increase in TRPM-2 expression was found to be present during liver regeneration following partial hepatectomy. These data together with the observation that TRPM-2 mRNA is highly expressed in normal rat liver, seem to suggest that this gene is not involved in liver apoptosis although at least in one case, CHX-induced apoptosis, increased levels of TRPM-2 mRNA were observed at a time of occurrence of cell death [44]. We have recently investigated the role of p53 in hepatic cell death. Again the results are quite unsettling. Increased levels of p53 mRNA were indeed observed in CHX induced apoptosis, but not during physiologically programmed cell death occurring during involution of rat liver following hyperplasia caused by the primary mitogens LN and cyproterone acetate. Moreover, we have observed a striking increase in hepatic p53 mRNA levels in severe lytic necrosis induced by carbon tetrachloride.

So far, the gene more closely associated with liver apoptosis appears to be the tissue transglutaminase gene (tTG). Transglutaminases are a class of enzymes that catalyze a Ca^{++} dependent acyl transfer reaction in which gamma-carboxide groups of peptid-bound glutamine residues serve as acyl donors and primary groups of several compounds function as acceptor substrates [18]. As far as the biological role of tTG is concerned, it appears that their general function is connected with the protection of cell and tissue integrity. Although no definite role for tTG has yet been established, recent studies indicate that the cytosolic form of this enzyme may be involved in apoptosis occurring in various organs and established cell lines [50]. As to the liver, experiments in vivo and in vitro have shown that the tTG mRNA levels increase during the proliferative phase that precede the apoptotic wave, and decrease in parallel with the onset of phagocytosis [50]. These results suggest that tTG gene induction occurs at the transcriptional level in pre-apoptotic hepatocytes, and its products (mRNA and protein) are degraded together with the apoptotic bodies in the phagocytes. However, no evidence is as yet available on whether tTG is involved in the causation of cell death. It could still be argued that tTg as well as other gene products, are associated with the biochemical and morphological changes characteristic of apoptosis, and may be important for the efficient disposal of the dying cells, rather than in the causation of apoptosis. Furthermore, at least in one experimental model, thioacetamide-induced apoptosis, an almost complete inhibition of tTG activity was observed during apoptosis [38].

CONCLUSIONS

A large body of evidence has now been accumulated demonstrating the existence of a highly organized type of cell death (apoptosis). The discovery that a finite number of cells, 131, systematically die at a certain stage of the life of the nematode *C. elegans,* and that unique sets of genes are involved in the negative and positive regulation of this process is perhaps the best example of a process of cell death highly active and controlled by several molecular events [51]. The finding of a certain degree of homology between the nematode ced-9 gene and the human gene bcl-2, together with the observation that human Bcl-2 can protect ced-9 mutant *C. elegans* from cell death, clearly indicates that despite the evolutionary distance, genetic control of cell death shows considerable similarity between very distant phyla. If the phenomenology and concept of apoptosis, as a process distinct from cell necrosis, seems to be fairly well established in the case of developmentally programmed cell death, the overall picture is, however, very fragmented and poorly defined in the cases of physiological and biochemically programmed cell death. Moreover, it is also evident that in many circumstances the subtle differences between apoptosis and necrosis are far from being understood. This is particularly true when cell death is induced by toxic agents. In fact, under these conditions a clear distinction between these two forms of cell death has not yet been found. This is perhaps not surprising, given the complexity of the biological and patho-

logical processes which have been so far studied, in which apoptosis may be only one of a large set of cell responses to the agents and conditions employed in the studies. An example of such a complex response comes from recent studies showing that a rise in calcium elicited by addition of oxidized low density lipoproteins to lymphoblastoid cells is a trigger mechanism for both apoptosis and necrosis [52]. In the same study, it was shown that apoptosis and necrosis, despite the common initial event, can be discriminated by selective inhibitors in that, while proteolysis and necrosis (but not apoptosis) are blocked by proteinase inhibitors, DNA fragmentation and apoptosis are inhibited by inhibitors of endonucleolytic cleavage of DNA. This type of approach is highly desirable, and more experimental studies aimed to elucidate the differences in mechanisms of the process of cell death induced by cytotoxic agents, in in vivo conditions, should be pursued in view of their potential significance in terms of tissue response.

Another critical question that has not been adequately addressed is what determines the reaction of an organ to cell death; why, for example massive apoptosis occurring during regression of hyperplasia is not accompanied by necrosis, and does not lead to compensatory regeneration, while in other conditions (administration of toxic agents), the initial apoptotic wave is rapidly followed by extensive necrosis and inflammation, which in turn elicit a prompt regenerative response? Is the difference simply due to the diversity in the mechanisms related to cell death or a different capacity of the tissue to handle the process may be responsible for the different consequences of an initial (common?) triggering event? A characteristic feature associated with apoptosis occurring during regression of LN-induced liver hyperplasia is that hepatocytes, in addition to Kupffer cells, are actively engaged in the uptake of dead cells; this phagocytic activity is paralleled by a receptor modulation, the pattern of modulation being dependent on the cellular type [53]. It is possible that specific changes in the phagocytic activity of cells, other than Kupffer cells, might play a role in preventing the triggering of the inflammatory response. It could be important to establish whether these changes are specific for physiologically or developmentally programmed cell death, while being inhibited in conditions where the initial apoptotic wave is followed by necrosis.

In conclusion, the development of reliable markers that could help in assessing whether and to what extent apoptosis and necrosis are indeed separate entities, appears to be the most critical need when studying complex (in vivo) systems. Studies of the mechanisms leading to cell death should be coupled with attempts to understand the basic principles modulating the response of the tissue to the stimulus responsible for triggering cell death.

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