Caspase-Independent Pathways of Programmed Cell Death: The Unraveling of New Targets of Cancer Therapy?

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Abstract: In the past few years, accumulating evidence in the literature supports the existence of pathways of caspase-independent programmed cell death (CI-PCD). These pathways are likely to be acting as ‘death backup systems’ that ensure effective removal of defective cells from the organism. Similar to classical apoptosis i.e. caspase-dependent programmed cell death (CD-PCD), the mitochondrion is the main organelle orchestrating the series of events which are required for the induction of CI-PCD. In addition, the pro-apoptotic proteins Bax and Bid are also key participants in CI-PCD. However, contrary to CD-PCD, CI-PCD involves executioners other than the caspases which include the cathepsins, the calpains and serine proteases. The protein AIF may also play an important role in the induction of CI-PCD. In this review we report current knowledge on CI-PCD and provide evidence for its regulation by chemotherapeutic agents currently used in the clinic and under investigation in clinical trials. Lastly, we discuss how the study of natural and synthetic agents triggering CI-PCD may help in the pharmacological design of a new generation of more effective chemotherapeutic drugs. The use of such drugs activating both CD-PCD and CI-PCD pathways should achieve a more successful eradication of carcinogenic cells and the attainment of lower levels of tumor resistance.

Keywords: AIF, apoptosis, caspase-independent cell death, chemotherapy, programmed cell death.

INTRODUCTION

Human tumors harbor mutations which render them resistant to the programmed cell death (PCD) mechanisms that the organism has evolved to kill and remove defective cells. Examples of such mutations are those of the tumor suppressor protein p53 which are present in more than 50% of cancer patients [1]. Chemotherapeutic drugs commonly used in the clinic may trigger one or more pathways of PCD. However, the lack of knowledge concerning the identity of the whole spectrum of these death triggering mechanisms has limited the design of more potent chemotherapeutic agents that could target all possible pathways of PCD.

Classical apoptosis, i.e. caspase-dependent programmed cell death (CD-PCD) is only one form of PCD while other ‘backup death pathways’ also exist in the cell [2-8]. Emerging evidence in the literature provides support for the existence of PCD in the absence of caspase activation [5-8]. Caspase-independent programmed cell death (CI-PCD) is likely to consist of several different pathways that converge in the commonality of induction of cell death in the absence of caspase activation. Even though the exact mechanism(s) of CI-PCD has not yet been elucidated, it is worth further investigation in light of the evidence that it is commonly triggered by several effective chemotherapeutic drugs in clinical use and natural agents with anti-tumorigenic properties. In this review, we will briefly describe the molecular events which take place in classical CD-PCD but mainly focus on the molecular mechanisms regulated by the newly emerging pathways of CI-PCD. We will also discuss how advancing knowledge of the signaling pathways / mechanisms induced by CI-PCD can be directly translated into the pharmacological design of novel chemotherapeutic agents with higher specificity and anti-tumorigenic potency.

THE APOPTOSIS VS. NECROSIS DICHOTOMY

Initially, two main forms of cell death had been identified: apoptosis and necrosis [9]. Apoptosis was defined as a process requiring a specific group of proteases known as caspases and characterized by morphological features such as cytoplasmic shrinkage, chromatin condensation in the nucleus, phosphatidyserine exposure, plasma membrane blebbing and disintegration of the cell into small fragments (apoptotic bodies) that are engulfed by nearby cells [10, 11]. Necrosis on the other hand was described as uncontrolled cell death characterized by cellular edema, disruption of the plasma membrane, release of cellular components and induction of an inflammatory response [12]. For almost three decades apoptosis was considered as synonymous with PCD but emerging evidence in the literature suggests that this is not the case. Therefore, cell death cannot be divided into the simple dichotomy of apoptosis or necrosis since other forms of PCD also exist.

CASPASE-DEPENDENT VS. CASPASE-INDEPENDENT PCD

As already mentioned, caspase-mediated apoptosis is not the only form of PCD but other forms of PCD also exist in the cell. Examples of other forms of PCD include autophagy (self-digestion by a cell through the action of enzymes originating within the same cell), cornification (specialized cell death of keratinocytes), mitotic catastrophe (cell death that occurs during mitosis due to deficient cell-cycle checkpoints that lead to DNA damage), anoikis (apoptosis triggered by...
loss of contact with extracellular matrix), paraptosis (apoptosis in the absence of cellular fragmentation), excitotoxicity (damage and death of neurons mediated by receptors for excitatory neurotransmitters), Wallerian degeneration (a form of degeneration occurring in nerve fibers as a result of their division) and programmed necrosis [2-8]. These types of cell death fall within the category of PCD and differ from accidental necrosis. Although caspase activation is a requirement for classical caspase-dependent apoptosis, this group of proteases is not necessary for other forms of PCD. Therefore, PCD can be divided into classical apoptosis i.e. CD-PCD (Caspase-dependent PCD) and CI-PCD (Caspase-independent PCD) (Fig. (1)).

THE MOLECULAR PATHWAYS OF CD-PCD (CLASSICAL APOPTOSIS)

The classical apoptotic cascade can be induced by several stimuli such as irradiation, growth factor deprivation and chemotherapeutic drugs. CD-PCD can be initiated by two main pathways known as the extrinsic (receptor-mediated) and the intrinsic (mitochondrial-mediated) pathways of CD-PCD. The caspases (cysteine aspartic acid-specific proteases) are the main participants of CD-PCD and consist of a set of cysteine proteases that are activated specifically in classical apoptosis [13]. Following is a discussion of the two main signaling pathways which are known to be regulated during the induction of CD-PCD (Fig. (2)).

The extrinsic (receptor mediated) pathway of CD-PCD is initiated by receptor (e.g. Fas and TRAIL receptors DR4 and DR5) ligation and trimerization, and recruitment of adapter molecules such as FADD and TRADD to the death domain (DD). This complex is known as the death inducing signaling complex (DISC). Procaspase-8 binds to the death effector domains (DED) of the adaptor molecules and this is followed by oligomerization and activation of caspase-8. Caspase-8 then either directly activates the executioner caspases -3 and -7 or activates the cytosolic (BH3-only pro-apoptotic Bcl-2 family member) protein Bid which translocates to the mitochondria and causes the release of cytochrome c (Fig. (2)) [14].

The intrinsic (mitochondrial) pathway of apoptosis is initiated when cellular stresses including DNA damage, heat shock and oxidative stress cause release of cytochrome c or Smac/DIABLO from the mitochondrial intermembrane space to the cytosol. Release of cytochrome c may be achieved by members of the Bcl-2 family (e.g. Bax, Bak, Bid), which translocate to the mitochondria and/or oligomerize within mitochondrial membranes, possibly forming pores that permit release of cytochrome c from the mitochondrial intermembrane space. Released cytochrome c then partici-

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pates in the formation of a high molecular weight caspase-activating complex known as the apoptosome. The apoptosome is a heptamer comprised of seven Apaf-1 adaptor molecules, each bound to one molecule of cytochrome c and a dimer of the initiator caspase-9. Formation of the apoptosome results in the activation of caspase-9, which then cleaves and therefore activates the effector caspases.

In a process known as karyorrhexis. Lastly, the cell breaks down into small fragments (apoptotic bodies) that are engulfed by nearby cells [10, 11].

THE MOLECULAR PATHWAYS OF CI-PCD

Until recently, the caspases were considered to be the only executioners of PCD. However, new data suggest that PCD can occur in the absence of caspase activation [15, 16]. One of the first observations of CI-PCD was reported by Xiang et al., who showed that the inhibition of caspase activation in Jurkat cells did not inhibit the induction of cell death triggered by the expression of the pro-apoptotic protein Bax [17]. Additional studies showed that typical apoptotic morphology (DNA fragmentation, DNA strand breaks, pycnotic nuclei, chromatin condensation) could take place in
the absence of caspase activation, supporting the regulation of a caspase-independent pathway of apoptosis [18-22].

Several intracellular molecules such as IFN-γ, Fas/APO-1, TNF-α, nitric oxide and molecular Iodine (I2) are known to be involved in the triggering of CI-PCD [19-22]. Interestingly, the stimuli inducing CI-PCD are not necessarily different from those inducing CD-PCD. In fact the two pathways may be activated simultaneously. Many reports in the literature have shown that the inhibition of CD-PCD by a caspase inhibitor may lead to the activation or unraveling of an already triggered CI-PCD pathway [23]. In addition, an increasing number of natural compounds are also capable of triggering CI-PCD. Examples of such compounds are selenium, lipic acid, thiosulfates from Allium tuberosum L., vitamin D and Resveratrol [18, 24-27]. The mechanism of action of representative natural compounds is examined in the last section of this review.

In addition to the in vitro studies, evidence is beginning to accumulate from in vivo systems that supports the existence of CI-PCD pathways. For example, the treatment of mice with TNF-α in the presence of a caspase inhibitor does not prevent induced toxicity suggesting that the inhibition of caspase activity can be overcome by other pathways that ensure induction of cell death [20]. Furthermore, treatment of mice inoculated with HA22T cells with the promising chemotherapeutic agent CHM-1 inhibits tumor growth via the activation of a caspase-independent pathway of apoptosis [28].

The evolutionary importance of having several pathways of cell death is reinforced by evidence proposing the existence of caspase-independent pathways of cell death in yeast. Many factors such as Aif1p (an ortholog of mammalian AIF), Nuc1p (an ortholog of mammalian endonuclease G), AMID and cyclophilin D play important roles in the triggering of cell death in the absence of caspase activation in yeast, which must be crucial for metazoan development [29, 30]. Hence, it is expected that future research on yeast cell death will define in detail the specific pathways involved in the regulation of both caspase-dependent and caspase-independent apoptosis. The latter should in turn lead to a better understanding of the CD-PCD and CI-PCD pathways regulated in mammals.

**Upstream Signaling Pathways Regulating CI-PCD**

Even though the upstream events triggering CI-PCD are not known in detail, there is evidence in the literature to support the involvement of some signaling pathways and key proteins in the induction of this type of cell death. More specifically, in addition to its well known role in activation of CD-PCD, the tumor suppressor protein p53 is involved in the triggering of CI-PCD. For example, p53 activates CI-PCD in rat embryo fibroblasts and cervical carcinoma HeLa Hep-2 cells [31, 32]. Furthermore, the phosphorylation of p53 on Ser-15, Ser-20 and Ser-392 occurs during the induction of CI-PCD in MCF-7 human breast cancer cells [24]. Although p53 activates CI-PCD in several cellular systems, this form of cell death is also triggered in T47D breast cancer cells lacking functional p53, indicating that the tumor suppressor is not necessary for the induction of CI-PCD [18].

Recently it was determined that the GSK-3 pharmacological inhibitors lithium and SB-415286 induce CI-PCD in B65 neuroblastoma cells accompanied by the up-regulation of cyclins D, E, A, cdk4 and cdk2, the phosphorylation of cdc2 and cell cycle arrest. Since the lithium and SB-415286–induced apoptosis cannot be prevented by z.VAD.fmk, the inhibition of GSK-3 and the phosphorylation of cdc2 must be involved in the induction of caspase-independent apoptosis [33].

The p38 MAPK pathway has also been shown to be involved in the induction of CI-PCD. For example, treatment of the cervical cancer cell line HeLa Hep-2 with selenium induces caspase-independent cell death via activation of p38 MAPK [32]. The important roles of p38 MAPK, Erk and CAMK-II in the induction of CI-PCD have been reported by several studies, proposing that the activation of the upstream signaling pathways may largely depend on the nature of the stimulus and cell line under investigation [34, 35]. Therefore, further studies need to be performed to unravel in detail the upstream signaling pathways that are involved in CI-PCD.

Contrary to the missing evidence regarding the upstream signaling mediators, there is sufficient information in the literature regarding the downstream events involved in CI-PCD. Following is a discussion of the main organelles, molecules and signaling pathways which are known to be regulated during the induction of CI-PCD (Fig. (3)).

**Mitochondria as the Mediators of CI-PCD**

While the mitochondrion is considered to be vital for the induction of classical caspase-mediated apoptosis, it is becoming evident that this organelle is also necessary for the induction of CI-PCD. Many of the events which are known to take place and activate CD-PCD are also involved in CI-PCD (e.g. Bax translocation from the cytoplasm to the mitochondria, cleavage of Bid and translocation to the mitochondria) while other molecules differ (e.g. AIF plays a role in CI-PCD but not in CD-PCD) (Fig. (3)).

The pro-apoptotic protein Bax plays a pivotal role in the induction of CI-PCD [36]. In fact, even though some differences may exist in different cell systems, Bax up-regulation and translocation from the cytoplasm to the mitochondria seems to be a requirement for the initiation of the caspase-independent response. The role of Bax in the induction of CI-PCD has been revealed by experiments in which the expression of Bax was shown to be sufficient to induce cell death despite the inhibition of caspases [17]. The translocation of Bax from the cytoplasm to the mitochondria is believed to be causing a fall in the mitochondrial membrane potential which causes an increase in the permeability of the outer membrane of the mitochondria and therefore a release of Smac/DIABLO and AIF [37] (Fig. (3)). An example of Bax upregulation and its involvement in a caspase-independent pathway is observed following treatment of the cervical cell line HeLa Hep-2 with selenium [32]. Nevertheless, similar to the case of CD-PCD pathways, the ability of Bax to induce CI-PCD may be counteracted by the anti-apoptotic protein Bcl-2. For example, Bcl-2 prevents the caspase-independent cell death induced by vitamin D3, suggesting that down-regulation of the anti-apoptotic protein...
Bcl-2 must be a necessary event for the activation of CI-PCD [21]. The vital role of the phylogenetically ancient flavoprotein AIF in the activation of CI-PCD is nowadays acknowledged [38]. In the absence of apoptotic stimuli, AIF resides in the intermembrane space of the mitochondrion co-localized with Hsp60 [39]. In response to an apoptotic stimulus, the 62 kDa AIF is cleaved in the mitochondria into a soluble pro-apoptotic protein of 57 kDa. This cleavage is achieved by calpains and cathepsins. Calpains activate AIF in a Ca\(^{2+}\) dependent context whereas cathepsins activate AIF in a Ca\(^{2+}\) independent context [8]. Subsequently, the soluble form of AIF (sAIF) is released from the mitochondria and it is believed that Bax is responsible for the mitochondrial membrane permeabilization that is necessary for this release) [37]. sAIF then translocates to the nucleus where it induces probably together with endonuclease G chromatin condensation and high molecular weight (approximately 50 kb) DNA fragmentation [38, 40-42]. The latter leads to the induction of CI-PCD [8, 38, 43]. The mitochondrio-nuclear redistribution of AIF is prevented by Bcl-2 specifically targeted to mitochondrial membranes (Fig. (3)) [39].

AIF was the first protein shown to be directly involved in the induction of CI-PCD. Since then numerous papers continue to report the involvement of this protein in the induction of this type of cell death. Most studies have shown that the release of AIF is independent of caspases [42] and that the caspase inhibitor z.VAD.fmk does not prevent the mitochondrial nuclear translocation of AIF or the induction of cell death that is caused by this protein [41]. In particular it has been shown that the extra-mitochondrial expression of AIF induces CI-PCD and caspase-independent DNA fragmentation in isolated nuclei [38]. Nevertheless, a few studies have shown that the mitochondrial release of AIF may require caspase activation and release of cytochrome c suggesting a crosstalk between CD-PCD and CI-PCD in particular cell systems [38, 43, 45, 46]. Interestingly, it has been reported that AIF can be transcriptionally positively regulated by p53. Therefore, p53 may activate CI-PCD via increasing the levels of AIF [47]. On the other hand, Hsp70

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**Fig. (3). Model of caspase-independent programmed cell death (CI-PCD).** The main regulator of caspase independent cell death is the mitochondrion which orchestrates a series of events causing the death of the cell in the absence of caspase activation. In response to an apoptotic stimulus, the pro-apoptotic protein Bax translocates to the mitochondrion. The translocation is brought about by activated cathepsins and calpains. Cathepsins are released from the lysosomes and calpains are activated following Ca\(^{2+}\) influx in the cell triggered by ER stress. Cathepsins and calpains are also involved in the cleavage and translocation of Bid to the mitochondrion and the cleavage of AIF. The presence of Bax and cleaved Bid at the mitochondrial membrane causes loss of mitochondrial potential and increased membrane permeability thereby causing the release of cleaved AIF (sAIF) and Smac/DIABLO. sAIF translocates to the nucleus where it induces probably together with endonuclease G chromatin condensation and high molecular weight (approximately 50 kb) DNA fragmentation.
inhibits the activity of AIF by inhibiting permeabilization of the lysosomal membrane [48].

In most studies investigating CI-PCD it has been shown that the increased permeability of the mitochondrial membrane, induced by the translocation of Bax from the cytoplasm to the mitochondrion, causes the release of Smac/DIABLO [31]. While several studies have supported a sole requirement for Smac/DIABLO, other reports have shown the release of cytochrome c to be necessary for the induction of CI-PCD [49-50]. Therefore, the contributions of Smac/DIABLO and cytochrome c towards CI-PCD are not yet clear and further studies should be performed to investigate this matter in more detail. In addition to the release of AIF, cytochrome c and Smac/DIABLO, endonuclease G and Htra2/Omi are also released from the mitochondria during CI-PCD [51, 52].

Other Organelles and Proteases Involved In CI-PCD

In CI-PCD, proteases other than the caspases are capable of inducing cell death with morphology similar to that visible during CD-PCD. The three main groups of enzymes involved in CI-PCD are the cathepsins, the calpains and serine proteases [53].

The cathepsins and the calpains are regulated by the lysosomes and the ER. The calpains may not only trigger CI-PCD but also cooperate with caspases in classic apoptosis (CD-PCD), further providing evidence for the existence of a cross-talk between the two types of PCD [16, 53]. Although the cathepsins and the calpains are clearly activated in many apoptotic instances, and have sometimes been shown to be triggered prior to MOMP, it has not yet been unequivocally shown if they are really triggers of mitochondria-mediated signaling [54-58].

Cathepsins are lysosomal proteases which were originally considered to be involved solely in non-specific protein degradation. Nevertheless, accumulating evidence provides strong support for the role of cathepsins in the regulation of CI-PCD. For example, cathepsins have been shown to be responsible for the translocation of Bax from the cytosol to the mitochondrion [56], the cleavage and translocation of Bid also from the cytoplasm to the mitochondrion [57-59] and for the direct cleavage of AIF (Fig. (3)) [8] (Fig. (3)). Specifically, it is mostly cysteine cathepsin B and aspartate cathepsin D which are linked to CI-PCD. For example, cathepsin B is involved in the regulation of CI-PCD triggered by bile salt in rat hepatocytes [60] and vitamin D in MCF7 cells [18]. Furthermore, cathepsin D induces CI-PCD in response to apoptotic stimuli such as IFN-γ, Fas/APO-1 and TNF-α [61]. AIF release independent of the caspase cascade and AIF mediated cell death in Apaf 1-/- and caspase 3 -/- cells are triggered by cathepsin D [56, 62]. Nevertheless, in addition to their ability to activate CI-PCD, cathepsins are also capable of activating the caspases, thereby inducing CD-PCD [63].

The calpains (calcium activated neutral proteases) normally reside in the cytosol as inactive zymogens and are activated by intracellular calcium influx caused by ER stress [64]. They contain a cysteine-protease domain that includes a conserved catalytic sequence Cys-His-Arg combined with a calmodulin like Ca2+ binding site [8]. Calpains are capable of activating pro-apoptotic members of the Bcl-2 family such as Bax and Bid [65, 66] but also induce the release of lysosomal cathepsins [67]. Similar to the cathepsins, the calpains can also cause the cleavage of AIF in the complete absence of caspase activation thereby contributing to the induction of CI-PCD (Fig. (3)) [8]. Further evidence for the role of calpains in the induction of CI-PCD comes from studies which showed that treatment of MCF7 cells with vitamin D causes calpain-mediated cell death in the absence of caspase activation [68, 69]. These results therefore propose that in addition to lysosomes, the ER is another organelle which is likely to play key role in CI-PCD and more details concerning this matter are likely to be the focus of further research.

There is also sufficient evidence in the literature to support an important role of serine proteases in CI-PCD. Interestingly, several studies have shown that serine proteases may have an upstream, initiating effect before the activation of MOMP [70, 71]. The most common serine proteases involved in CI-PCD are granzymes A and B [53]. The latter has actually been shown to cause cleavage and activation of the pro-apoptotic protein Bid to generate the 14-kD granzyme B-truncated product (gtBid) that translocates to the mitochondria. In turn, gtBid recruits Bax to the mitochondria through a caspase-independent mechanism where it becomes integrated into the membrane and induces cytochrome c release [58, 59]. Other serine proteases include Omi/Htra2 and apoptotic protease 24 (AP24) [72, 73]. Omi/Htra2 in combination with Smac/Diablo causes cleavage of IAP proteins. This cleavage is independent of caspase activity and can take place in CI-PCD. Nevertheless, the down-regulation of IAP by Omi/Htra2 has been shown to cause increased caspase activity in HeLa cells thereby triggering CD-PCD [74].

THE IMPORTANCE OF CI-PCD IN THE DEVELOPMENT OF NOVEL CHEMOTHERAPEUTIC TREATMENTS

In the past few years pharmacological therapies to treat cancer have focused on classical caspase-mediated apoptosis and the main regulators of this pathway i.e. the caspases. Therefore, many chemotherapeutic drugs have been designed to activate the intrinsic (mitochondrial) or the extrinsic (receptor mediated) pathways of classical apoptosis [75, 76]. Even though in theory the induction of CD-PCD by chemotherapeutic agents should lead to the treatment of cancer, the anti-tumorigenic potency of several such candidate drugs has been shown to be low [77-81]. The latter provides an explanation for the observation that many CD-PCD activating agents (e.g. the human monoclonal antibody to TRAIL-R1 and Bcl-2 targeted antisense therapy) are still in preclinical development [82-84]. The low efficiency of the CD-PCD activating drugs could be attributed to the fact that human tumors often harbor mutations in proteins involved in the extrinsic and intrinsic pathways of CD-PCD [85, 86]. Furthermore, tumor cells are often resistant to agents that activate the caspases because of over-expression of anti-apoptotic proteins like IAPs, survivin and Hsp70 [77, 87]. Due to the limitations of the conventional apoptotic agents as anti-cancer drugs, it is becoming essential to identify new agents that induce CI-PCD [88]. The latter could be used in
combinations with CD-PCD activating agents for the development of more effective therapeutic approaches.

Activation of CI-PCD by Conventional Cancer Chemotherapeutic Drugs

Several conventional cancer chemotherapeutic agents used currently in the clinic seem to function, at least in certain environments, through not only the activation of CD-PCD but also the activation of CI-PCD pathways [18, 89, 90]. Doxorubicin induces CI-PCD in the cardiomyocytes NerCaMs, but CD-PCD in HUVECs and A2780 cells, suggesting a role for the cellular environment on the decision of which type of cell death is induced by this agent [91]. Several other agents used in the clinic such as the topoisomerase inhibitor camptothecin, paclitaxel, cladribine and cisplatin are also capable of inducing cell death in hepatocellular carcinoma, ovarian carcinoma, leukemic and prostate carcinoma cells respectively [23, 44, 92-95]. The induction of cell death by these compounds takes place in the absence of detectable caspase activity and consistently occurs in the presence of the caspase inhibitor z.VAD.fmk. Most importantly however, these chemotherapeutic agents regulate AIF activity via causing the translocation of this molecule from the mitochondrion to the nucleus [23, 44, 92-95]. The latter suggests that AIF may not only play a central role in the induction of CI-PCD but also that it could be a possible target for future design of anti-tumorigenic agents.

Clinical Trials with Anti-Cancer Agents Inducing CI-PCD

The poor sensitivity of certain forms of cancer to current therapeutics urge the constant search for new drugs. Several drugs currently in clinical trials are known to induce CI-PCD. For example, BZL101 (Scutellaria barbatae) is a plant drug that has been developed by BioNovo that induces CI-PCD via the translocation of AIF from the mitochondria to the nuclei. This drug has been tested in a phase I clinical trial in advanced breast cancer patients where it has been shown to have an efficient anti-tumor activity [96]. A phase II clinical study for advanced breast cancer is underway but this drug is also being tested in preclinical studies for its possible use in the treatment of pancreatic cancer [8]. The completion of a recent study on the molecular mechanism involved for the induction of CI-PCD by this agent on breast cancer cells has shown that BZL101 induces a death pathway that involves oxidative stress, DNA damage and activation of death-promoting genes [97].

Similarly, another drug known as Atiprimod (azaspirane), a novel cationic amphiphilic compound with anti-inflammatory, anti-neoplastic and anti-angiogenic properties, is being tested for its possible use in the treatment of metastatic carcinoid tumors (Phase II) and refractive myeloma (Phase I/II) [50]. Atiprimod has been shown to induce apoptosis of MCL cell lines and freshly isolated primary tumor cells in vitro via activation of JNK and up-regulation of Bax, Bad and phosphorylated Bcl-2, resulting in the release of AIF and cytochrome c from mitochondria and the activation and cleavage of caspase-9, caspase-3 and PARP [50]. However, the observation that an AIF inhibitor, but not a pan-caspase inhibitor, completely abrogated Atiprimod-induced apoptosis, demonstrated that Atiprimod induces cell death mainly via activation of CI-PCD [50].

Flavopiridol is a synthetic N-methylpiperidinyl chloro-phenyl flavone used in a large number of Phase I and Phase II clinical trials. This drug has a potent anti-carcinogenic activity and is being tested in clinical trials for the treatment of solid tumors and leukemia. Flavopiridol is a cyclin-dependent kinase inhibitor that inhibits the in vitro and in vivo growth of several solid malignancies such as renal, prostate and colon cancers via decreasing the expression of cyclin D1, CDK4 and p21. This agent induces CI-PCD via the down-regulation of Bcl-2, release of cytochrome c and translocation of AIF to the nucleus [98-101].

Since cathepsins are involved in the induction of CI-PCD, there is considerable interest to develop drugs that could be used by these proteases to trigger this death pathway. Such an example is paclitaxel poliglumex (PPX) (CY-103; XYOTAX), a macromolecular taxane which was designed to increase the therapeutic index of paclitaxel. PPX enters tumor cells and is metabolized via cathepsin B to paclitaxel. The design of this molecule may have significant therapeutic implications as cathepsin B is upregulated during the induction of CI-PCD and has been shown to be present in high levels in cancer cells [102]. PPX is currently in a phase II clinical trial where in combination with estradiol it is tested for the treatment of AR-prostate cancer. Furthermore, a phase III clinical trial has been initiated to test the potency of PPX in patients with advanced non-small cell lung carcinoma [103].

New Synthetic and Natural Agents Inducing CI-PCD

In addition to the agents currently being tested in clinical trials, cellular proteins as well as several other synthetic and natural molecules, induce cell death in cancer cell lines via activation of CI-PCD. The following section provides a brief discussion of promising compounds for the treatment of cancer whose main mode of action is the induction of CI-PCD.

Bobel 24 (2,4,6-triodophenol) has been reported to induce CI-PCD in several human pancreatic carcinoma cell lines via reactive oxygen species (ROS) production, mitochondrial depolarization, cytochrome c release, AIF nuclear translocation and lysosomal cathepsin release. It has been suggested that the CI-PCD mechanism induced by Bobel 24 may overcome the resistance to apoptosis observed in pancreatic carcinoma when treated with current genotoxic drugs [104].

CHM-1, (2’-fluoro-6,7-methylenedioxy-2-phenyl-4-quinolone a synthetic 6,7-substituted 2-phenyl-4-quinolone) has been identified as a potent and selective anti-tumor agent in human hepatocellular carcinoma which is highly chemoresistant to currently available chemotherapeutic agents [28]. CHM-1 has also been shown to be very effective in hepatocellular carcinoma cell lines without activation of the caspase cascade, as determined by the inability of z.VAD.fmk to abolish CHM-1-induced cell death. Apparently, CHM-1 activates CI-PCD by inducing the translocation of AIF from the mitochondria to the nucleus. Therefore, both Bobel 24 and CHM-1 may be promising chemotherapeutic agents for treating cancer, especially pancreatic and
hepatocellular carcinomas respectively, worthy of further development into clinical trial candidates [28].

Given the toxicity of chemotherapeutic agents, there is a considerable interest in the use of natural agents with anti-tumorigenic properties for cancer treatment. So far several natural compounds have been reported to have anti-tumorigenic properties and, interestingly, many of these compounds have been reported to induce CI-PCD. Selenium is an example of a natural compound capable of inducing CI-PCD in the cervical cancer cell line HeLa Hep-2 cell line. The pathway involves activation of p53, accumulation of Bax and release of AIF and Smac/DIABLO without activation of caspases; cell death is observed despite co-treatment with the caspase inhibitor z.VAD.fmk, confirming the activation of CI-PCD by the compound [32]. In a similar manner, selenocystine (a naturally occurring selenoamino acid) induces CI-PCD in MCF7 breast cancer cells via the phosphorylation of p53 and translocation of AIF [24]. Vitamin D also activates CI-PCD in MCF7 breast cancer cells by a pathway that does not require p53 or the caspases but activates cathepsin D and can be inhibited by Bcl-2 [18]. Lipidic acid activates CI-PCD in HL-60 leukemia cells via down-regulation of Bcl-2, up-regulation of Bax, release and translocation of AIF and cytchrome c from the mitochondria to the nucleus [25]. The thiosulfimates from Allium tuberosum L. activate both CD-PCD and CI-PCD in P35 prostate cancer cells via decreasing the expression of Bcl-2 and increasing the expression of Bax and AIF [26]. Lastly, Resveratrol seems to trigger CI-PCD in MCF-7 breast cancer cells without induction of CD-PCD. The latter is achieved via down-regulation of Bcl-2, decreased mitochondrial membrane potential, increased reactive oxygen species and nitric oxide production and inhibition of NF-kB [27].

Similar to the potential use of CI-PCD activating synthetic and natural agents in chemotherapeutic treatments, intracellular proteins activating CI-PCD could be used in gene therapy. RB94 is a molecule which lacks the N-terminal 112 amino-acid residues of the full-length retinoblastoma protein (RB110). RB94 is a more potent inhibitor of cancer cell growth than RB110 and is expected to be used for gene therapy. Although it was initially thought that RB94-induced cell death was caspase-dependent, it is currently believed that this agent initially triggers CI-PCD (induced by AIF translocation from the nucleus to the mitochondrion) followed by activation of CD-PCD [105].

In summary, central to the induction of CI-PCD by the proposed natural and synthetic compounds is the translocation of AIF from the mitochondrion to the nucleus. Furthermore, the inhibition of Bcl-2, the translocation of Bax to the mitochondria and the release of Smac/DIABLO are also key events in the induction of CI-PCD by these agents. The contribution of each of these molecules in the induction of CI-PCD is proposed in the model presented in this review (Fig. (3)).

The Possible Role of AIF as a New Target for Anti-Cancer Therapy

The previous description of the molecular pathway of CI-PCD has clearly revealed that AIF plays an important role in the induction of this type of cell death [23, 38-48, 50, 93-95, 97-101]. The importance of AIF in the avoidance of tumor development has been shown by several studies. For example, in non-small cell lung carcinoma (NSCLC) cells where the caspase-dependent pathway is less efficient in comparison to the small cell lung carcinoma cells, the triggering of an AIF-mediated caspase-independent mechanism by staurosporine circumvents the resistance of NSCLC to conventional anti-cancer drugs [43]. Furthermore, recent studies have revealed that the inhibition of AIF may cause chemoresistance of non-small-cell lung carcinomas [106] and radiation resistance in human T cell lymphoma [107] and in other types of human cancer [108]. These results therefore suggest that AIF could be a candidate target for the development of chemotherapeutic therapies [8].

Nevertheless, there are many problems that should be carefully considered concerning the development of such therapies based on AIF. The main difficulty arises from the fact that AIF is a crucial survival factor in mitochondria through its NADH oxidoreductase activity. Evidence for the function of AIF as a survival factor comes from studies in which embryonic lethality is observed in AIF deficient mouse models [109-112]. Therefore, one of the main difficulties in designing a drug to activate AIF would be to find ways to activate the pro-apoptotic but not the survival arm of AIF. It seems that the localization of AIF is the critical factor determining which of the two pathways is followed. In the mitochondrion AIF performs a vital normal function in energy production. In the nucleus however it is involved in the triggering of chromatin condensation and high molecular weight DNA fragmentation which lead to CI-PCD. Therefore, in order to trigger the induction of CI-PCD by AIF the design of any chemotherapeutic drugs should trigger the translocation of this factor selectively to the nucleus of cancerous cells [8, 88, 109-112, 113].

FURTHER INSIGHTS INTO THE MOLECULAR MECHANISMS OF CI-PCD

In the past CI-PCD was commonly considered as a delayed death process which unravels when caspases are inhibited or slowed down [70]. However, newly emerging evidence from the literature suggests that this may not be the case [23, 38-48, 50, 93-95, 97-101] and further studies should focus on understanding the molecular pathways involved in CI-PCD in greater detail. While the main molecular events triggered during the induction of CI-PCD have been identified with the use of caspase inhibitors (such as z.VAD.fmk and Q-VD), a common concern has been that these inhibitors may not be able to inhibit several unidentified caspases or penetrate the cells completely to achieve their inhibitory effects. To separate the molecular events triggered during CI-PCD from those activated during CD-PCD, it would be desirable to generate a mouse that has genetically knocked-out all caspases. The latter would provide vital information on the ability of induction of CI-PCD in the complete absence of any caspase activity and would directly identify the key players involved in this pathway.

CONCLUDING REMARKS

Reports challenging the simple classical pathways of apoptosis began to appear in the scientific literature about 10
years ago. It is now apparent that besides using the caspase-mediated apoptotic pathways, cells have alternative methods of undergoing PCD. This understanding provides new opportunities for the development of novel, efficacious anti-tumor agents. Although drugs designed against targets of CD-PCD have provided some benefit to cancer patients, chemotherapeutic agents activating CI-PCD seem equally promising [84, 86, 88]. Natural or synthetic compounds are continuously being developed against the new molecular targets that have been revealed from these studies. In particular, the recent realization of the role of AIF in CI-PCD opens new cancer chemotherapeutic approaches to the research community and the pharmaceutical industry. The observation of a complex crosstalk between CD-PCD and CI-PCD, is of great importance, as it suggests that the use of a drug, or a combination of drugs, targeting both pathways could lead to more efficacious cancer treatment protocols and lower levels of tumor resistance.

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CONFLICT OF INTEREST

Dr Constantina Constantinou and Dr Konstantinos A. Papas are employed by Yasoo Health Ltd and Yasoo Health Inc respectively which market dietary supplements.

ABBREVIATIONS

AIF = Apoptosis Inducing Factor
AR = Androgen Receptor
CD-PCD = Caspase-Dependent Programmed Cell Death
CI-PCD = Caspase-Independent Programmed Cell Death
DD = Death Domain
DED = Death Effector Domain
DISC = Death Inducing Signalling Complex
GSK-3 = Glycogen synthase kinase -3
Hsp60 = Heat shock protein 60
Hsp70 = Heat shock protein 70
MOMP = Mitochondrial Outer Membrane Permeability
PCD = programmed Cell Death
PS = Phosphatidyl Serine
z.VAD.fmk = Benzoyloxy carbonyl-Val-Ala-Asp-fluoromethylene}

REFERENCES


