Minireview

Modulation of Drug-induced Apoptosis by Interruption of the Protein Kinase C Signal Transduction Pathway: A New Therapeutic Strategy

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Introduction

The seminal work of Skipper and Schabel in the 1950s laid the groundwork for what is generally considered the scientific approach to combination chemotherapy. Most of the principles established during this period continue to serve as guidelines for the development of modern chemotherapeutic regimens. These concepts include administration of drugs at maximally tolerated doses, combination of cycle-active and -inactive agents, inclusion of drugs with disparate mechanisms of action and/or non-overlapping toxicities, and exclusion of agents that lack activity against the tumor of interest. The success of regimens such as cyclophosphamide-Adriamycin-vincristine-prednisone for large cell lymphoma (1), ara-C for acute myelogenous leukemia (2), and nitrogen mustard-vincristine-procarbazine-prednisone/Adriamycin-bleomycin-vinblastine (3) for Hodgkin’s disease attests to the validity of this approach, at least in the case of hematological malignancies.

However, aside from adherence to the principles outlined above, the design of such regimens was largely empirical in that interactions between individual antineoplastic agents remained poorly defined. A more rational approach to combination chemotherapy, developed by Martin and others, was subsequently termed biochemical modulation. This strategy was based upon the premise that significant biochemical differences exist between normal and neoplastic cells and that such differences can be exploited to improve the therapeutic selectivity of effective antineoplastic agents. Examples of regimens whose design evolved from this concept include sequential methotrexate and 5-fluorouracil (4), 5-fluorouracil and leucovorin (5), and thymidine and ara-C (6). Although several of these continue to show promise, it is uncertain whether this general strategy will prove superior to the more empirical approaches that preceded it.

More recently, a new therapeutic strategy has evolved, based upon emerging appreciation of the process known as programmed cell death, or apoptosis. Apoptosis represents a genetically regulated sequence of events in which a cell commits itself to a program of self-destruction (reviewed in Refs. 7–10). It is characterized by certain stereotypical morphological features, such as cell shrinkage, nuclear condensation, and karyolytic degeneration of the nucleus, resulting in the formation of membrane-bound nuclear fragments referred to as apoptotic bodies (11). It is also frequently, although not invariably, accompanied by the degradation of genomic DNA into integer multiples of 180–200-bp fragments by one or more of a family of Ca2+-Mg2+-dependent endonucleases (12). Alternatively, apoptosis may occur in the absence of internucleosomal DNA cleavage, but accompanied by the formation of larger (i.e., 50- or 300-kbp) fragments, presumably corresponding to DNA loopes or rosettes, respectively (13). Early studies of apoptosis focused on its role in embryonic development (14), thymic involution (15), and steroid-induced lymphocytolysis (16). However, it is now apparent that diverse chemotherapeutic agents kill neoplastic cells by inducing apoptosis, at least in vitro (17). The potential in vivo significance of this finding is underscored by the detection of apoptotic cells in the peripheral blood of leukemic patients who have received cytotoxic chemotherapy (18).

It is now recognized that the status of various signal transduction pathways influences the susceptibility of cells to apoptosis induced by adverse conditions, such as growth factor deprivation (19). It is also clear that the same pathways may, to a significant extent, determine the vulnerability of neoplastic cells to cytotoxic drugs. For example, mutations in the p53 tumor suppressor/G1 checkpoint gene confer resistance to both chemotherapeutic drugs and ionizing radiation (20). Similarly, overexpression of the bcl-2 oncogene provides cells with a survival advantage over their wild-type counterparts and at the same time renders them more resistant to multiple cytotoxic agents (21). Furthermore, expression of the bcr-abl p140 tyrosine kinase by the chronic myelogenous leukemia-derived erythroleukemia cell line K562 inhibits apoptosis and permits cells to survive otherwise lethal exposures to a variety of chemotherapeutic drugs (22). Thus, in addition to classical mechanisms of drug resistance (e.g., decreased drug uptake or activation, increased expression of P-gp and so forth), neoplastic cells may evade the effects of chemotherapy through intrinsic defects in the cell-death machinery itself. The implication of these findings is that perturbations in signal transduction pathways that contribute to neoplastic transformation by antagonizing apoptosis may at the same time protect cells from eradication by cytotoxic agents. A corollary of this hypothesis is that interventions at the level of signal transduction pathways could potentially reverse drug resistance by overcoming a block in one or more distal steps in the cell death process.

Protein Kinase C Signaling and Apoptosis

PKC is a Ca2+- and lipid-dependent serine/threonine kinase consisting of at least nine isoforms differing in Ca2+-
dependence, sensitivity to diglyceride or phorboids, substrate specificity, subcellular localization, and tissue distribution (23). This isoenzyme family has been implicated in the regulation of diverse cellular functions, including proliferation, differentiation, transformation, and stimulus-secretion coupling, among others (24). The bulk of evidence suggests that PKC functions to oppose apoptosis, particularly in hematopoietic cells. For example, the tumor-promoting phorbol diester phorbol 12-myristate 13-acetate, which targets various isoforms of PKC, prevents hematopoietic cells from undergoing apoptosis in response to growth factor deprivation (25). Moreover, diverse PKC inhibitors, including staurosporine, H-7, calphostin C, and chelerythrine, are potent inducers of apoptosis in human leukemic cells (26–28). Finally, treatment of leukemic cells with phorbol 12-myristate 13-acetate reduces their sensitivity to drug-induced apoptosis (29), although this effect may be indirect and related to induction of differentiation.

The mechanism by which PKC opposes apoptosis remains obscure, although several hypotheses have been proposed. For example, PKC phosphorylates numerous kinases comprising growth factor-related signaling pathways, such as Raf-1 (30). Consequently, antagonism of PKC may predispose both normal and malignant cells to apoptosis by interrupting signaling cascades intimately involved in cell survival. Alternatively, interference with PKC may lead to changes in intracellular pH, which may in turn serve as a trigger for apoptotic events. In this context, one of the actions of PKC is to activate the Na+ /H+ antiporter, leading to an increase in intracellular pH (31). Antagonism of PKC would therefore be expected to induce intracellular acidification, which could facilitate activation of acid-sensitive endonucleases (e.g., DNAse II) implicated in the induction of apoptosis (32).

More recently, attempts to understand the role of PKC in the regulation of apoptosis have focused on interactions between PKC and the antiapoptotic protein Bcl-2. For example, it has recently been shown that induction of apoptosis by Taxol in human lymphoblastic leukemia and prostatic cancer cells is associated with phosphorylation of Bcl-2, suggesting that such an action may interfere with the protective functions of this protein (33, 34). Furthermore, Raf-1, which is a substrate for PKC, has been implicated in this phenomenon (35). Thus, perturbations in PKC may act indirectly through the Raf-1 pathway to modulate Bcl-2 function. On the other hand, studies involving normal hematopoietic cells suggest that PKC acts directly to phosphorylate Bcl-2 on serine residues and that this action has the net effect of antagonizing apoptosis (36). The discrepancy between this and the previous findings may stem from differences in the behavior of normal and neoplastic hematopoietic cells. In any event, the possibility that PKC may regulate apoptosis through modulation of the function of Bcl-1 and/or related proteins should provide a fertile area for future investigation.

Finally, the ability of PKC inhibitors to induce apoptosis may involve factors other than, or in addition to, interference with the PKC pathway. For example, induction of apoptosis in lymphoid cells by staurosporine (37) and 7-OH staurosporine (38) has been associated with dephosphorylation of cyclin-dependent kinase 1 (p34cdc2). Whether the latter event is primarily responsible for apoptosis in cells treated with these agents remains open to question.

Interactions between Ceramide- and Diglyceride-dependent Pathways

Multiple lipid effectors are known to be involved in the regulation of cell survival. Recently, increased intracellular availability of ceramide has been shown to promote apoptosis in mammalian cells (39, 40). Ceramide stimulates, among several subcellular targets, a membranal proline-directed serine/threonine protein kinase (CAPK; Ref. 41), that may be involved in the lethal actions of ceramide. Ceramide generation has been implicated in lethal responses to diverse stimuli, including: (a) activation of neutral and/or acid sphingomyelinases by cytoxic receptor systems (e.g., CD120a; reviewed in Ref. 41); (b) environmental stresses such as ionizing radiation (42); and (c) antineoplastic agents such as anthracyclines (43) and ara-C (44).

In contrast, PKC seems to oppose the putative lethal actions of CAPK. PKC activity is reciprocally regulated by diglyceride and sphingosine, which exert stimulatory and inhibitory effects, respectively, on this enzyme. Thus, the apoptotic effects of ceramide are opposed by diglyceride and by acute exposures to certain pharmacological PKC activators (e.g., PDB; Ref. 45). Conversely, ceramide-mediated lethality is potentiated by sphingosine and pharmacological inhibitors of PKC (e.g., calphostin C, chelerythrine; Ref. 46). The opposing actions of PKC and ceramide in the regulation of apoptosis have recently been confirmed by others (47). Together, these findings suggest that cell survival and death may be determined by the balance that exists between the respective cytoprotective and cytotoxic influences of PKC and CAPK. In this regard, the reciprocal effects of diglyceride and ceramide may mimic the actions of Bcl-2 and Bax in determining the fate of drug-treated cells. Interestingly, overexpression of Bcl-2 protects cells from ceramide-mediated apoptosis (48), thereby providing another mechanism through which this protein might protect cells from drug-mediated lethality.

Potentiation of ara-C Action by PKC Inhibitors

The nucleoside analogue ara-C represents a prototypical target drug, the apoptotic actions of which may be modulated by perturbations in PKC-dependent signaling. After conversion to its lethal derivative, ara-CTP, it is incorporated into DNA, leading to premature chain termination, interference with chain elongation and initiation, DNA fragmentation, and, ultimately, apoptosis (49). The response of neoplastic cells to ara-C has been extensively characterized, and there are numerous potential mechanisms by which this agent might interact with signal transduction pathways. For example, as noted previously, treatment of leukemic cells with ara-C leads to the generation of both ceramide (through sphingomyelinase activation; Ref 44) as well as diglyceride (through reversal of phosphotidylycholine synthase activity; Ref. 50). Exposure of leukemic cells to ara-C has also been associated with up-regulation of c-jun and c-fos (49), increases in total cellular PKC activity (51), activation of stress-activated protein kinase enzymes (52), and proteolytic degradation of nPKC8 (53). As shown in Fig. 1, the susceptibility of cells to ara-C-induced apoptosis may therefore depend upon the balance that exists between the protective effects of the PKC pathway and the proapoptotic actions of ceramide, both of which are subject to perturbations by ara-C itself.

Initial efforts to characterize the effects of PKC inhibitors
on ara-C-induced apoptosis were prompted by the observation that agents such as H7 and staurosporine could block the up-regulation of c-jun associated with ara-C-mediated cell death (51). Because interference with c-jun function (e.g., by antisense oligonucleotides) has previously been shown to block growth factor deprivation-induced apoptosis in cells of lymphoid origin (54), it was postulated that if increased expression of c-jun represented an essential component of the apoptotic response in myeloid leukemia cells, prevention of such a response would antagonize the lethal actions of ara-C. However, several subsequent studies demonstrated that PKC inhibitors increase rather than antagonize ara-C-induced apoptosis (55, 56); moreover, this effect was in some cases accompanied by increased expression of c-jun (56). Taken in conjunction with the finding that highly specific inhibitors of PKC (e.g., chelerythrine, calphostin C) also induce c-jun up-regulation in association with apoptosis (57), it now seems more likely that under these circumstances, increased expression of c-jun represents a response to rather than a cause of apoptotic DNA damage by these agents. In addition, the capacity of PKC inhibitors to sensitize leukemic cells to ara-C-induced apoptosis may result, at least in part, from their ability to antagonize the protective actions of PKC.

**Bryostatin 1 as a Potentiator of Apoptosis**

An alternative approach to the modulation of PKC activity has evolved from studies of the macrocyclic lactone bryostatin 1. Bryostatin 1, like phorbol diesters, activates PKC but lacks their tumor-promoting properties (58). It also induces differentiation in some, but not all, human leukemic cell lines (59) and, in fact, blocks phorboid-associated activities that it does not itself possess (60). The basis for the unusual spectrum of bryostatin 1 activity remains unknown but has been postulated to result from unique patterns of isofrom activation (61). In view of its documented antitumor activity in vitro (62) and in vivo (63), Phase I trials of bryostatin 1 have recently been initiated (64).

Pretreatment of differentiation-unresponsive HL-60 cells with bryostatin 1 leads to an increase in their susceptibility to ara-C-induced apoptosis (65). Although this finding seems to be in conflict with evidence that PKC activation opposes apoptosis, it is important to note that whereas acute exposure of cells to PKC activators results in increases in enzyme activity, chronic exposure leads to PKC down-regulation, presumably a consequence of enzyme degradation by certain interleukin-converting enzyme β-like proteases (e.g., calpain; Ref. 66). In this regard, bryostatin 1 is known to be a particularly potent down-regulator of PKC (67). Interestingly, acute exposure (e.g., 1–2 h) of HL-60 cells to bryostatin 1, which is associated with increased PKC activity, leads to antagonism of ara-C-mediated apoptosis; conversely, chronic exposure (e.g., 24 h), which is associated with substantial enzyme down-regulation, results in potentiation of ara-C-induced cell death (68). Moreover, down-regulation of PKC activity in these cells by bryostatin 1 increases the susceptibility of cells to ceramide-mediated apoptosis (46). Based upon these and earlier findings, a model has been proposed to explain the relationship between ara-C-induced apoptosis and the PKC signal transduction pathway (Fig. 2). In this model, ara-C administration produces, in addition to DNA damage, elevations in intracellular levels of ceramide and dihydrglycerol. The balance between the reciprocal effects of these lipid second messengers may contribute to the decision of the cell to survive or undergo apoptosis. This balance can also be modified by the administration of PKC inhibitors, which promote cell death. Alternatively, apoptosis may be facilitated by the chronic administration of agents such as bryostatin 1, which lead to PKC down-regulation rather than inhibition. The products of other genes (e.g., bel-2, c-myc, c-jun, and so forth) may also contribute to this decision-making process, perhaps through mechanisms also involving the PKC pathway. One implication of this model is that not only may the status of signal transduction pathways influence the apoptotic response of neoplastic cells to cytotoxic agents but that perturbations in such pathways might theoretically increase drug efficacy.

**Interactions with other Agents**

The initial rationale for using PKC inhibitors in conjunction with cytotoxic agents was based upon attempts to overcome P-gp-mediated drug resistance. For example, the protective effects of P-gp have been shown to be at least partially dependent upon PKC-induced phosphorylation (69). In addition, the response of cells to agents such as Adriamycin can be modulated by phorbol diesters (70). Moreover, staurosporine and some staurosporine analogues have been shown to reverse, at least partially, multidrug resistance in human leukemia cells (71, 72). However, recent studies indicate that perturbations in the PKC pathway may increase the susceptibility of malignant cells to a broad range of antineoplastic agents, perhaps through a variety of mechanisms. For example, bryostatin 1 has been shown to increase the susceptibility of cervical carcinoma cells to cis-diaminedichloroplatinum(II) by increasing intracellular drug accumulation (73). Bryostatin 1 has also been reported to increase the in vivo activity of vincristine and melphelan against a Waldenstrom's macroglobulinenia tumor line (74). More recently, Schwartz and coworkers demonstrated that exposure of p53-deficient gastric carcinoma cells to the PKC inhibitor safin-
1918 PKC Signal Transduction Pathway Modulates Drug-induced Apoptosis

Fig. 2 A proposed model to account for interactions between PKC and ara-C-induced apoptosis in leukemic cells. The bifurcating arms of the PKC signal transduction pathway involve enzyme activation and calcium mobilization, which can be independently stimulated (e.g., by diradylglycerols and by IP$_3$ or calcium ionophore, respectively). In this model, PKC opposes apoptosis, an action that can be directly antagonized by PKC inhibitors or by enzyme down-regulation after chronic stimulation (e.g., by bryostatin 1). PKC may exert its effects by altering intracellular pH or by modulating the expression or function of genes implicated in the regulation of cell death (e.g., Bcl-2). Cells exposed to ara-C may also exhibit an increase in diradylglycerols (DRG), leading to an increase in PKC activity. Alternatively, ara-C may augment ceramide levels, thereby antagonizing the protective effect of PKC. Lastly, activation of PKC may lead to induction of leukemic cell differentiation, which, despite its association with programmed cell death as a late event, can antagonize drug-induced apoptosis on an acute basis. PIP$_2$, phosphatidylinositol 4,5-bisphosphate; Ptd Cho, phosphatidylcholine; PLC, phospholipase C; IP$_3$, inositol 1,4,5-trisphosphate.

gol (the L-threo enantiomer of dihydrosphingosine), an agent currently under clinical investigation, substantially increased apoptosis after exposure to the alkylating agent mitomycin C (75). The significance of these findings lies in the possibility that: (a) modulation of PKC may potentiate apoptosis in cells of nonhematopoietic origin; and (b) interruption of the PKC pathway may enhance the cytotoxicity of diverse classes of antineoplastic agents.

Modulation of Drug-induced Apoptosis by Inhibition of PTKs

Signaling pathways other than PKC exist that offer additional opportunities for therapeutic intervention. In particular, the activity of PTKs have also been implicated in the regulation of apoptosis. Like PKC inhibitors, PTK inhibitors are potent inducers of programmed cell death (76). In addition, PTK inhibitors such as quercetin (77) and genistein (78) have been shown to potentiate the lethal actions of ara-C and Taxol in leukemic cells, presumably by facilitating apoptosis. More recently, it has been demonstrated that a group of PTK inhibitors known as tyrphostins increase the susceptibility of small cell lung cancer cells to various cytotoxic agents (79). The implication of these findings is that diverse agents capable of interrupting signaling pathways in neoplastic cells may not only exert direct cytotoxic effects but may also modulate apoptotic responses of established chemotherapeutic agents.

Summary

There is an accumulating body of evidence that interference with the PKC (and possibly other) signal transduction pathways may increase the susceptibility of neoplastic cells to drug-induced apoptosis, at least in vitro. In view of the ongoing clinical development of agents that inhibit (safingol, 7-OH staurosorpin) or down-regulate (bryostatin 1) PKC activity, it seems likely that attempts to combine such agents with established chemotherapeutic drugs will be feasible in the relatively near future. From a broader perspective, compounds that perturb signal transduction pathways, may, in addition to their direct antineoplastic effects, ultimately prove most useful as modulators of cytotoxic drug action. Based upon past experience in the field of biochemical modulation, demonstration of superior efficacy for this strategy will not be a trivial matter. For example, facilitation of drug-induced apoptosis in neoplastic cells by PKC inhibitors may not lead to an increase in the therapeutic index; moreover, the role of apoptosis, as opposed to alternative forms of cell death, in determining responsiveness to chemotherapy in vivo remains to be clearly established. Nevertheless, the intense interest that has recently been directed at developing agents that interfere with signaling pathways, including PKC, should provide virtually unlimited opportunities for the design of novel combination chemotherapeutic strategies. Identification of regimens exhibiting improved in vivo efficacy and selectivity represents an important challenge for the future.

References


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PKC Signal Transduction Pathway Modulates Drug-induced Apoptosis

1920


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