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REVIEW

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The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review

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Abstract *Purpose*: Microtubules are important cytoskeletal components involved in many cellular events. Antimicrotubule agents including polymerizing agents (paclitaxel and docetaxel) and depolymerizing drugs (vincristine, vinorelbine, and estramustine phosphate) are widely used either alone or in combination with other anticancer drugs. These antimicrotubule agents are promoters of apoptosis in cancer cells. In this review, we discuss the role of bcl-2 family genes in the regulation of apoptosis, and summarize effects of microtubule targeting agents on apoptotic signal transduction pathways. *Conclusion*: Disruption of microtubule structure by antimicrotubule drugs results in induction of tumor suppressor gene p53 and inhibitor of cyclin-dependent kinases, p21WAF1/CIP1 (p21), and activation/inactivation of several protein kinases including Ras/Raf, PKC/PKA I/II, MAP kinases, and p34cdc2. These protein kinases are associated directly or indirectly with phosphorylation of bcl-2. Phosphorylation of bcl-2 and the elevations of p53 and p21 lead to apoptosis. New pathways of antitumor agents could be directed at this p53, p21 and bcl-2/bax function, and may enhance the effect of existing agents.

Key words Apoptosis · Antimicrotubule agents · Bcl-2 phosphorylation · Protein kinases · Signal transduction pathways

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Introduction

Maintenance of the integrity of an organism and its tissues depends upon a delicate balance between proliferation, differentiation, and programmed cell death or apoptosis. Uncontrolled growth at a stage of incomplete maturation or as a consequence of a disorder in differentiation and/or as dysfunctional apoptosis results in significant changes of this balance. Ideally, cancer cell growth could be controlled by reactivating pathways leading to cell cycle arrest, cellular differentiation, or apoptosis.

Microtubules, the self-assembly of α,β tubulin heterodimers, are important cytoskeletal components involved in the regulation of cell proliferation, differentiation, and apoptosis. These molecules are formed by 13 parallel protofilaments (microtubule wall) to which a variety of microtubule-associated proteins (MAPs) and motor proteins bind. Both α and β subunits exist in several isotypic forms and undergo a variety of post-translational modifications [39, 40, 86]. Polymerization and depolymerization of tubulin basically regulates microtubular dynamics. Numerous ligands bind to tubulin, affecting its assembly properties. Microtubule targeting agents are important ligands which have been shown to have utility as chemotherapeutic drugs for the treatment of various types of tumors [11, 18, 24, 29, 47, 56, 62, 66, 67, 68]. Taxanes interact with polymerized tubulin and prevent depolymerization, while vinca alkaloids interact with monomeric tubulin and prevent polymerization. Studies have demonstrated that these microtubule targeting agents promote apoptosis in cancer cells [21, 26, 82]. This review focuses on the apoptotic signal transduction pathways induced by antimicrotubule agents.

Regulation of apoptosis by bcl-2 gene family

Regulation of apoptosis involves a large number of genes that can be classified into three broad categories as

illustrated in Table 1. These include genes that primarily suppress apoptosis, genes that act as promoters of apoptosis, and genes upstream of apoptosis [9, 10, 12, 17, 19]. Apoptosis in mammalian cells is controlled by an equilibrium between suppressor and promoter gene products [59].

The bcl-2 gene family plays a critical role in the regulation of apoptosis and contributes to the pathogenesis of many diseases. Aberrant expression of bcl-2 is believed to contribute to malignant cell expansion via an antiapoptotic effect which enhances cell survival rather than by accelerating cell proliferation. Constitutive expression of bcl-2 in some lymphomas and leukemias allows escape of these cells from physiological apoptosis in the germinal centers and appears to be a major determinant of their pathogenesis [61]. Inhibition of bcl-2 with antisense oligonucleotides induces apoptosis in acute myelogenous leukemic blasts and increases their sensitivity to chemotherapeutic agents [41]. The HTLV-1 tax protein induces apoptosis which is believed to be blocked by bcl-2 [88].

Bcl-2 family members, both apoptosis suppressors and promoters, interact with each other at the level of protein-protein interactions. Bcl-2 protein binds to other proteins with which it has amino acid sequence homology, including bax, bcl-X_L, bcl-X_S, mcl-1, bik, and bad [9, 10, 18, 60, 80]. The functional significance of many of these bcl-2 family protein-protein interactions at present remains unclear. A previous report has suggested that the heterodimerization of bcl-2 with bax appears to be critical in preventing bax-mediated apoptosis [60]. For example, since bax appears to antagonize bcl-2 function thus abrogating the ability of bcl-2 to prolong cell survival, a high level of bcl-2 relative to bax promotes survival. In contrast, an excess of bax relative to bcl-2 results in cell death [60]. Similar observations have been reported for bcl-2 and bak [12]. However, more recent studies indicate that bcl-2 and bax can independently regulate apoptosis [31, 43]. This conclusion is based on the observations that (1) the bcl-2 transgene blocks apoptosis with comparable efficiency in the absence of bax, (2) bax can clearly promote apoptosis in the absence of bcl-2, and (3) bax deficiency and bcl-2 expression (endogenous or transgene) result in an additive effect on apoptosis [43]. Moreover, a recent report also suggests that the complex interaction between bcl-2 and bax may not be an in vivo phenomenon [36]. These findings thus argue that bcl-2 and bax can each function independently of their physical interaction with each other.

Table 1 Genes that regulate apoptosis

Category	Genes	References
Suppressors	Bcl-2, Bcl-X_L, Bcl-w, Bfl-1, Brag-1, Mcl-1, A1, Ced-9, BHRF-6, LMW5, CREB, Caspase-9b	9, 12, 15, 19, 23, 30, 32, 45, 48, 51, 57, 72
Promoters	Bcl-xS, Bax, Bak, Bad, Bid, Bik, HRK, Apaf-1, BOD, ZK1, Caspase-1, Caspase-3	10, 12, 37, 60, 80, 88
Upstream genes	Fas/Fas ligand, p53, p63, Myc, WAF1/CIP1	4, 76, 85

The role of bcl-2 phosphorylation in the regulation of apoptosis remains controversial. Previous reports have suggested that bcl-2 function is regulated through its phosphorylation [26]. Studies using inhibitors of phosphatases, such as okadaic acid, have shown that bcl-2 can be phosphorylated at serine residues 70 and 87, and that bcl-2 phosphorylation is associated with loss of its antiapoptotic function [5, 26]. A compelling correlation between bcl-2 phosphorylation and induction of apoptosis in lymphoid cells has been reported [26], suggesting that phosphorylation/dephosphorylation of bcl-2 could be a molecular determinant of cell survival or death. However, this model is challenged by recent observations that phosphorylation of bcl-2 is a marker of M phase events, and not a determinant of apoptosis [52]. Bcl-2 phosphorylation was closely associated in time with M phase arrest, accumulation of cyclin B1, and activation of cdc2/cyclin B1 kinase, but not with apoptosis, when continuous exposure to paclitaxel was evaluated in HeLa cells. Apoptosis was first detected at 12 h and steadily increased thereafter until the termination of the experiments at 48–60 h [52]. Since bcl-2 phosphorylation was detected as early as 6 h after HeLa cells were exposed to paclitaxel, the question remains whether bcl-2 phosphorylation is an early signal leading to programmed cell death.

Induction of bcl-2 phosphorylation and bax expression by microtubule damaging agents

Apoptosis in cancer cells can be selectively triggered by various stimuli, which include ionizing radiation, osmotic shock, cytokines, Fas antigen, and anticancer drugs [33, 38, 56, 70]. TNF-α, whose receptor is related to Fas, can induce apoptosis in myelogenous leukemia and other cancer cells [70]. Antimicrotubule agents, both polymerizing agents and depolymerizing drugs, have apoptosis-inducing activity [21]. These drugs induce apoptosis by disorganization of microtubule structure. The effect of this antimicrotubule action is believed to result in the inactivation of bcl-2 function through phosphorylation [26].

Several studies have demonstrated that bcl-2 phosphorylation can be specifically induced by drugs that affect microtubule depolymerization or prevent microtubule assembly. This effect is not seen by DNA-damaging agents [26, 28, 73]. Induction of bcl-2 phosphorylation at serine-70 and serine-87 is required for microtubule-damaging drug-induced apoptosis [5]. Our recent studies have demonstrated that a significant increase of bcl-2 phosphorylation occurs at 3 h, and achieves peak levels at 16 h after exposure of MFC-7 cells to vinorelbine or estramustine [82]. This change occurs much earlier than observable cell death. Bcl-2 phosphorylation thus is an early signal. The induction of bcl-2 phosphorylation is known to be followed by loss of

its ability to form heterodimers with bax. We have recently reported a significant decrease of the formation of bcl-2/bax heterodimers after induction of bcl-2 phosphorylation, even though the level of bax protein is increased in MCF-7 cells exposed to vinorelbine [82]. A decrease in bax immunoprecipitated with bcl-2 antibody has also been reported in paclitaxel-, vincristine-, or vinblastine-treated cells [73]. Similarly, treatment of prostate cancer cells with these agents causes induction of the bcl-2 phosphorylation followed by a decrease of heterodimer complex formation with bax during apoptosis [27].

Apoptotic signal transduction pathways mediated by antimicrotubule agents

The effects of antimicrotubule agents on cellular function are complex and involve several apoptotic signal transduction pathways as illustrated in Fig. 1.

c-Raf-1/Ras/Bcl-2 pathway

Raf kinases and the ras GTP-binding protein are important regulators of cellular proliferation, transformation, differentiation, cell cycle progression, and apoptosis [42, 44, 56, 64, 69, 71, 85, 87]. Raf-1, the cellular homolog of the v-raf oncoprotein, is a ubiquitously expressed serine/threonine kinase which serves as a central interface in the transmission of mitogenic signals from the cell membrane to the nucleus [64, 69, 71, 85, 87]. In addition, ras protein is an important effector of growth factor receptors and activates different signal transduction cascades [3, 20, 66, 75]. A protein p23 R-ras is associated with bcl-2 activity [23, 26]. Overexpression of p23 R-ras cannot transform cells but can induce apoptosis [25]. Recent studies indicate that apoptosis induced by microtubule targeting agents may require the c-Raf-1/Ras/Bcl-2 pathway [7, 26, 28, 82]. Treatment of tumor cells with paclitaxel results in apoptosis and in c-raf-1 activation which occur simultaneously with bcl-2 phosphorylation [7]. Raf-1 activation requires the interaction of paclitaxel with tubulin, and raf-1 is markedly diminished in paclitaxel-resistant cell sublines. An association has been observed, using paclitaxel analogues and other microtubule targeting agents, between tubulin polymerization, raf-1 activation, bcl-2 phosphorylation, and apoptosis [7]. Thus a model can be proposed that envisions a pathway of raf-1 activation and bcl-2 phosphorylation following disruption of the microtubular architecture, serving a role similar to p53 induction following DNA damage [8].

p53/p21WAF1/CIP 1 pathway

As upstream genes of apoptosis, both p53 and p21 participate in the apoptotic process triggered by microtubule targeting agents [4, 6]. Cell cycle progression is regulated by cyclin-dependent kinases (Cdks) counterbalanced by cdk inhibitors. p21WAF1/CIP 1 (p21) is a potent inhibitor of cdk2 and cdk4. Treatment of cells with microtubule-targeting agents results in induction of p21 and p53 expression [4, 6]. Paclitaxel induces concentration- and time-dependent accumulation of p21 in both p53 wild-type and p53-null cells, although the degree of induction is greater in cells expressing wild-type p53. Coincident with the elevation of p53 and p21, paclitaxel altered the electrophoretic mobility of c-raf-1 and stimulates mitogen-activated protein (MAP) kinase. Previous depletion of c-raf-1 brought about by treatment of 3T3 cells with the benzoquinone ansamycin GA results in inhibition of both the p21 and p53, as well as the activation of MAP kinase. These findings suggest that induction of p21 and p53 by paclitaxel requires c-raf-1 activity, but apoptosis is not strictly dependent on wild-type p53 [6]. Elevation of p21 expression also parallels the inhibition of p34cdc2 activity [4]. High

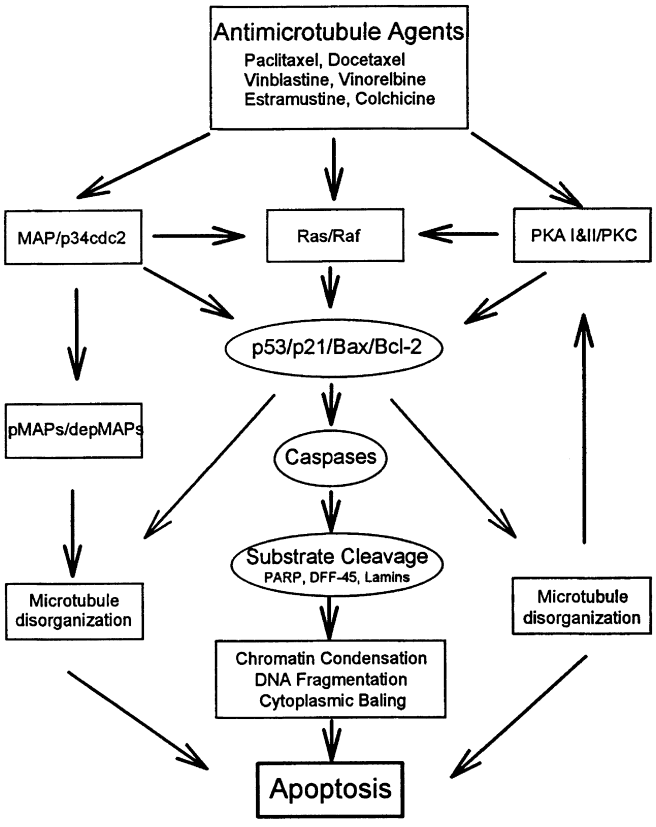

Fig. 1 The effect of antimicrotubule agents on signal transduction pathways of apoptosis. Exposure of cells to antimicrotubule drugs, such as taxanes, vinca alkaloids, colchicine, 2-methoxyestradiol, results in rapid alterations of protein kinase activities (c-raf-1/ras, MAP kinases, p34cdc2, PKA I and II, PKC) and induction of bax. These protein kinases are directly or indirectly responsible for bcl-2 phosphorylation, p53 and p21 stabilization or expression. Overexpression of bax causes G_2-M-phase arrest, tubulin polymerization, and bcl-2 phosphorylation. The increased tumor suppressor protein p53, cdc kinase inhibitor p21, and apoptosis promoter bax, as well as loss of bcl-2 function together with other apoptosis promoters form the death signals leading to apoptosis

levels of p21 protein have been found to be associated with inactive p34cdc2/cyclin B protein complex after treatment with paclitaxel [4].

Protein kinase A (PKA) pathways

The actions of cAMP in the regulation of various cellular functions, including cell proliferation, differentiation, and gene induction through the activation of PKA are well known [18, 46]. Apoptosis induced by the cAMP analog 8-Cl-cAMP and the RI-a antisense oligonucleotide parallels the degree of downregulation of PKA type I and the level of upregulation of PKA type II in several cancer cell lines [13, 14, 49, 73]. Synergistic inhibition of growth and induction of apoptosis by 8-Cl-cAMP and paclitaxel, cisplatin, or retinoic acid in several human cancer cells has been demonstrated [72, 77]. In addition, intracytoplasmic microinjection of purified PKA catalytic subunit promotes cell death [79]. PKA type II has been found to be associated with mammalian centromeres [78], and upon microtubule disruption by paclitaxel, vincristine, or vinblastine, activated PKA causes bcl-2 phosphorylation, leading to apoptosis. In contrast, the drug nocodazole acts analogously to vincristine to block microtubule polymerization but does not activate PKA, does not induce increase of bcl-2 phosphorylation, and consequently does not lead to apoptosis [74]. Hence, disruption of microtubule polymerization does not necessarily lead to apoptosis unless downstream elements are activated. These studies indicate that activation of PKA due to microtubule damage is an important event in bcl-2 phosphorylation and induction of apoptosis.

MAP kinase/p34cdc2-cyclin A and B kinase/bcl-2 pathways

Microtubules are important cytoskeletal components involved in many cellular processes [16, 22]. Recent evidence suggests that the functions of microtubule-associated proteins (MAPs) and filaments in microtubular assembly are regulated through their phosphorylation by MAP kinases and/or p34cdc2 [65]. MAP kinase subfamilies contain three members which include extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 [83, 84]. MAPs are good substrates for ERKs, and phosphorylation weakens their stabilizing effects on microtubules [35]. A differential effect of microtubule-targeting agents on MAP kinase activity during induction of apoptosis has been reported. Previous studies have demonstrated that paclitaxel induces apoptosis through its inhibition of MAP kinase and p34cdc2 kinase activation at G2/M phase in PC-9 and PC-14 cell lines [58]. This decrease in MAP kinase and p34cdc2 kinase activities parallels the increased complex formation between microtubule associated proteins with α and β tubulin with an increase in the amount of MAP-2 phosphorylation [58]. Paradoxically, activation of MAP kinase induced by apoptosis has also been reported [51]. A possible explanation may be a differential effect on MAP kinases depending upon whether or not the antimicrotubule agent promotes polymerization or depolymerization. In addition, these effects may be cell line specific.

We have observed in MCF-7 cells that MAP kinase can be activated rapidly by vinorelbine, but is inhibited by paclitaxel, and estramustine [83]. Approximately a threefold increase of MAP kinase activity was observed within 30 min when exponentially grown MCF-7 cells were treated with 0.2 μM vinorelbine. The maximal activation occurred at 1 h with a fourfold increase, and the enzyme activity was maintained at high levels up to 24 h. In contrast, treatment of MCF-7 cells with estramustine resulted in a significant decrease of MAP kinase activity within 30 min, and the maximal inhibition occurred at 1 h followed by partial recovery. Consistent with the previous report [58], inhibition of MAP kinase in MCF-7 cells by paclitaxel was also observed. This differential effect of the antimicrotubule agent on MAP kinase activity may be due to different binding sites of these drugs to microtubules which then triggers distinct damage of microtubule structures and signal transduction pathways leading to apoptosis.

Whether or not activation of the MAP kinases is involved in bcl-2 phosphorylation is currently unclear. In our recent study, no bcl-2 phosphorylation was observed when immunopurified bcl-2 reacted directly with pure MAP kinase [82]. However, MAP kinases may indirectly be responsible for bcl-2 phosphorylation [2]. 2-Methoxyestradiol, a natural estrogen metabolite, exhibits a similar inhibition of microtubule dynamics to paclitaxel. Exposure of the erythromyeloid leukemia cell line K562 to 2-methoxyestradiol results in stabilization of microtubules which is accompanied by phosphorylation and inactivation of bcl-2. Raf-1 is also phosphorylated in response to 2-methoxyestradiol, but this effect occurs later than bcl-2 phosphorylation, suggesting that raf-1 is not the kinase involved in the phosphorylating bcl-2. On the other hand, a rapid, but transient activation of JNK/SAPK occurs following 2-methoxyestradiol treatment. This finding indicates that overexpression of JNK/SAPK may indirectly lead to bcl-2 phosphorylation [3]. Recent studies have also demonstrated that microtubule targeting agents activate JNK/SAPK through both Ras and apoptosis signal-regulating kinase (ASK1) pathways in a variety of human cells. This activation requires interactions with microtubules [83].

Conclusion

Based on the observations mentioned above, apoptosis induced by antimicrotubule agents is a very complex process associated with many protein kinase signal pathways. Many questions remain to be clarified, such

as the roles of bcl-2 phosphorylation, and protein-protein interactions between apoptosis promoters and suppressors in the regulation of apoptosis. The relationships among various protein kinases in apoptosis pathways affected by antimicrotubule agents also need to be better defined. For example, c-raf-1 is a central component of signal transduction pathways simulated by various factors, protein kinase C [4], and other protein kinases. C-raf-1 is also linked to MAP kinase activity [53, 89]. PKC is involved in apoptosis induced by phorbol 12-myristate 13-acetate (TPA) [62]. Stimulation of tubulin polymerization by MAP-2 is controlled by PKC-mediated phosphorylation at specific sites in the microtubule-binding region [1]. Activation of c-raf-1 by TPA is believed to be via stimulation of protein kinase C-dependent phosphorylation. It is conceivable that different microtubule binding sites, and different disruptions (polymerization or depolymerization) of antimicrotubule agents may trigger different protein kinase signal pathways. Thus, different protein kinase signal pathways and possible crosstalk among those kinases involved in the apoptosis mediated by microtubule-targeting agents need to be better defined. Understanding these signal transduction pathways may reveal a unique avenue that may be useful in either preventing or controlling cancer.

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