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Melanoma and other skin neoplasms

Apoptosis and melanoma Hersey

Apoptosis and melanoma: how new insights are effecting the development of new therapies for melanoma

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Purpose of review

Melanoma has proven resistant to most available chemotherapy and immunotherapy.

Despite a range of different biochemical targets, most agents kill cancer cells by
induction of apoptosis.

Recent findings

Investigation of this process has provided insights into the resistance mechanisms in cancer cells and to development of a range of new agents that target apoptosis pathways. These include agents which inhibit antiapoptotic B cell lymphoma-2 family proteins and inhibitor of apoptosis proteins. In addition a range of signal pathway inhibitors have become available that are able to inhibit signal pathways known to be associated with resistance to apoptosis.

Summary

Evaluation of most of these reagents are at a preclinical level but studies on some pathway inhibitors have passed from phase II into phase III studies. Similarly, evaluation of antisense reagents are at an advanced stage. These early trials show much promise and suggest this approach to development of new therapies will lead to much needed advances in treatment of this disease.

Keywords

apoptosis, Bcl-2 family, inhibitors of apoptosis, melanoma, pathway inhibitors

Abbreviations

Bcl B cell lymphoma protein

BH3 Bcl-2 homology region 3

BIR Baculovirus IAP repeat

IAP inhibitor of apoptosis protein

NF- κ B nuclear factor- κ B

TNF tumor necrosis factor

TRAIL tumor necrosis factor-related apoptosis inducing ligand

Introduction

There has been little progress over the past 30 years in the medical treatment of metastatic melanoma. It is largely unresponsive to available chemotherapy [1*] and shows low response rates to a number of different biologic agents such as interleukin-2, interferon- α 2 and melanoma vaccines [2]. Although different forms of chemotherapy have a number of biochemical targets, it is generally believed that chemotherapy kills cancer cells by induction of a final common pathway that leads to programmed cell death or apoptosis [3–5]. Better understanding of this process is gradually providing insights into the resistance of melanoma to treatment [6,7] and how this may lead to development of new treatment approaches based on activation of the apoptotic pathways.

The agents that are commonly used against melanoma act by damaging cellular components – for example, by methylating guanine (dacarbazine/temozolomide), crosslinking DNA (cisplatin) or damaging microtubules (taxols, vinblastine) – to such an extent that apoptosis is induced. Traditionally, two principal pathways to apoptosis have been recognized the transmembrane 'extrinsic' pathway and the mitochondrial 'intrinsic' pathway [8,9]. Both depend on activation of cysteine proteases that cleave at aspartate residues (caspases). These enzymes are synthesized as proenzymes that become activated by adaptor proteins. Initiator caspases once activated can activate effector caspases, which act on a wide range of substrates to cause apoptosis. The initiator caspases for the extrinsic pathway are caspase 8 and 10, whereas caspase 9 and 2 are initiator caspases for the intrinsic pathway. The effector caspases are believed to be similar for both pathways: 3, 6 and 7.

Irrespective of whether apoptosis is initiated by the extrinsic or intrinsic pathway, apoptosis of most solid carcinomas is dependent on changes in mitochondria. Mitochondria play a central role in energy-generating processes within the cell but, in addition, constitute a crucial source of products which facilitate apoptosis. These include the release of cytochrome c and other aptogenic proteins such as Smac/DIABLO or OMI [10*]. Cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) and this complex (the apoptosome) activates caspase 9 [11,12]. Smac/DIABLO and OMI on the other hand facilitate apoptosis by binding to inhibitor of apoptosis proteins (IAPs), which bind to and inhibit effector caspases.

Members of the B cell lymphoma protein (Bcl)-2 family are critical regulators of the mitochondrial pathway to apoptosis and include both pro-survival (e.g. Bcl-2, Bcl-XL, Mcl-1) and pro-apoptotic proteins [13,14]. The anti-apoptotic members of the Bcl-2 family function as guardians of mitochondrial integrity. Their anti-apoptotic activity can be inhibited by heterodimerization with some pro-apoptotic members. Multiple apoptotic stimuli have been shown to regulate the activity of Bcl-2 family members by different mechanisms involving transcription, proteolytic cleavage or phosphorylation [4,5]. The pro-apoptotic proteins include Bax and Bak multidomain proteins, which share two to three Bcl-2 homology regions with the anti-apoptotic Bcl-2s and Bcl-2 homology region 3 (BH3) sensor proteins, which share only the BH3 homology region with the anti-apoptotic proteins. Bax and Bak appear critical for induction of apoptosis in that Bax⁻¹⁻ Bak⁻¹⁻ cells do not undergo apoptosis in response to BH3 proteins [15,16]. Bax/Bak can mediate release of cytochrome c by forming a pore in the mitochondrial membrane or by binding to the voltage-dependent anion channel and adenine nucleotide translocase components of the mitochondrial permeability transition pore [10*]. Some members of

the BH3 sensor proteins are transcriptionally regulated by p53 (Noxa, PUMA, Bad) whereas others, such as Bim, are released from the cytoskeleton by chemotherapy or upregulated by p53 independent transcription factors such as Foxo 3a [17,18]. Recent concepts suggest that there are two functionally different groups of BH3 proteins. Bid and Bim are believed to be able to directly bind and activate Bax/Bak in the mitochondrial membrane (Bid, Bim) whereas others such as Noxa, PUMA, Bad and Bik bind to the antiapoptotic proteins Bcl-2, Bcl-XL or Mcl-1 [19–21], which causes release of the activating BH3 proteins (Bid, Bim) bound to them. Cytosolic p53 may also function as a BH3 only protein and directly activate Bak in the mitochondrial membrane [22]. Noxa also appears to have specificity for binding to Mcl-1 and thereby activation of Bak [23*].

The extrinsic pathway is activated by binding of members of the tumor necrosis factor (TNF) family, such as TNF-related apoptosis inducing ligand (TRAIL) to receptors on the cell surface, which results in binding of adaptor proteins containing death effector domains. The latter activates initiator caspase 8 (or 10) and this results in a cascade of caspase activation that can lead to direct activation of effector caspases such as caspase 3 [8,9,24,25]. More commonly, however, in melanoma and other solid cancers the extrinsic pathway leads to apoptosis via the mitochondrial pathway due to activation (cleavage) of the BH3 only protein Bid to tBid. The latter then binds to and activates Bax in the mitochondrial membrane. Granzyme B released from the granules of cytotoxic T cells also activates Bid and induces apoptosis in a similar way [26,27]. These concepts are illustrated in Fig. 1. Many of the TNF family members may also trigger other signal pathways that activate anti-apoptotic pathways, for example, TRAIL was shown to activate nuclear factor- κ B (NF- κ B) [28] (which regulates several anti-apoptotic proteins)

and prosurvival pathways such as the mitogen activated protein kinase ERK1/2 pathway [19].

Apoptosis is also regulated by another family of proteins referred to as IAPs [30–32] . These include IAP 1 and 2, XIAP, ML-IAP and Survivin. In general they bind to caspases and prevent their activation (caspase 9) or inhibit their effector function (caspase 3, 7). They also have other roles as E3 ligases and in ubiquitination of proteins for degradation by proteasomes [33*], as discussed elsewhere. Binding of IAPs to caspases is competitively inhibited by Smac/DIABLO and OMI released from mitochondria and this allows effector caspases to induce apoptosis. This mechanism was shown to be the principal pathway in TRAIL induced apoptosis of melanoma [34].

It is apparent from the above that there is a considerable amount of information about induction of apoptosis in cancer cells and this has posed a challenge as to how this knowledge can be used to develop new treatment approaches against melanoma and other cancers. The following sections summarize the advances made and approaches being taken in this field, with particular emphasis on human melanoma (Table 1).

Targeting the B cell lymphoma-2 family of anti-apoptotic proteins

Given the importance of Bcl-2 anti-apoptotic proteins in regulation of the mitochondrial pathway, much effort has been focused on agents that may decrease their activity. One of the first approaches was based on antisense methods against mRNA for the proteins. Nuclease resistant antisense against Bcl-2 in melanoma was shown by Jansen *et al.* [35] to sensitize melanoma xenografts to chemotherapy with dacarbazine. Subsequent phase I studies in patients with the antisense agent oblimersen suggested it was associated with

similar effects in melanoma patients [36]. In view of this, Genta Inc. initiated a large randomized phase III study on 771 patients with metastatic melanoma, which was completed in August 2003. This showed a significant prolongation of progression free survival and overall response rate in patients treated with dacarbazine and oblimersen. The primary endpoint of overall survival was not significantly different between the groups when all patients were included in the analysis but was significant when the analysis was confined to patients with normal lactate dehydrogenase levels (A. Bedikian *et al.*, in preparation) [37]. (The latter measure is an indicator of more advanced disease.) This particular trial was criticized on several procedural matters but more importantly it did not target treatment to patients with melanoma known to express Bcl-2. This was important as Bcl-2 expression is low or absent in many metastatic melanomas. In contrast to Bcl-2, the Mcl-1 protein was shown in immunohistological studies to be increased in metastatic melanoma compared with primary melanoma (Fig. 2). Again the reasons for upregulation of this protein and differential regulation of Bcl-2, Bcl-XL and Mcl-1 are not well understood (Zhuang *et al.*, in preparation). Nevertheless, these results suggest that downregulation of Mcl-1 and perhaps Bcl-XL may be more effective in sensitizing melanoma to chemotherapy than targeting Bcl-2.

Antisense oligonucleotides have been developed against Mcl-1 [38,39]. Bcl-XL [40-42] and both Bcl-2 and Bcl-XL [43]. Studies on melanoma xenografts in severe combined immunodeficient mice showed antisense to Mcl-1 to be effective in sensitizing melanoma to dacarbazine chemotherapy [38]. Similarly, antisense to Bcl-XL enhanced sensitivity of melanoma to chemotherapy [41]. Antisense to both Bcl-2 and Bcl-XL was also reported to have an anti-angiogenic effect [43]. Phase I/II studies are now required to assess their safety and pharmacological effects.

Small molecular weight inhibitors of B cell lymphoma 2 and XL proteins

The BH3 α -helix in the BH3-only proteins binds to a large hydrophobic pocket on the Bcl-2 anti-apoptotic proteins. Cell permeable peptides constructed from the BH3 only protein Bid, were shown to directly induce apoptosis in human leukemia cells *in vitro* and in xenografts [44]. Nuclear magnetic resonance screening of BH3 mimic compounds resulted in production of several non-peptide inhibitors [45] of Bcl-2. One such inhibitor, ABT-737, produced by Idun and Abbot Pharmaceuticals, was able to induce killing of lymphoma and small cell lung carcinoma lines and to sensitize cancer cells to chemotherapy. It had weak binding activity to Mcl-1 [46*]. Additional studies are needed on specific inhibitors of Mcl-1 and A1 as inhibitors of Bcl-2 and Bcl-XL have only weak activity against Mcl-1 and A1.

Gossypol is a naturally occurring compound in cotton seeds which was also shown to bind to the BH3 binding pocket of Bcl-2 and Bcl-XL. It may therefore be a promising agent to sensitize cells to chemotherapy and other agents [47].

Inhibitors of inhibitor of apoptosis protein

The IAPs, particularly XIAP, suppress cell death by inhibiting the activity of caspases via the zinc binding Baculovirus IAP repeat (BIR) domains in the IAPs. Smac/DIABLO promotes apoptosis by binding to the BIR domains of the IAPs. The binding of Smac/DIABLO to XIAP is via the four aminoterminal amino acids (AVP1), which bind to the BIR3 domain of XIAP [48,49]. Binding of XIAP to caspase 3 appears to involve a region between the BIR2 and BIR1 domains of XIAP. Several groups have used caspase activation assays to screen for small molecular weight mimics of Smac/DIABLO that

reverse binding of XIAP to the caspases [50]. A class of polyphenyl ureas was found to increase caspase activity and this was associated with sensitization of cancer cells to chemotherapy and retardation of growth of experimental tumors in mice [51*]. Similar results have been reported with other mimics of Smac/DIABLO [52–54].

Signal pathway inhibitors: mitogen activated protein kinase signaling

The approaches discussed above are based on direct interventions against proteins regulating apoptosis. Signal pathways regulated by the phosphorylation status of proteins in the pathway are also known to have a major influence on apoptosis. Regulation of these pathways are consequently an alternative treatment approach. We [29] and others have shown that activation of the MEK ERK1/2 pathway was associated with resistance to TRAIL induced apoptosis of human melanoma. Resistance was reversed by inhibitors of MEK (U0126) and this was associated with increased activation of Bax and release of Smac/DIABLO from mitochondria. There was also a decrease in Mcl-1 and Bcl-2 but these effects were relatively late and unlikely to be the main mechanism of resistance. Studies by others have drawn attention to suppression of Bim by the ERK1/2 pathway. Bim is regulated transcriptionally as well as post-translationally. There are at least three major transcripts: EL, L and S. EL and L are sequestered to the cytoskeleton by binding to the microtubule dynein motor complex under non-apoptotic conditions, while S has high killing potency and is usually only expressed at the onset of apoptosis. Phosphorylation of Bim-EL directly by ERK1/2 at Serine 69 results in rapid degradation via the proteasome pathway. Additionally, phosphorylation at Threonine 56 by the c-Jun NH2-Terminal kinase (JNK) releases Bim-EL from the microtubular dynein motor complex enhancing apoptosis [55,56]. ERK may also phosphorylate the BH3 protein Bad and inhibit its pro-apoptotic function.

Approximately two-thirds of melanomas have activating mutations of BRAF [57] and N-Ras (18%) [58]. In addition, this pathway is activated by multiple mechanisms unrelated to mutation. Several inhibitors of this pathway have been produced, such as the Onyx/Bayer 43-9006 agent (sorafenib) [59] and the Pfizer compound CI-1040 [60]. In the phase II studies with sorafenib as a single agent there was only one response in 34 patients but in combination with carboplatin and paclitaxel in studies on 54 patients there were 20 partial responses and 26 with stable disease. The response rate in 23 previously untreated patients was 48% (Flaherty et al, presentation at World Congress on Melanoma, Vancouver, September 2005). These results are now being tested in a randomized trial in previously untreated patients (Eastern Cooperative Oncology Group (ECOG) trial 2603) and in previously treated patients (Onyx/Bayer (117118 protocol)).

Ras is upstream of Raf and requires a farnesyl group to be attached for membrane anchorage. It may therefore be possible to inhibit the pathway with inhibitors of farnesyl transferase. These have shown antitumor activity in preclinical studies [61,62] and sensitized human melanoma cells to cisplatin [63] but further evaluation is needed.

Phosphoinositide 3 kinase and Akt inhibitors

Phosphoinositide 3 kinase (PI3K) is activated by tyrosine kinase receptors such as the insulin receptors and leads to phosphorylation of Akt on threonine 308 via PDK1 [64] or serine 473 by the Rictor-mTOR (target of rapamycin) complex [65].

Akt is constitutively activated in many melanoma cells [66,67] and is able to suppress apoptosis via a number of mechanisms including phosphorylation of Forkhead transcription factors, which regulate several pro-apoptotic proteins such as Bim and Fas ligand. The phosphorylated Forkhead proteins are trapped in the cytosol and cannot enter the nucleus. Akt also phosphorylates and inactivates several pro-apoptotic proteins such as Bad and caspase 9 [64,68]. Importantly, it activates I κ B kinase (IKK) and thereby activates the transcription factor NF- κ B, leading to transcription of several anti-apoptotic proteins such as Bcl-XL, AI and XIAP [69].

Relatively few studies have been carried out with inhibitors of this pathway. PX-866 is a specific inhibitor of PI3K which was shown to have single agent activity and to enhance chemotherapy and radiation in preclinical studies [70]. Heat shock protein 90 (HSP90) is a chaperone for a number of signal proteins, including Akt and Raf. A geldanamycin derivative (17AAG) was shown to deplete Akt and cyclin D1 in melanoma lines [71,72]. Phase I studies have been conducted in patients with advanced malignancies and phase II studies on melanoma patients in the Memorial Sloan Kettering Institute are in progress. A more soluble preparation, referred to as KOS-953, is about to enter clinical trials (Kosan Biosciences Inc.).

CCI-779, a rapamycin analogue, was tested in 33 patients with melanoma. Only one partial response was seen [73] but studies in combination with apoptosis inducing agents may be needed. Rapamycin was found to inhibit activation of NF- κ B by doxorubicin but the mechanism of action appeared independent of PI3K [74]. Specific inhibitors of NF- κ B activation do not appear to have been clinically evaluated but proteasome inhibitors such as PS-341/bortezomib have been thought to act by inhibiting activation of NF- κ B

and account for its effects in potentiating chemotherapy [75] and radiotherapy [76]. Nevertheless, proteasome inhibitors affect a wide range of apoptosis regulators. One study in fact found no effect on NF- κ B activity but instead apoptosis appeared to be due to upregulation of Noxa [77]. A number of agents inhibit NF- κ B activation *in vitro*, such as curcumin [78], but are yet to be tested *in vivo*.

Activating the extrinsic pathway

The agents discussed above are also applicable to attempts to treat melanoma by agents such as TRAIL or Fas Ligand. These pathways have several additional obstacles that may need to be overcome. Principal among these is the low or absent death receptor expression on many melanomas, particularly on fresh isolates [79]. The main death receptor for TRAIL, TRAIL-R2 (DR5), was shown to be transcriptionally regulated by p53 and non-p53 dependent mechanisms as reviewed elsewhere [80]. In melanoma mRNA for the death receptors appeared at normal levels and non-transcriptional events appeared more important in regulation [81]. It was shown in TRAIL resistant colon carcinoma that TRAIL-R1 appeared located in the golgi and treatment with tunicamycin resulted in upregulation of TRAIL-R1 [82]. Similarly, tunicamycin was shown to upregulate TRAIL-R2 in prostate carcinoma cells [83]. These findings have been reproduced in cultured melanoma cells. Further studies are needed to investigate clinical applicability.

Conclusion

New treatment initiatives for metastatic melanoma prior to 2000 were largely based on testing new chemotherapy agents or combination of agents or various biological agents with or without chemotherapy. Research on apoptosis and other death pathways over the

past decade has, however, generated a wide range of new reagents which are designed primarily to enhance cell death induced by chemotherapy, radiation or immunotherapy. Evaluations of these new agents are at an early stage and many are at the preclinical level. This applies particularly to small molecular weight mimics of the Bcl-2 family and Smac/DIABLO proteins.

The first agents in large clinical trials were based on antisense strategies and randomized phase III studies have been completed with antisense (oblimersen) against Bcl-2. This drug had relatively small effects in patients with stage IV melanoma but unfortunately the trial design may have limited the outcome, particularly its failure to target treatment to particular patients with known Bcl-2 positive melanoma. Nevertheless, much was learnt from this trial, such as the need to target therapy to particular patients and careful design of trials to establish significance of prolongation of progression free survival. It is hoped that treatment of melanoma with antisense against other more important anti-apoptotic proteins in melanoma (such as Mcl-1) will follow.

Arguably the most exciting results in melanoma trials are those reported from phase II studies of chemotherapy with the RAF kinase/VEGF kinase inhibitor (sorafenib).

Ongoing phase III trials are needed to confirm these results but the frequency of responses in previously treated patients is encouraging. Again, these trials have helped establish another principle: that this new class of agents is unlikely to be effective when given as single agents. In the case of the signal pathway inhibitors, this may be because of extensive cross talk between the pathways which limit direct apoptotic or antiproliferative effects of the drug. These results with sorafenib are likely to apply to

studies against other pathway inhibitors such as the Akt and Farnesyl transferase inhibitors.

This review has focused on apoptosis as the cell death pathway but there is an increasing appreciation that necrosis may play an equally important role in control of tumor growth in cells that become resistant to apoptosis. This was shown in animal models with knockout of Bax and Bak, and was reproduced in human melanoma cells resistant to apoptosis induced by cisplatin [84]. The mechanism is likely to be ATP depletion by activation of polyADP-ribose polymerase (PARP), as reported elsewhere [88**]. Again, these observations may help design strategies that influence necrotic cell death such as inhibition of Akt. In summary, the availability of a number of new agents that target the apoptotic process promise to allow the development of targeted therapy in combination with agents that initiate apoptosis. Studies over the next few years should allow selection of effective combinations and their use in ways which minimize damage to normal tissue.

References and recommended reading

Papers of particular interest, published within the annual period of review have been highlighted as

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Additional references related to this topic can also be found in the Current World

Literature section in this issue (pp. 000–000)

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Table 1 Apoptosis targets in treatment of melanoma

Target/action	Drug	Study Reference
<u>Anti-apoptotic Bcl-2 proteins</u>		
BH3 mimics	ABT-737 (Abbot)	[46]
	GX015-070, BL-193	[45]
	Gossypol	[47]
Bcl-2 Antisense	Oblimersen (Genta)	[36,37]
Bcl-XL Antisense		[40–42]
Mcl-1 Antisense		[39,40]
<u>Inhibitor of apoptosis proteins</u>		
Smac/DIABLO mimics	IDN-13389 (Idun Pharmaceuticals)	[51–54]
<u>RAF/MEK/ERK kinases</u>		
	Bay-43-9006 (sorafenib; Bayer/Onyx)	[59]
	CI-1040 (Pfizer)	[60]
<u>RAS–farnesyl transferases</u>		
	Bim-46228	[62]
	R-115777 (Zarnestra)	[61]
<u>PI3K, Akt kinases</u>		
	PX-866 (ProlX Pharmaceuticals)	[70]
HSP90 inhibitor	KOS-953 (Kosan Biosciences)	[71]
Rapamycin analogue	CCI-779	[73]
<u>Nuclear factor κB</u>		
	PS-341/bortezomib	[75,77]
	Curcumin	[78]

Death receptors

Inhibition of glycosylation

Tunicamycin

[83]

Bcl, B cell lymphoma protein; PI3K, phosphoinositide 3 kinase; HSP90, heat shock protein 90.

Figure 1

Title: Schematic Outline of Apoptosis Mediated Through the Mitochondrial Pathway

Figure 1

(1), (2) and (3) indicate BH3 sensor proteins involved in apoptosis initiating apoptosis in response to agents which (1) target DNA, or (2) are activated by the immune system, or (3) respond to agents targeting the cytoskeleton or in response to cytokine withdrawal (Bim, Hrk) or anoikis (Bmf).

Figure 2

Mean percentage of cells positive for the antiapoptotic proteins by immunohistology showing an increase in expression of Mcl-1 but a decrease in Bcl-2 with progression of melanoma.

[Figures not available]