Death Receptor-Independent Apoptosis in Malignant Melanoma Induced by the Small-Molecule Immune Response Modifier Imiquimod

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Bypassing molecular mechanisms of apoptosis deficiency may be of great utility for the successful treatment of malignant tumors. We have discovered that imiquimod, a small-molecule immunomodulator, exerts rather tumor-selective direct pro-apoptotic activity in vivo and in vitro towards cutaneous metastases of malignant melanoma, an aggressive skin tumor. This pro-apoptotic activity was not detectable with resiquimod, a closely related structural analogue whose pro-inflammatory activity is even greater than that of imiquimod. Unresponsiveness of some melanoma metastases to imiquimod in vivo corresponded to resistance towards imiquimod-induced apoptosis in vivo and in vitro. At the molecular level, the pro-apoptotic activity of imiquimod was independent of membrane-bound death receptors, but depended on Bcl-2 expression as demonstrated by overexpression of Bcl-2 in melanoma cells. Imiquimod is the first topical compound with the potential to bypass molecular mechanisms of apoptosis deficiency, a concept that may be relevant for other tumors as well.

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Successful treatment of metastatic melanoma represents one of the most challenging, but often frustrating tasks in today’s cancer management. This is largely due to the tumor’s aggressive growth and its resistance to many therapeutic regimens (Armstrong and Kricker, 1994; Chang et al, 1998). Although various chemo- and/or immunotherapies, such as IFNα, interleukin-2, or tumor-directed vaccination, have been evaluated in clinical trials and/or are in current clinical use, all of these approaches, unfortunately, met with rather limited success (Becker et al, 2000; Schneeberger et al, 2000). Thus, novel approaches selectively targeting melanoma cells are highly desirable. Among the most appealing strategies are those that bypass mechanisms developed by malignant tumors to resist pro-apoptotic stimuli. Similar to other malignant tumors, examples for such mechanisms described for melanoma cells include mutations of the p53, Mcl-1 or Bcl-X(L) genes, or alterations of NF-κB whose downregulation may sensitize melanoma cells to TRAIL- and TNF-mediated apoptosis. In addition, elevated expression of various proteins such as survivin and other IAPs may inhibit apoptotic signals in melanoma cells (Hussein et al, 2003). Several other mechanisms also have been implicated in apoptosis deficiency of malignant melanoma, such as mutations in the PITSRLE protein kinases resulting in diminished Fas- or TNF-mediated apoptosis (Bayaert et al, 1997; Ariza et al, 1999; Nelson et al, 1999). Of particular note, many mechanisms of apoptosis deficiency described for melanoma involve resistance in death receptor-mediated pathways (Griffith et al, 1998; Shin et al, 1999; Zhang et al, 2000; Franco et al, 2001; Uğurel et al, 2002; Uğrühart et al, 2002). Given that apoptosis deficiency of malignant melanoma appears to be associated with drug resistance in many cases (Helmbach et al, 2003), it is conceivable that specifically bypassing mechanisms of apoptosis resistance will result in an improvement of the as yet poor prognosis of advanced malignant melanoma (Kaufmann and Earnshaw, 2000).

In clinical trials, imiquimod (1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine), a topical compound of Mr = 240.3, has demonstrated high efficacy in the treatment of basal cell carcinomas (Miller et al, 1994; Reiter et al, 1994; Weeks and Gibson, 1994; Gibson et al, 1995; Wagner et al, 1997) or actinic keratoses (premalignant intraepidermal keratinocyte neoplasias) (Miller et al, 1994; Megyeri et al, 1995). Interestingly, recent clinical observations also indicate that imiquimod may be efficacious for topical treatment of some melanoma metastases (Steinmann et al, 2000; Bong et al, 2002; Uğurel et al, 2002). It is thought that imiquimod exerts its effect through elicitation of a strong cell-mediated antitumoral immune response. Its action is mediated, at least in part, through TLR-7-dependent regulation of the transcription factor NF-κB, which upon

Abbreviations: IFN, interferon; IL, interleukin; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MM, malignant melanoma; TNF, tumor necrosis factor.
activation migrates to the nucleus and upregulates transcription of various cytokines (Miller et al, 1994; Reiter et al, 1994; Tomai et al, 1995; Wagner et al, 1997; Hemmi et al, 2002). In addition, imiquimod activates dendritic cells (resident Langerhans cells in the skin) possibly resulting in prolonged protective Th1-skewed immunity against viral infections and malignant tumors (Miller et al, 1994; Tomai et al, 1995). In addition, imiquimod has shown direct pro-apoptotic activity towards epithelial cancer cells (Schön et al, 2003). Recently, resiquimod (4-amino-dimethyl-2-ethoxymethyl-1H-imidazo[4,5-c]quinoline-1-ethanol), a closely related compound of M_r = 314.4, has been demonstrated to exert similar, in some experimental settings even more pronounced, pro-inflammatory effects as its parent analogue, imiquimod (Weeks and Gibson, 1994; Gibson et al, 1995; Wagner et al, 1997; Wagner et al, 1999). There are, however, no published clinical data thus far regarding the efficacy of resiquimod in skin tumors. Overall, the molecular basis of the antitumoral activity of imiquimod is only poorly understood. Of note, it is generally thought that neither imiquimod nor resiquimod exhibits direct antineoplastic activities.

Intrigued by recent clinical observations that imiquimod is efficacious in the treatment of some cutaneous metastases of malignant melanoma (Steinmann et al, 2000; Bong et al, 2002; Ugurel et al, 2002) and that its antitumoral activity extends beyond the known immunomodulatory mechanisms (Schön et al, 2003), we initiated investigations to unravel molecular mechanisms underlying the mode of action of imiquimod and resiquimod towards melanoma cells. To our surprise, we found that imiquimod, but not resiquimod, consistently induced apoptosis in melanoma cells in vitro and in vivo in a rather tumor-selective manner. Our data provide evidence for a direct antineoplastic activity of imiquimod by directly inducing death receptor-independent apoptosis in melanoma cells, which may synergistically enhance the profound immunomodulatory effects in the treatment of malignant melanoma. This principle may be relevant for other malignancies as well.

Results

Imiquimod, but not resiquimod, induces apoptosis in melanoma cell lines. In our initial experiments, four different human melanoma cell lines as well as NHM (established from five different donors) were cultured in the presence of imiquimod or resiquimod at concentrations of up to 50 µg per mL, which is within the range of concentrations reached in vivo (i.e., 3-logs below the marketed formulation, which is 50 mg per g). Unexpectedly and not discernible from previously published data, markedly diminished cell numbers were consistently observed in the melanoma cell lines A375 and Mel-HO in a concentration-dependent manner (Fig 1a). The reduction of cell numbers after incubation with 50 µg per mL imiquimod for 48 h reached 54% (± 11.0) and 75% (± 2.4), respectively, as compared with vehicle-treated control cultures (p < 0.01 in both cases, Fig 1a, left panel). The reduction of cell numbers after treatment with imiquimod was considerably less pronounced in normal melanocytes and two other melanoma cell lines, MeWO and Mel-2A (Fig 1a). No reduction of cell numbers was observed when the cells were cultured in the presence of resiquimod (Fig 1a, right panel). Since direct antitumoral effects of imiquimod have not been reported (Miller et al, 1994; Tomai et al, 1995), this was a surprising observation and we sought to analyze the underlying mechanisms.

In order to distinguish between necrosis and apoptosis, we first assessed the direct cytotoxic effects of both imiquimod and resiquimod on normal melanocytes and four different melanoma cell lines (Mel-HO, Mel-2A, A375, MeWo) using the LDH release method. Neither imiquimod nor resiquimod exerted direct cytotoxic effects when applied at concentrations ranging from 0 to 50 µg per mL (Fig 1b). In contrast, when apoptosis was assessed by ELISA measuring histone-bound DNA fragments, imiquimod exerted dramatic pro-apoptotic effects in the two sensitive melanoma cell lines (Mel-HO and A375) in a concentration-dependent manner. After 24 h of incubation with 50 µg per mL imiquimod, apoptosis was increased by 250%–900% as compared with vehicle-treated control cultures (p < 0.01 in all cases, Fig 1c). In some experiments, induction of apoptosis was even as high as 1400% as compared with untreated controls. In contrast, when normal melanocytes (i.e., cultures established from five different donors) or the melanoma cell lines Mel-2A or MeWo were studied, induction of apoptosis with imiquimod was markedly weaker, if detectable at all, thus suggesting relative resistance to imiquimod-induced apoptosis in these cell populations (Fig 1a). Resiquimod did not induce apoptosis in either of the cell lines studied (Fig 1c).

In order to confirm the pro-apoptotic effect of imiquimod on the single-cell level by a second method, modified TUNEL assays were performed using cultured A375 or Mel-HO cells incubated with normal culture medium, 50 µg per mL resiquimod, or 50 µg per mL imiquimod for 24 h (Fig 2a). In control cultures and in resiquimod-treated cultures, the percentage of apoptotic (TUNEL positive) cells was 0.7% (± 0.7) and 1.3% (± 0.6), respectively. In contrast, when the cultures were incubated with imiquimod, 15.8% (± 3.0) of the cells were apoptotic after 24 h (Fig 2a, right panel). Thus, it appeared that the diminished in vitro cell numbers in imiquimod-treated cultures of melanoma cells were due to apoptosis induced by imiquimod. In contrast, resiquimod again did not exert detectable pro-apoptotic effects in this experimental system (Fig 2a, left panel).

In vivo induction of apoptosis by imiquimod in cutaneous metastases of malignant melanomas. In order to examine directly whether apoptosis was induced during clinical use of imiquimod, thus being relevant in vivo, three patients with multiple cutaneous melanoma metastases were treated topically with imiquimod (Aldara 5% cream) as described recently (Bong et al, 2002). From each patient, several cutaneous melanoma metastases were biopsied and processed for histopathological analysis prior to initiating the treatment, and several additional metastases were excised when signs of local inflammation were clinically apparent. Overall, seven untreated metastases and seven metastases treated topically with imiquimod were analyzed. Utilizing a modified TUNEL assay, paraffin-
embedded sections of these melanoma metastases were subjected to BrdU-labeling of DNA fragments, and bound BrdU was detected by immunohistochemistry. Whereas few, if any, apoptotic cells were detected in untreated metastases, the number of apoptotic, BrdU-positive cells within the tumor tissue were greatly increased after treatment with imiquimod (Fig 2b). Abundant apoptotic cells were detected within the tumor nests, but not within the inflammatory infiltrate surrounding the tumors (apoptotic tumor cell visualized at high power magnification in Fig 2c).

Corresponding to the clinical response of some but not all cutaneous metastases in clinical trials, however, and similar to the divergent responses of different melanoma cell lines in vitro, not all of the treated metastases were equally sensitive to treatment with imiquimod: profound induction of apoptosis was detected in five of seven metastases, whereas two of seven metastases showed only a few apoptotic cells following topical treatment with imiquimod. This finding is consistent with the clinical observation that some (the majority) of cutaneous metastases resolve under topical imiquimod treatment, whereas others appear to be resistant, although skin inflammation developed in either case (Steinmann et al, 2000; Bong et al, 2002; Ugurel et al, 2002). Thus, it is conceivable that additional factors besides induction of a cellular immune response, such as apoptosis of tumor cells, are involved in the clinical outcome of imiquimod-treated melanoma metastases.

Although our data suggest that susceptibility to apoptosis may be relevant for the clinical response of melanoma metastases to topical imiquimod-treatment in patients, this hypothesis is difficult to verify: the necessity to excise metastases in order to assess the presence of apoptotic cells prevents the follow-up needed to evaluate the clinical response. In addition, only a minority of cutaneous melanoma metastases do not resolve under topical imiquimod treatment, and the response cannot be predicted from clinical features alone. Hence, in order to overcome these imminent problems at least in part, we established long-term cultures from three metastases (obtained from two patients), which proved imiquimod-resistant during clinical use of imiquimod (Fig 3a). Of note, a prominent imiquimod-induced cutaneous and peritumoral inflammatory reaction was present in all cases, but apoptotic tumor cells were conspicuously absent in vivo (i.e., in the excised, imiquimod-treated metastases) as determined by TUNEL assays performed on representative sections of the metastases (Fig 3b). This observation provided circumstantial evidence for an association of clinical unresponsiveness of some
Imiquimod-induced apoptosis is independent of membrane-bound death receptors, but depends on caspase activation. Two major pathways have been described through which cytostatic drugs may induce apoptosis. The first involves activation of membrane-bound death receptors, including the CD95 (Fas/APO-1), TNF, or TRAIL receptor systems, and the other is dependent on mitochondrial cytochrome c release, both resulting in apoptotic cell death (Thompson, 1995; Kaufmann and Earnshaw, 2000; Dirsch et al., 2001). The latter pathway is largely dependent on members of the Bcl-2 family of pro- and anti-apoptotic proteins (Raisova et al., 2001). Thus, at least two mutually not exclusive hypotheses can be delineated concerning the molecular mechanisms involved in imiquimod-induced apoptosis of melanoma cells: first, imiquimod could act directly on membrane-bound death receptors, thus initiating the apoptotic signal transduction cascade. Second, imiquimod could influence the Bcl-2-dependent mitochondrial pathway of apoptotic cell death.

In order to evaluate the first hypothesis, CD95 (Fas/APO-1) expression by melanoma cell lines susceptible to imiquimod-induced apoptosis was examined in cultures incubated with imiquimod (50 μg per mL for 0–48 h). Alterations in expression were not detected by FACS or Western blot analysis (data not shown). In order to assess whether imiquimod might activate CD95 without altering its expression, we compared apoptosis induced by imiquimod with apoptosis induced by the CD95-activating CH-11 mAb. It was found that apoptosis of Mel-HO melanoma cells was similarly increased four- to seven-fold by imiquimod or the CH-11 mAb (p < 0.01). But when the cells were incubated with the CD95-directed function-blocking ZB4 mAb, CH-11/CD95-mediated apoptosis was completely abrogated, whereas the pro-apoptotic effect of imiquimod was not affected (p < 0.01 comparing CH-11/ZB4-treated cells and imiquimod/ZB4-treated cells, Fig 4a). Similar results were obtained with the melanoma cell line A375 (data not shown). These findings were
confirmed by TUNEL assays: although only 1.7% of Mel-HO cells were TUNEL-positive in untreated control cultures, in cultures incubated with imiquimod or a combination of imiquimod and the ZB4 mAb, the proportion of TUNEL-positive cells reached 17.3% and 13.7%, respectively (data not shown). Thus, apoptosis induced by imiquimod appeared to occur independent of CD95 (Fas/APO-1).

In the next series of experiments, the effects of imiquimod on TRAIL (tumor-necrosis-factor-related apoptosis-inducing ligand) receptors, TRAIL-R1 to –R4 (DR4, DR5, DcR1, and DcR2), were assessed. As demonstrated by FACS analysis, TRAIL receptors 1 and 2 (DR4 and DR5) were expressed by both Mel-HO and A375 melanoma cells at moderate levels (MFI 30–60; data not shown). Expression of TRAIL-R3 and TRAIL-R4 (DcR1 and DcR2) could not be detected by means of FACS-analysis. Whereas the ligand, TRAIL, binds to all four receptors, only TRAIL-R1 (DR4) and TRAIL-R2 (DR5) confer intracellular proapoptotic signals via activation of initiator caspases (e.g., caspase-8 (Cryns and Yuan, 1999)). In contrast, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) have been termed decoy receptors that do not transmit pro-apoptotic signals and may even have anti-apoptotic effects due to lacking or non-functional cytoplasmic death domains (Abe et al, 2000; Sheikh and Fornace, 2000). Similar to the effect observed with the stimulating anti-CD95 mAb, CH-11, it was found that activation of TRAIL receptors in Mel-HO cells and in A375 cells by the natural ligand (TRAIL, 10 ng per mL for 24 h) resulted in a marked and significant 3–6-fold induction of apoptosis (p < 0.01 as compared with untreated control cultures; Fig 4b). This pro-apoptotic activity of TRAIL was significantly, albeit not completely diminished by either of the function-blocking antibodies against TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (reduction by 41% and 58%, respectively; p < 0.05 in both cases as compared with TRAIL-treated cultures; Fig 4b). As expected, blocking of TRAIL-R3 (DcR1) or TRAIL-R4...
(DcR2) did not inhibit the pro-apoptotic effect of TRAIL stimulation (data not shown). In contrast, apoptosis induced in Mel-HO and A375 cells by 25 or 50 μg per mL imiquimod was not inhibited by TRAIL receptor-blocking antibodies (Fig 4b), indicating that imiquimod-induced apoptosis was independent of the TRAIL receptor system.

Finally, the effect of TNF-receptor inhibition on imiquimod-induced apoptosis was assessed in Mel-HO and A375 cells. As demonstrated by FACS analysis, TNF-R2 was almost absent on the surface of either cell line, whereas TNF-R1 was expressed at low levels (MFI approximately 15–25, data not shown). Consistent with this finding, incubation of the cells with TNF-α resulted in only weak, if any, induction of apoptosis (Fig 4c). In addition, function-blocking antibodies to TNF-R1 or TNF-R2 did not significantly affect the imiquimod-induced apoptosis (Fig 4c).

In order to assess whether imiquimod affected caspase-mediated apoptotic pathways downstream of membrane-bound death receptors, we have inhibited all known caspases using the pan-caspase inhibitor zVAD-FMK. In addition, we have blocked distinct caspase-mediated pathways using specific fluoromethyl (FMK)-conjugated oligopeptide inhibitors irreversibly blocking functional motifs of the respective caspases. When the susceptible melanoma lines A375 or Mel-HO were incubated with the pan-caspase inhibitor zVAD-FMK, imiquimod-induced apoptosis was completely abrogated (Fig 4d). Likewise, blocking the functions of caspases-3, -4, -6, -8, -9 or -10 also resulted in significant inhibition of the imiquimod-induced apoptosis in the two melanoma lines (p < 0.02 in all cases as compared with imiquimod-treated controls). In contrast, inhibitors of caspases-1, -2, and -13 did not significantly affect imiquimod-induced apoptosis (Fig 4d). These results suggested that, on the one hand, imiquimod-induced apoptosis of melanoma cells involved activation of caspases mediating death receptor-initiated and/or mitochondrial pathways.

Figure 4
The pro-apoptotic effect of imiquimod in melanoma cells is independent of death receptor functions, but involves caspase activation. (a) Subconfluent Mel-HO melanoma cells were incubated for 24 h with normal culture medium (control), 50 μg per mL imiquimod, 1 μg per mL of the Fas(CD95)-activating CH-11 mAb, 1 μg per mL of the function-blocking Fas(CD95)-directed ZB4 mAb, or combinations of imiquimod or the CH-11 mAb with the ZB4 mAb as indicated. Apoptosis of the cells (± SD) was determined as outlined in Materials and Methods. The experiment shown is representative of three independent experiments showing similar results. * indicates p < 0.01 (as compared with cells treated with the respective stimulus alone). Similar results were obtained with the melanoma line A375. (b) Subconfluent cultures of Mel-HO melanoma cells were incubated for 24 h with normal culture medium, imiquimod (50 μg per mL), TRAIL (TNF-related apoptosis-inducing ligand, 10 ng per mL), function-blocking mAbs (50 ng per mL) against the TRAIL receptors DR4 or DR5, or combinations of TRAIL or imiquimod with either of the TRAIL-blocking mAbs as indicated. Apoptosis was assessed as in (a). * indicates p < 0.05 (as compared with the TRAIL-treated control). The experiment shown is representative of two independent experiments showing similar results. Similar results were obtained with the melanoma line A375. (c) Mel-HO melanoma cells were incubated for 24 h with normal culture medium, imiquimod (50 μg per mL), TNF-α (1 ng per mL), function-blocking mAbs (3 μg per mL) against the TNF receptors TNF-R1 or TNF-R2, or combinations of TNF-α or imiquimod with either of the TNF-R-blocking mAbs as indicated (± SD). The experiment shown is representative of three independent experiments showing similar results. Similar results were obtained with the melanoma line A375. (d) Mel-HO melanoma cells were cultured for 24 h in normal medium (black bar), in the presence of 25 μg per mL imiquimod (open bar), or combinations of imiquimod and specific oligopeptide inhibitors of caspases as indicated (crosshatched bars, zVAD inhibits all caspases through binding of a shared functional motif). Apoptosis was assessed by determining histone-bound DNA fragments as outlined in Materials and Methods. * indicates p < 0.02. The experiment shown is representative of two independent experiments using the Mel-HO cell line and one experiment using the A375 cell line, all showing similar results.
Direct inhibition of membrane-bound death receptors, on the other hand, did not influence the imiquimod-induced apoptosis in melanoma cells. Thus, it appeared that imiquimod bypassed the signal transduction pathways initiated by membrane-bound death receptors in melanoma cell lines susceptible to imiquimod-induced apoptosis.

The pro-apoptotic effect of imiquimod is inversely associated with Bcl-2 expression Since involvement of membrane-bound death receptors in imiquimod-induced apoptosis could not be detected, we next tested our second hypothesis, involvement of Bcl-2 in the imiquimod-induced apoptosis in melanoma cells. Given that the release of cytochrome c and subsequent apoptotic cell death is governed by the relative amounts and associations of anti-apoptotic (Bcl-2, Bcl-xL) and pro-apoptotic (Bax, Bak, Bid, etc.) proteins in the outer membrane of the mitochondria (Raisova et al., 2001), we hypothesized that imiquimod affected, at least in part, the mitochondrial Bcl-2-dependent apoptotic pathway. In order to test this, we sought to assess the expression of two counteracting proteins of the Bcl-2 family, Bcl-2 and Bax. The imiquimod-susceptible melanoma lines Mel-HO and A375 as well as the apoptosis-resistant lines Mel-2A and MeWo were incubated with normal medium, imiquimod, resiquimod, or the CD95-stimulating antibody, CH-11. Mitochondrial lysates of these melanoma cells were analyzed by Western blot, and antibodies against both proteins, Bcl-2 and Bax, were used subsequently on the same membrane. In the lysates of Mel-HO and A375 cells treated with vehicle, the expression of Bcl-2 was about 5–10-fold higher than the expression of Bax. The same was true for cells treated with resiquimod. In contrast, in lysates of cells treated with imiquimod the level of the pro-apoptotic Bax was increased relative to the Bcl-2 expression level. A similar shift in Bcl-2 versus Bax expression was seen in CH-11-treated Mel-HO and A375 cells (Fig. 5a, top panel). In marked contrast to the melanoma lines susceptible to imiquimod-induced apoptosis, vehicle-treated Mel-2A and MeWo cells revealed a strong constitutive Bcl-2 signal but only a very weak mitochondria-associated signal for Bax. Of note, treatment with imiquimod, resiquimod, or the CD95-stimulating antibody CH-11 did not alter this relation between the highly expressed Bcl-2 and the weakly expressed Bax (Fig. 5a, bottom panel). Thus, these results suggested a participation of proteins of the Bcl-2 family in apoptosis induction by imiquimod in the sensitive cell lines, and might be part of an explanation as to why Mel-2A and MeWo cells were resistant against induction of apoptosis by imiquimod.

In order to evaluate this important finding in more detail, we sought to further analyze the influence of Bcl-2 on induction of apoptosis by imiquimod. In proof-of-principle experiments, Mel-HO cells (a cell line susceptible to imiquimod-induced apoptosis) were transfected with a murine bcl-2 construct (thus resembling the Bcl-2 situation in the imiquimod-resistant lines) or the pIRES vector alone (Müller-Wieprecht et al., 2000), and overexpression of Bcl-2 was confirmed by western blot analysis (data not shown). This resulted in suppression of the imiquimod-induced shift of mitochondrial Bax-expression (Fig 5b). In order to determine the relevance of Bcl-2 overexpression to imiquimod-driven apoptosis, we again assessed DNA-fragmentation

Figure 5: Imiquimod induces apoptosis in melanoma cells in a Bcl-2-dependent manner. (a) Mel-HO cells (i.e., a melanoma cell line susceptible to imiquimod-induced apoptosis, upper panel) and Mel-2A cells (i.e., a melanoma line resistant against imiquimod-induced apoptosis, bottom panel) were incubated for 24 h in normal medium, the Fas-stimulating mAb CH-11, imiquimod, or resiquimod as indicated. Thereafter, mitochondria were purified, and lysed and Bcl-2 and Bax were assessed by western blot analysis as outlined in Materials and Methods. Imiquimod and the CH-11 mAb, but not resiquimod, induce marked shifts of the Bcl-2/Bax ratio in Mel-HO cells, but not in Mel-2A cells. (b) Mel-HO/bcl-2 cells were treated identical to the nontransfected cells depicted in (a), and mitochondrial Bax expression was assessed by western blot analysis. (c) Mock-transfected Mel-HO cells (Mel-HO/pIRES, black bars) and Mel-HO cells overexpressing Bcl-2 (Mel-HO/bcl-2, crosshatched bars) were incubated with normal medium, imiquimod, resiquimod, or the Fas-stimulating CH-11 mAb as indicated. After 24 h, apoptosis was assessed by determining histone-bound DNA fragments. Values represent means of three independent cultures (± SD). * indicates p < 0.01.
in the Bcl-2 overexpressing and the mock transfectants. Mel-HO/pIRES and Mel-HO/bcl-2 transfectant cells were incubated with normal medium, imiquimod, resiquimod, or the CH-11 mAb for 24 h, and apoptosis was determined as before. As expected, both imiquimod and the CH-11 mAb induced marked apoptosis in the mock-transfected Mel-HO/pIRES cells by 493% and 468%, respectively, whereas resiquimod did not induce apoptosis (Fig 5c). In contrast, both the baseline level of apoptosis as well as the imiquimod-induced apoptosis were significantly reduced in untreated cultures of Mel-HO cells overexpressing Bcl-2 as compared with their mock-transfected counterparts (p < 0.02, Fig 5c). Resiquimod did not exert any pro-apoptotic effects in either of the cell lines (Fig 5c).

These findings suggested that imiquimod-induced apoptosis in melanoma cells is independent of membrane-bound death receptors but is, at least to a considerable extent, dependent on Bcl-2 expression.

Discussion
Preferential induction of apoptosis in tumor cells by a small-molecule compound is an exciting anticarcinogenic concept that may greatly benefit the management of some of the most problematic tumors, such as malignant melanoma. We demonstrate that imiquimod, a small-molecule topical compound of the imidazoquinoline family of immune response modifiers, was able to induce apoptosis in malignant melanoma cells both in vitro and in vivo in a rather tumor-selective manner. Given that imiquimod-induced apoptosis in melanoma cells could not be antagonized by functional blockade of various death receptors, including Fas/APO-1 (CD95), TRAIL receptors, and TNF receptors, this action was exerted downstream or independent of death receptor activation. Thus, imiquimod-mediated apoptosis appears to be relevant for effectively bypassing mechanisms of malignant melanomas to resist death receptor-mediated apoptotic stimuli (Griffith et al., 1998; Shin et al., 1999; Zhang et al., 2000; Franco et al., 2001; Urquhart et al., 2002). Our results provide evidence for such direct antineoplastic activity of imiquimod.

Whereas it is possible that in vivo cytokines, such as TNFα or other pro-apoptotic soluble factors, pro-oncotic factors such as reactive oxygen species, or cytoktotic T cells participate in imiquimod-induced tumor destruction, it is reasonable to expect that a direct pro-apoptotic activity may contribute, given that the concentration of drug in the marketed formulation is 3 logs greater than the concentration shown to cause apoptosis in vitro. In addition, the pro-apoptotic effect occurred independent of T cell- or dendritic cell-derived cytokines, as dendritic cells, the primary drug-responsive cell type, and the principal source of released cytokines (Reiter et al., 1994; Gibson et al., 1995; Burns et al., 2000; Suzuki et al., 2000; Hemmi et al., 2002), as well as other leukocytes were not included in the cultures. The finding that resiquimod did not lead directly to apoptosis of melanoma cells does not necessarily predict how this compound might act in a clinical setting, where cytokines and leukocytes would be involved. But the formulation of resiquimod currently under development is only 1.3 logs higher in drug concentration than the highest doses tested in vitro in our study (Spruance et al., 2001), and thus a direct pro-apoptotic effect is unlikely to occur in vivo.

Of note, the clinical response of cutaneous melanoma metastases to imiquimod treatment appeared to correspond to the presence or absence of apoptosis both in vivo and in vitro. This observation strongly suggests that imiquimod-induced apoptosis is an important component of the overall antitumoral activity of this compound. Given that a profound inflammatory reaction was present in all cases, i.e., metastases that resolved under treatment with imiquimod as well as metastases that were resistant or even progressed under such treatment, one may even speculate that susceptibility to imiquimod-induced apoptosis is necessary for the desired net result, the eventual demise of the tumor.

Two frequent mechanisms by which tumor cells achieve chemoresistance have been described: the first involves mutations in the p53 gene, and the second is associated with alterations in pro- and/or anti-apoptotic proteins of the Bcl-2 family. On the one hand, imiquimod was able to induce apoptosis in both p53 wild-type and p53-mutated melanoma lines (Mel-HO and A375) (Raisova et al., 2001; Riebeling et al., 2002). It appears, therefore, that mutations in the p53 gene do not primarily determine the sensitivity of melanoma cells to imiquimod-induced apoptosis. This does not formally rule out that p53 mutations may be involved in some cases of resistance to imiquimod-induced apoptosis in melanoma cells, since wild-type p53 has been shown to upregulate Bax (Miyashita et al., 1994; Miyashita and Reed, 1995), thereby creating a functional link between Bax/Bcl-2 expression and the mutational status of p53. Loss of functional p53 alone, however, cannot serve as a sufficient explanation for susceptibility or resistance to imiquimod-induced apoptosis in melanoma cells, at least under the conditions used in our study.

On the other hand, the status of Bcl-2 appeared to be involved in the susceptibility of melanoma cells to imiquimod-induced apoptosis, since imiquimod-resistant melanoma lines exhibited markedly higher constitutive Bcl-2 expression than the susceptible lines, and imiquimod was able to shift the mitochondria-associated Bcl-2/Bax ratio in susceptible but not apoptosis-resistant melanoma lines. The functional involvement of Bcl-2 in imiquimod-induced apoptosis of melanoma cells was further confirmed when Mel-HO transfectant cells overexpressing Bcl-2 were analyzed: these cells were significantly less susceptible to imiquimod-induced apoptosis as compared with their mock-transfected counterparts. Blocking of caspases, however, which are thought to mediate death receptor-initiated apoptotic pathways, e.g., caspase-8, also resulted in inhibition of imiquimod-induced apoptosis, thus suggesting that imiquimod-induced apoptosis of melanoma cells is not exclusively mediated by the mitochondrial pathway.

Although imiquimod exhibited profound pro-apoptotic activity on several melanoma lines, its analogue, resiquimod, showed very little, if any, such activity. The mechanisms underlying this striking difference between the biological activities of these structurally closely related compounds have not been clarified yet. But given that both compounds exert similar effects on NF-κB-mediated and
NF-κB-independent cytokine induction (Tomai et al, 1995; Imbertson et al, 1998; Wagner et al, 1999), it is conceivable that different structural motifs are required for the pro-apoptotic and the pro-inflammatory activity, respectively. Additional side chains on resiquimod, as compared with imiquimod, may cause steric hindrance or binding to molecular motifs not involved in apoptosis. In addition, differential entry into living cells may contribute to the distinct biological effects observed with the two compounds (Schön et al, 2003). Further molecular modifications may unravel minimal structural requirements for either of these functions. Based on our observations that imidazooquinolines of the resiquimod type exert profound pro-inflammatory but no pro-apoptotic effects, although compounds of the imiquimod type have both effects, it is conceivable that in the future compounds of this family may become available, which have preferential pro-apoptotic effects while exerting only little inflammation. Such compounds may overcome the profound systemic side-effects observed when imiquimod was administered orally for treatment of progressive cancers (Savage et al, 1996).

Overall, our results demonstrate that it is possible, in principle, to use a topical small-molecule compound to induce apoptosis directly and selectively in malignant melanoma. By inducing apoptosis in the tumor cells, a synergistic enhancement of the profound immunomodulatory effects of imiquimod may enable or at least speed the clearance of melanoma metastases.

Materials and Methods

Cells, culture conditions, and Bcl-2 overexpression Four established human melanoma cell lines were used in this study: A375 (Giard et al, 1973), Mel-HO (Holzmann et al, 1988), Mel-2A (Bruggen et al, 1981), and MeWo (Bean et al, 1975). Three additional long-term melanoma cell populations (MM-1, MM-2, and MM-3) were newly established from patients with metastatic melanoma. Tissue specimens were obtained from metastases, which proved clinically resistant to topical imiquimod. The metastases were carefully freed from the surrounding connective tissue, cut into small pieces (2–3 mm), and viable tumor cells were obtained by gentle mechanical teasing of the tissue fragments. Additional tumor cells were released from the tissue pieces by subsequent Trypsin treatment (0.25% at 37°C for 4 h). The identity of the newly established melanoma cultures was confirmed by FACS analysis demonstrating expression of the melanoma-associated antigens HMB-45 and Mel-CAM (CD146) in all three populations, and by melanin production in two of the new populations (melanin was macroscopically visible in the culture medium and microscopically within intracellular granules). All melanoma cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U per mL penicillin, and 100 μg per mL streptomycin (all from Gibco/Life Technologies, Karlsruhe, Germany).

Cultures of normal human melanocytes (NHM) were established from five donors and were grown in MCDB 153 medium supplemented with 5 μg per mL insulin, 10 μg per mL transferrin, 0.4% bovine pituitary extract, 2 ng per mL BFGF, 1 mM cholaer toxin, and 50 μM hydrocortisone (all from Biochrom, Berlin, Germany).

The generation of Mel-HO transfectant cells showing stable overexpression of functional murine Bcl-2 using the pIRES/mcbl-2 expression construct was performed as described recently (Raisova et al, 2001). Selection with 1 mg per mL geniticin (G418, Gibco/Life Technologies) was performed, and stable expression of murine Bcl-2 was routinely confirmed by western blot analysis.

Monoclonal antibodies Murine monoclonal antibodies (mAb) specific for human CD95 (Fas/APO-1; clones CH-11 and ZB4, Chemicon, Hofheim, Germany) were used in functional assays at final concentrations of 1 μg per mL. For assays assessing the function of TRAIL receptors 1–4, the following function-blocking antibodies were used at final concentrations of 50 ng per mL: anti-TRAIL-R1 affinity-purified goat IgG (#AF347, lot BWF 01), anti-TRAIL-R2 affinity-purified goat IgG (#AF631, lot BVO 02), anti-TRAIL-R3 affinity-purified goat IgG (#AF630, lot BTO 02), and anti-TRAIL-R4 affinity-purified goat IgG (#AF633, lot CFL 02, all from R&D Systems, Wiesbaden, Germany). TNF-R1 was blocked by mAb 225 (clone 16803, R&D Systems, 3 μg per mL), and TNF-R2 was inhibited by mAb 226 (clone 22221.311, R&D Systems, 3 μg per mL). For western blot analysis, anti-murine Bcl-2 mAb (clone 100/DS, Novocastra, Dossenheim, Germany) was used at a 1:1000 dilution, anti-human Bcl-2 mAb (clone 124, DAKO, Hamburg, Germany) and anti-human Bax mAb (clone 2D2, Biozol, Eching, Germany) was used at a 1:1000 dilution. Isotype-matched mouse or goat immunoglobulins served as negative control.

Cytotoxicity assays Direct cytotoxicity was determined using a lactate dehydrogenase-(LDH)-release detection kit according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany).

Flow cytometric analysis (FACS) Adherent cells were detached from the tissue culture plastic ware using 0.25% trypsin/0.02% EDTA in PBS. Cells were incubated at 4°C for 45 min with 10 μg per mL of the primary mAb, washed three times in PBS, and incubated with PE-labeled goat anti-mouse IgG or FITC-labeled mouse antigoat IgG for 45 min at 4°C. The cells were washed again three times in PBS, fixed in 2% paraformaldehyde, and analyzed using a fluorescence activated cell sorter (FACScan, Becton Dickinson, Heidelberg, Germany) and the Cell Quest software (Becton Dickinson).

Western blot analysis Cultured cells were solubilized in lysis buffer (1% Triton-X-100, 50 mM Tris, pH 7.4, 150 mM NaCl supplemented with Complete protease inhibitor (Boehringer Mannheim, Mannheim, Germany). A Bradford assay was performed to determine the protein concentrations in the lysates. Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Proteins of interest were detected by specific mAbs following by peroxidase-labeled goat anti-mouse IgG or peroxidase-labeled goat anti-mouse IgM and a chemiluminescence reaction (ECL from Amersham Pharmacia, Buckinghamshire, GB). Signals were quantified densitometrically (Fluor-S Multimager, BioRad, München, Germany).

Subcellular fractionation Cytosolic and mitochondrial extracts were prepared as described recently (Raisova et al, 2001). Briefly, after incubation with imiquimod or resiquimod, cells were detached from the culture dishes, harvested in PBS, dissolved in hypotonic buffer containing Complete protease inhibitor, and incubated on ice for 15 min. Cells were then homogenized using a syringe (28 gauge), and centrifuged for 10 min at 4°C and 10,000 × g. The cytosolic fractions were collected in the supernatants, and the mitochondrial pellets were solubilized in lysis buffer (1% Triton-X-100, 50 mM Tris, pH 7.4, 150 mM NaCl containing Complete protease inhibitor). Western Blot analysis to detect Bcl-2 and Bax within the mitochondrial fractions was performed as described (Raisova et al, 2001).

Inhibition of caspase activation Cells that were induced by imiquimod to undergo apoptosis were cultured in the presence or absence of caspase inhibitors (caspase-specific oligopeptides conjugated to fluoromethyl ketone), which bind to the active site of the proteases, thereby irreversibly inhibiting the catalytic activity of the enzymes. The following inhibitors have been used: Z-VAD-FMK
embedded sections from treated and untreated metastases, respectively, were subjected to apoptosis detection by a modified TUNEL method (DermaTACS) according to the manufacturer’s instructions (Roche Diagnostics). TUNEL-positive cells were visualized with TACS Blue Label (Roche Diagnostics) generating an intense blue staining in cells with DNA fragmentation. In each experiment, one section from each tissue was treated with TACS-nuclease (in order to generate fragmentation of DNA in all cells, positive control), and one section from each tissue was used in which TdT had been omitted (negative control).

**Statistical analyses** All statistical analyses were performed using the Excel software (Microsoft GmbH, Munich, Germany). Data are displayed as mean ± SD; p values were determined using the two-tailed t test, and p values < 0.05 (confidence interval of 95%) were considered statistically significant. All statistical tests were two-sided.

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