Indomethacin Sensitizes TRAIL-Resistant Melanoma Cells to TRAIL-Induced Apoptosis through ROS-Mediated Upregulation of Death Receptor 5 and Downregulation of Survivin

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Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has attracted considerable attention owing to its selective killing of tumor cells but not normal cells. Melanoma shows weak response to TRAIL because of its low level of TRAIL death receptors. Here, we investigated whether indomethacin, a nonsteroidal anti-inflammatory drug, can potentiate TRAIL-induced apoptosis in melanoma cells. We showed that indomethacin was capable of promoting TRAIL-induced cell death and apoptosis in A375 melanoma cells. Mechanistically, indomethacin induced cell surface expression of death receptor 5 (DR5) in melanoma cells and also in various types of cancer cells. DR5 knockdown abolished the enhancing effect of indomethacin on TRAIL responses. Induction of the DR5 by indomethacin was found to be p53 independent but dependent on the induction of CCAAT/enhancer-binding protein homologous protein (CHOP). Knockdown of CHOP abolished indomethacin-induced DR5 expression and the associated potentiation of TRAIL-mediated cell death. In addition, indomethacin-induced reactive oxygen species (ROS) production preceded upregulation of CHOP and DR5, and consequent sensitization of cells to TRAIL. We also found that indomethacin treatment downregulated survivin via ROS and the NF-κB-mediated signaling pathways. Interestingly, indomethacin also converted TRAIL-resistant melanoma MeWo and SK-MEL-5 cells into TRAIL-sensitive cells. Taken together, our results indicate that indomethacin can potentiate TRAIL-induced apoptosis through upregulation of death receptors and downregulation of survivin.


INTRODUCTION

Malignant melanoma is a highly aggressive and treatment-resistant cancer, with increasing incidence and high mortality rates worldwide. The long-term survival rate for patients with metastatic melanoma is only 5% (Cummins et al., 2006). Primary melanoma without any evidence of metastases is mostly treated by surgery. Chemotherapeutic agents such as dacarbazine and temozolomide (alkylating agents), vemurafenib (targeting the BRAF V600E mutation), ipilimumab (targeting CTLA-4), interferon Alfa-2b, and IL-2 for melanoma are presently evolving (Julia et al., 2012; Ma and Armstrong, 2013); however, they are not suitable for many patients because of toxicity, lack of the BRAF V600E mutation, and/or development of resistance (Julia et al., 2012; Ma and Armstrong, 2013). Therefore, other treatment strategies are still required.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has attracted considerable attention owing to its selective killing of tumor cells but not normal cells (Pan et al., 1997). Apoptosis induction by TRAIL is mediated by its interaction with cognate death receptors, TRAIL-R1 (DR4) and -R2 (DR5) (Abdulghani and El-Deiry, 2010). Melanoma cells were previously reported to be variably susceptible to TRAIL-induced apoptosis (Griffith et al., 1998; Nguyen et al., 2001). However, intrinsic resistance to TRAIL, because of the low TRAIL receptor expression levels, appears to be a major barrier for the development of efficient therapies for melanoma (Nguyen et al., 2001; Ivanov et al., 2003; Zhang et al., 2004). Moreover, Zhuang et al. (2006) revealed that decreased expression of TRAIL receptors was associated with thick primary melanoma and metastatic melanoma. Therefore, therapeutic initiatives designed to upregulate TRAIL death receptor expression levels have been proposed to be used in combination with TRAIL for treating melanoma.

A number of reports have suggested that induction of endoplasmic reticulum (ER) stress response within cancer cells...
could sensitize cancer cells to TRAIL through the induction of DR5 expression (Mellier and Pervaiz, 2012; Siegelin, 2012). It has been documented that CCAAT/enhancer-binding protein homologous protein (CHOP), a transcription factor that is upregulated following multiple stimuli, directly controls DR5 expression via a CHOP-binding site in DR5 promoter region (Yamaguchi and Wang, 2004). Since that time, various reports with different stress-inducing compounds have been shown to modulate TRAIL-mediated apoptosis in different cancer cells (Mellier and Pervaiz, 2012; Siegelin, 2012). Given that indomethacin induces CHOP upregulation (Tsutsumi et al., 2004; Okamura et al., 2008; Franceschelli et al., 2011), the current study focused on addressing whether indomethacin can induce DR5 expression in TRAIL-resistant melanoma cells and, if it does, whether indomethacin can also enhance TRAIL responses. We describe here that indomethacin can potentiate TRAIL-induced apoptosis in TRAIL-resistant melanoma cells through upregulating death receptor DR5 via ROS-mediated CHOP activation and through the downregulation of survivin.

RESULTS

Indomethacin sensitizes melanoma cancer cells to TRAIL-mediated apoptosis

We first examined the cytotoxic effects of indomethacin alone or in combination with TRAIL. A375 melanoma cells displayed low rates of TRAIL-induced cell death (Figure 1b, top). However, pretreatment with indomethacin significantly enhanced TRAIL-induced cytotoxicity, as determined by MTT assay (Figure 1b, bottom) and crystal violet staining (Supplementary Figure S1a online).

We also investigated whether indomethacin increases TRAIL-induced activation of caspase-3, -8, and -9 and

Figure 1. Indomethacin sensitizes TRAIL-induced apoptosis in A375 melanoma cells. (a) Chemical structure of indomethacin. (b) Cell viability was assessed by the MTT assay. (c) Western blotting of lysates from A375 cells pretreated with indomethacin for 24 hours followed by treatment with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) for 3 hours using the indicated antibodies. (d) Caspase activities in A375 cells that had been treated with indomethacin for 24 hours and then TRAIL for 8 hours. (e) Cells were stained with PI/Annexin V and then analyzed by FACS. *P<0.05 with a versus control, b versus indomethacin and c versus TRAIL. (b, d) At least two independent experiments with triplicates revealed comparable results. (c, e) At least two independent experiments revealed largely comparable results.
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consequent PARP cleavage. We found that indomethacin enhanced TRAIL-induced activation of all 3 caspases, which in turn led to increased PARP cleavage (Figure 1c). Combinatory treatment of A375 with indomethacin and TRAIL also strongly stimulated caspase-3 and caspase-8 activities (Figure 1d). In addition, we found that indomethacin enhanced TRAIL-induced cell death assayed by annexin V/PI staining (Figure 1e). Furthermore, pretreatment of A375 cells with indomethacin resulted in a markedly increased accumulation of sub-G1 phase cells and apoptotic nuclei (Supplementary Figure S1b and c online) upon TRAIL treatment. Taken together, these results suggest that indomethacin enhances TRAIL-induced apoptosis.

Indomethacin upregulates expression of death receptor DR5 and contributes to the enhancement of TRAIL-induced cell death

TRAIL mediates its activity through the receptors DR4 and DR5 but not DR4 in a dose- and time-dependent manner (Figure 2a). We also found that indomethacin was able to induce DR5 expression in various types of cancer cells (Supplementary Figure S2a online) and enhanced TRAIL-induced cytotoxicity in HepG2, HCT116, and HeLa cells (data not shown), indicating that DR5 induced by indomethacin is probably not cell-type specific. We next analyzed the cell surface expression of DR5 using flow cytometry analysis. After treatment of indomethacin, the level of DR5 on the cell surface increased (Figure 2b). Collectively, these results indicate that indomethacin upregulates the expression of DR5 on the cell surface.

Next we asked whether DR5 expression is induced by indomethacin at the transcriptional level. Using reverse transcriptase PCR (RT-PCR), we found that indomethacin poteniated TRAIL-induced apoptosis through the modulation of DR5 and DR4 expression. Treatment of A375 cells with indomethacin resulted in an increased protein expression of DR5 but not DR4 in a dose- and time-dependent manner (Figure 2c). We also found that indomethacin was able to induce DR5 expression in various types of cancer cells (Supplementary Figure S2a online) and enhanced TRAIL-induced cytotoxicity in HepG2, HCT116, and HeLa cells (data not shown), indicating that DR5 induced by indomethacin is probably not cell-type specific. We next analyzed the cell surface expression of DR5 using flow cytometry analysis. After treatment of indomethacin, the level of DR5 on the cell surface increased (Figure 2b). Collectively, these results indicate that indomethacin upregulates the expression of DR5 on the cell surface.

Figure 2. Indomethacin-induced DR5 upregulation is essential for sensitization of TRAIL-mediated apoptosis. (a) Western blotting of DR5 and DR4 in A375 cells. (b) Cell surface expression of death receptor 5 (DR5) in A375 cells treated with 300 μM indomethacin for 24 hours was measured by flow cytometry analysis. MFI, mean fluorescence intensity. (c) Top) Reverse transcriptase PCR (RT-PCR) product from A375 cells treated as indicated for 18 hours. (Bottom) Real-time PCR analysis of DR5 mRNA. *P<0.05 versus control. (d) Top) Cells were transfected with DR5 small interfering RNAs (siRNAs), and cell extracts were prepared for western blot analysis of DR5 and PARP. (Bottom) After transfection with death receptor 5 (DR5) siRNA, A375 cells viability was assessed by the MTT assay. *P<0.05. (e) siRNA-transfected cells were stained with PI/Annexin V and then analyzed by FACS. (a–e) At least two independent experiments revealed largely comparable results. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
markedly upregulated DR5 mRNA expression in a dose-dependent manner (Figure 2c), suggesting that indomethacin does modulate DR5 expression at the transcriptional level.

We next examined whether the suppression of DR5 by small interfering RNAs (siRNAs) could abrogate the sensitizing effects of indomethacin on TRAIL-induced cytotoxicity and apoptosis. Transfection of A375 cells with DR5 siRNAs reduced indomethacin-induced DR5 expression (Figure 2d). PARP cleavage and cytotoxicity induced by indomethacin plus TRAIL was significantly inhibited in cells transfected with DR5 siRNAs (Figure 2d). Moreover, the effect of indomethacin on TRAIL-induced cell death was effectively abolished in cells transfected with DR5 siRNAs (Figure 2e), whereas treatment with control siRNA had no effect.

The resistance of melanoma cells to TRAIL was also reported to mediate through downregulation of DR4 (Kurbanov et al., 2007), and apoptosis induced in A375 cells by TRAIL alone mainly depends on DR4 signaling (Kurbanov et al., 2005). We therefore also examined whether the silencing of DR4 in addition to DR5 could abolish the sensitizing effects of indomethacin on TRAIL-induced cell death. Cell death induced by TRAIL alone in A375 cells was strongly inhibited by DR4 siRNA treatment (Supplementary Figure S2b and c online), as expected from previously published data (Kurbanov et al., 2005). In contrast, the combination effect was particularly inhibited by DR5 siRNA (Supplementary Figure S2c online). Thus, indomethacin appeared to mediate a switch from mainly DR4 signaling to DR5. Taken together, these results suggest that indomethacin-induced DR5 induction is critical for TRAIL-enhancing effects in A375 cells.

Indomethacin activates DR5 transcription in a CHOP-dependent manner

It has been shown that the induction of DR5 can be mediated through the activation of CHOP (Yamaguchi and Wang, 2004). In this study, we found that the protein expression of CHOP was significantly increased by indomethacin treatment in a time-dependent manner (Figure 3a, top panel). We further found that indomethacin induced CHOP mRNA expression in a dose-dependent manner (Figure 3a, middle panel). Pretreatment of cells with transcriptional (actinomycin D) and translational (cycloheximide) inhibitors blocked indomethacin-induced CHOP expression (Figure 3a, bottom panel), indicating that CHOP induction occurs at both transcriptional and post-transcriptional levels. Furthermore, treatment with indomethacin was able to induce CHOP promoter activity (Supplementary Figure S3a online). Together, these results suggest that indomethacin regulates the transcription of CHOP.

To determine whether CHOP activates DR5 promoter activity, we first tested the effects of indomethacin on the transactivation of reporter constructs with different lengths of DR5 5′-flanking regions (Figure 3b, top). We observed that indomethacin did not increase the luciferase activity of pGL3-DR5 (+71, +150, and +293), whereas it did significantly induce the luciferase activity of pGL3-DR5 (−310, −80, +1) (Figure 3b, bottom), indicating that the region between +1 and +71 is essential for indomethacin-induced DR5 transactivation. Given that a CHOP-binding site is located in this region (Yamaguchi and Wang, 2004), we further examined the effects of indomethacin on the transactivation of DR5 reporter constructs carrying wild-type or mutated CHOP-binding sites. Whereas the promoter activity of pDR5-310 was significantly increased by indomethacin treatment, the promoter activity of pDR5-310ΔCHOP was not enhanced by indomethacin (Figure 3c). Moreover, knockdown of CHOP expression by siRNAs transfection significantly inhibited indomethacin-induced DR5 upregulation (Figure 3d, top left). In line with this, knockdown of CHOP significantly attenuated the cytotoxicity (Figure 3d, top right) and cell death (Figure 3d, bottom) induced by the combined treatment of indomethacin and TRAIL. Taken together, these results suggest that CHOP induction plays an essential role in both indomethacin-induced DR5 upregulation and indomethacin-mediated TRAIL-induced cell death enhancement.

Indomethacin potentiates TRAIL-induced apoptosis through ROS generation

Many reports have shown that ROS plays a critical role in both TRAIL-induced apoptosis (Mellier and Pervaiz, 2012; Siegelin, 2012). Thus, whether ROS is needed for potentiation of TRAIL-induced apoptosis by indomethacin was examined. Indomethacin strongly induced H2O2 production in A375 cells (Figure 4a). In contrast, pretreatment with antioxidant N-acetyl-L-cysteine (NAC) effectively blocked H2O2 production induced by indomethacin (Figure 4a, left panel). We also found that indomethacin increased superoxide (O2−) production (Figure 4a, right panel). Recently, reports have revealed that ROS induces upregulation of CHOP, leading to DR5 induction (Kim et al., 2008; Yokouchi et al., 2008; Moon et al., 2011). Therefore, we next determined whether ROS is involved in indomethacin-induced CHOP and DR5 expression. As shown in Figure 4b, pretreatment of cells with NAC reduced the indomethacin-induced CHOP and DR5 upregulation. Under these conditions, NAC also suppressed the enhancement effect of indomethacin on TRAIL-induced cell death (Figure 4c) and PARP cleavage (Figure 4d). To further confirm the critical role of H2O2 and O2− in indomethacin-induced DR5 expression, we performed DR5 promoter studies with the overexpression of catalase or SOD1. Indomethacin-mediated DR5 promoter activity was significantly blocked by the overexpression of catalase or SOD1 (Figure 4e and Supplementary Figure S4 online), suggesting the essential role of H2O2 and O2− induction in indomethacin-mediated DR5 upregulation. Taken together, these data clearly indicate that ROS generation induced by indomethacin is critical for the upregulation of
CHOP and DR5, contributing to indomethacin-stimulated TRAIL-induced cell death and apoptosis.

**Downregulation of survivin is involved in indomethacin-stimulated TRAIL-induced apoptosis**

A number of anti-apoptotic proteins such as survivin, XIAP, Mcl-1, cIAP-1, cIAP-2, cFLIP, Bcl-2, bax, bak, and Bcl-xL have been shown to be responsible for the TRAIL resistance (Dimberg et al., 2013). Therefore, we examined whether indomethacin sensitized TRAIL-induced cell death through modulation of these cell survival proteins. We found that indomethacin inhibited the expression of survivin but had no apparent effect on the expression of other anti-apoptotic proteins (Figure 5a). The downregulation of survivin by indomethacin was in a dose- and time-dependent manner (Supplementary Figure S5a online). We also found that indomethacin caused a significant decrease (0.581 fold) in survivin mRNA levels (data not shown). Previous studies found that survivin could confer TRAIL resistance in melanoma cells (Chawla-Sarkar et al., 2004; Li et al., 2005; Hilmi et al., 2008). Whether survivin downregulation is needed for potentiation of TRAIL-induced apoptosis by indomethacin was examined. Ectopic expression of survivin partially rescued indomethacin-stimulated PARP cleavage (Figure 5b) and caspase-3 activity (Supplementary Figure S5b online) in TRAIL-treated A375 cells, demonstrating that TRAIL-sensitizing effects of indomethacin are partially mediated by the downregulation of survivin.

To determine the mechanism of survivin downregulation, we first tested whether NAC can abrogate the indomethacin-
induced downregulation of survivin. We found that NAC partially reversed the indomethacin-induced suppression of survivin (Figure 5c), indicating the involvement of ROS in observed survivin downregulation. A previous study showed that NF-kB could transactivate survivin gene promoter (Kawakami et al., 2005). To test this possibility, we assessed whether indomethacin would suppress NF-kB activity. We observed that indomethacin inhibited nuclear translocation of
NF-κB p65 without altering total p65 levels (Figure 5d). Ectopic expression of p65 by transient transfection significantly blocked the downregulation of survivin induced by indomethacin treatment (Figure 5e). Interestingly, indomethacin treatment markedly reduced the NF-κB-dependent luciferase activity, and pretreatment with NAC did not reduce this inhibitory effect (Figure 5f). Taken together, these results suggest that indomethacin-induced ROS generation and NF-κB inhibition independently mediate survivin downregulation and contribute to indomethacin-stimulated TRAIL-induced apoptosis.

**Indomethacin sensitizes TRAIL-resistant melanoma cells**

It has been shown that some melanoma cells such as Mewo and SK-MEL-5 cells are completely resistant to TRAIL (Kurbanov et al., 2005; Chen et al., 2012). As expected, both MeWo and SK-MEL-5 cells had no responses to TRAIL treatment alone (Figure 6a). Pretreatment with indomethacin significantly enhanced TRAIL-induced cell death as determined by MTT assay (Figure 6a) and crystal violet staining analysis (Supplementary Figure S6a online). We also found that indomethacin induced upregulation of DR5 in these cells.

![Image of Figure 6](https://www.jidonline.org/1403)

**Figure 6. Indomethacin sensitizes TRAIL-resistant melanoma cells.** (a) Cell viability was assessed by the MTT assay. *a,b,c* *p*<0.05 with a versus control, b versus indomethacin and c versus TRAIL. At least two independent experiments with triplicates revealed comparable results. (b) Whole-cell extracts were analyzed for expression of DR5 by western blotting. (c) The cell surface expression levels of DR5. MFI, mean fluorescence intensity. (d) Western blotting of lysates using the indicated antibodies. (e) Cells were stained with propidium iodide (PI)/Annexin V and then analyzed by FACS. (f) Schematic diagram of the mechanism by which indomethacin potentiates TRAIL-induced apoptosis. (b-d) At least two independent experiments revealed largely comparable results.
(Figure 6b). Results of flow cytometry analysis also revealed the increase in DR5 cell surface expression in both cell lines (Figure 6c). Further, we found that pretreatment of both cell types with indomethacin resulted in a markedly increased accumulation of apoptotic nuclei (Supplementary Figure S6b online) and PARP, caspase-8 and caspase-3 cleavages (Figure 6d), and cell death (Figure 6e) under TRAIL treatment. Mechanistically, indomethacin strongly induced ROS production in MeWo and SK-MEL-5 cells (Supplementary Figure S6c online), and pretreatment of cells with antioxidant NAC reduced the indomethacin-induced CHOP and DR5 upregulation and rescued survivin downregulation (Supplementary Figure S6d online). Interestingly, indomethacin showed no DR5 induction and enhancement of cytotoxic effect in human epidermal melanocytes HEMn cells (Supplementary Figure S7 online). Overall, we found that indomethacin can also enhance TRAIL-induced apoptosis in TRAIL-resistant melanoma cells associated with the ROS–CHOP-mediated upregulation of DR5 expression.

DISCUSSION

Therapeutic strategies to restore TRAIL receptor levels are urgently needed to overcome resistance of melanomas to TRAIL. In this study, we demonstrate that indomethacin effectively sensitizes human melanoma cells to TRAIL-induced apoptosis through upregulation of DR5 via ROS signaling pathways. Indomethacin-induced ROS mediates the expression of CHOP and further upregulation of DR5 via the CHOP-binding element in the DR5 promoter. We also found that indomethacin downregulated anti-apoptotic protein survivin levels in a ROS- and NF-kB-dependent manner and therefore facilitated indomethacin-stimulated TRAIL-induced apoptosis (summarized in Figure 6f). Given that indomethacin is employed as an anti-inflammatory agent clinically, the combination of TRAIL and indomethacin may be an effective cancer therapy that warrants additional study in vivo.

Our results indicated that indomethacin enhanced TRAIL-induced apoptosis through ROS–CHOP–DR5 induction pathway. We observed that abrogation of DR5 or CHOP upregulation could not completely protect against TRAIL/indomethacin-induced cell death (Figures 2d and 3d). This effect was probably due to the cytotoxicity induced by indomethacin alone as silencing of DR5 had no effect on indomethacin-induced cell death (Figure 2d). The mechanism of indomethacin-induced cell death in melanoma cells requires further investigation. Our results clearly showed that silencing of DR5 or CHOP effectively blocked the cell death induced by TRAIL/indomethacin (Figures 2e and 3d), but the blocking effects were not completely. This phenomenon can be explained by (i) incomplete knockdown of DR5 in the cell surface receptor level, (ii) change of TRAIL receptor responsiveness by indomethacin/TRAIL treatment. It has been reported that basal TRAIL death receptor expression may not correlate with TRAIL sensitivity (Wagner et al., 2007). In this respect, enhancing effects targeting on TRAIL receptor modification such as O-glycosylation or other TRAIL-DISC components such as FADD, caspase-8 and FLIP are needed to be considered and further investigated in future. We found that ROS plays a critical role in the expression of DR5 induced by indomethacin. Interestingly, overexpression of catalase gave a lesser suppressive effect on DR5 promoter activity as compared to SOD1 overexpression (Figure 4e). This phenomenon can be explained by the following: (i) overexpression of catalase alone induced slight upregulation of DR5 promoter activity, (ii) superoxide generation may be more critical for CHOP/DR5 induction by indomethacin. Further investigation is needed to clarify the individual role of superoxide and hydrogen peroxide in indomethacin-induced ER stress and CHOP upregulation. CHOP is a typical ER stress–regulated protein that is involved in ER stress–induced apoptosis. Previous studies reported that indomethacin-induced ROS production and CHOP induction are involved in its apoptotic and anti-inflammatory activities (Tsutsumi et al., 2004; Okamura et al., 2008; Franceschelli et al., 2011). Our data indicate that indomethacin can trigger ER stress in melanoma cells. The mechanism underlying indomethacin-induced CHOP upregulation via the ROS pathway during ER stress needs further investigation. In previous studies, it has been suggested that induction of DR5 can be mediated through the p53 intronic binding site in DR5 promoter (Wu et al., 1997; Takimoto and El-Deiry, 2000; Burns et al., 2001). However, we found that DR5 was induced by indomethacin through a p53-independent mechanism, as determined using HCT116 p53 knockout cells (Supplementary Figure S3b online). Indeed, indomethacin did not upregulate, but rather slightly downregulated, the expression of p53 (Supplementary Figure S3b online). Moreover, indomethacin was able to induce DR5 expression in p53-mutated MeWo melanoma cells (Zölzer et al., 1995) (Figure 6b), p53-deleted cells (SKOV3), and in various p53-mutated (BxPC-3, OVCAR3, MDA-MB-231, HT-29) cell lines (Supplementary Table S1 online shows p53 status of cell lines used in this study), further suggesting that p53 is not involved in indomethacin-induced DR5 upregulation.

Inhibitors of apoptosis proteins (IAPs) such as XIAP, cIAP-1, cIAP-2, and survivin are important determinants of TRAIL-induced apoptosis in mitochondria-dependent type II cancer (Zhang and Fang, 2004). In this study, we found that indomethacin inhibited survivin expression (Figure 5a), and this contributed to the indomethacin-stimulated apoptotic effect of TRAIL (Figure 5b). Our data showed that transient transfection of Flag-tagged survivin only partially protected against indomethacin/TRAIL-induced PARP-1 cleavage (Figure 5b) and caspase-3 activity (Supplementary Figure S5b online). Further studies by stably overexpression of survivin in A375 cells are needed to confirm whether survivin can completely protect the indomethacin-induced TRAIL enhancement effect. It has been shown that downregulation of survivin by chemotherapeutic agents can be abolished by the antioxidant NAC (Prasad et al., 2010, 2011; Sung et al., 2012). Interestingly, we found that the effect of indomethacin on survivin was mediated through ROS and that NAC reversed the downregulation (Figure 5c). Indomethacin has been reported to reduce survivin expression in gastric mucosal and epithelial cells through proteasome degradation (Chiou et al., 2005; Chiou and Mandayam, 2007). Consistent with these findings, we found that survivin downregulation occurred at both
protein (Figure 5a) and mRNA levels (data not shown) in A375 melanoma cells. Further studies are, however, needed to determine the possible involvement of proteasome degradation of survivin by indomethacin in melanoma cells.

Many reports have shown that indomethacin can inhibit NF-kB activity (Takada et al., 2004; Preciado et al., 2005; Shen et al., 2007). Consistent with previous findings, in our system we found that indomethacin inhibited p65 nuclear translocation in A375 melanoma cells (Figure 5d). We also showed that survivin protein expression was downregulated by indomethacin treatment through inhibition of the NF-kB signaling pathways, in which the introduction of ectopic p65 restored the protein levels of survivin to almost basal levels in indomethacin-treated cells (Figure 5e). The detailed mechanism of how indomethacin inhibits NF-kB is still not fully understood. Using the NF-kB luciferase reporter assay, we determined that the NF-kB inhibitory effect by indomethacin was not caused by ROS signaling (Figure 5f). Collectively, the results of this study reveal that indomethacin-induced ROS generation and NF-kB inhibition mediate survivin downregulation by indomethacin and contribute to enhancement of TRAIL responses. Recently, many studies have shown sensitization effects of melanoma cells for TRAIL-induced apoptosis with different mechanisms including phosphorylation and activation of Bax (Berger et al., 2013a), RAF inhibition (Berger et al., 2013b), enhancement of both extrinsic and intrinsic apoptosis pathways (Berger et al., 2011), and SMAC release (second mitochondria-derived activator of caspases) (Quast et al., 2012). Moreover, NASIDs other than indomethacin such as acetylsalicylic acid, sodium salicylate, diclofenac, and hyaluronic acid have been shown to enhance TRAIL-induced apoptosis in skin-related cancer through downregulation of c-FLIP (Braun et al., 2012; Fecker et al., 2012). In this study, we found that NASID indomethacin enhanced DR5 expression and increased TRAIL-induced apoptosis. Therefore, it is interesting to investigate whether a combination of these TRAIL-sensitizing agents would provide further dramatic effects on TRAIL-induced apoptosis in melanoma cells. Moreover, we observed that indomethacin treatment caused a marginal decrease in the c-FLIP expression level (Figure 5a). As previous reports showed that downregulation of c-FLIP is sufficient to sensitize melanoma cells to TRAIL (Geserick et al., 2008; Braun et al., 2012; Fecker et al., 2012), it is possible that this minor reduction of c-FLIP level by indomethacin may also influence the TRAIL responses. More prudent experiments will be needed to investigate the role of c-FLIP in the TRAIL-sensitizing activity by indomethacin. We also found that indomethacin showed a basal cytotoxic effect in human melanocytes but no increase in both DR5 level and sensitivity to cell death in the presence of TRAIL (Supplementary Figure S7 online). Thus, it is interesting to determine the effects of indomethacin in the ROS–CHOP pathway in human melanocytes and whether a higher dose of indomethacin is needed to increase the DR5 levels of human melanocytes in future.

In conclusion, we show that indomethacin can effectively restore TRAIL sensitivity in melanoma cells by either: (1) upregulation of DR5 and/or (2) downregulation of survivin, a well-known inhibitor of apoptosis. Furthermore, ROS generated by indomethacin plays a critical role in CHOP-mediated DR5 upregulation, and ROS and NF-kB signaling pathways are important for the downregulation of survivin protein expression. As indomethacin is used clinically, animal studies are warranted to explore the combined effects of indomethacin and TRAIL, with the goal of providing safe and effective treatment regimens for melanoma.

**MATERIALS AND METHODS**

**Western blot analysis**

Whole-cell protein and nuclear lysates were prepared and analyzed by western blotting as described previously (Tse et al., 2005).

**Analysis of cell surface DR5**

Cells were detached with 0.5 mM EDTA and then stained with phycoerythrin-conjugated mouse monoclonal anti-human DR5 or isotype control, and incubated for 60 minutes at 4 °C. Surface expression of DR5 was determined by flow cytometry. Phycoerythrin-conjugated mouse anti-human DR5 (DJR2-4) and phycoerythrin-mouse IgG1 isotype control (MOPC-21) were purchased from BioLegend (San Diego, CA).

**MTT cytotoxicity assay and crystal violet staining**

Cytotoxicity was determined by the MTT uptake method (Yang et al., 2011). For crystal violet staining assay, cells (1 × 10^5) were seeded in 60 mm dishes, and then untreated or pretreated with indomethacin for 24 hours, washed with phosphate-buffered saline, and then treated with TRAIL. After 5 days, the cells were stained with crystal violet.

**siRNA treatment**

Silencing of DR4, DR5, or CHOP was achieved by transfecting siRNAs (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Gene silencing effect was evaluated by western blot analysis.

**Construction of plasmids, transient transfection, and luciferase activity assay**

All DR5 luciferase promoter regions were cloned into pG3-Luc vector. The NF-kB luciferase reporter (pNF-kB-Luc) was obtained from Clontech (Mountain View, CA). SOD1, catalase, and survivin full-length expression constructs were synthesized by PCR using A375 total mRNA and subcloned into the pcDNA3-Flag tagged vector. The plasmid transfection and luciferase assay were the same as described previously (Tse et al., 2005). Firefly and renilla luciferase activities were assayed according to the manufacturer’s protocol (Promega, Madison, WI). Firefly luciferase activity was normalized to renilla luciferase activity in cell lysate and expressed as an average of three independent experiments.

**Measurement of reactive oxygen species**

Cells were plated in black 96-well plates and allowed to attach for 24 hours. Then cells were loaded with fluorescent dyes, 6-Carboxy-2’,7’-dichlorofluorescein diacetate, or dihydroethidium, and further stimulated with 300 μM indomethacin with or without the pretreatment of 5 mM NAC for 1 hour. Fluorescence was measured using a fluorescence microplate reader (EnVision Multilabel Reader, PerkinElmer, Waltham, MA) at the indicated time point at 37 °C.
Asp-Glu-Val-Asp-ase (DEVDase) and Ile-Glu-Thr-Asp-ase (IETDase) activity assays
DEV Dase (caspase-3) or IETDase (caspase-8) activities were examined according to the protocol provided by caspase colorimetric quantipak assay kit (Enzo Life Science, Farmingdale, NY).

Annexin V/PI assay
The indicator of cell death and apoptosis was detected by using annexin V/PI binding kit (Abcam, Cambridge, MA). Briefly, A375 cells were pretreated with 300 μM indomethacin for 24 hours followed by treatment with TRAIL (25 ng/ml⁻¹ for A375 and 50 ng/ml⁻¹ for MeWo/5K-MEL-5) for 24 hours. Then, cells were trypsinized, stained with annexin V/PI, and then analyzed with flow cytometer.

Statistical analyses
All data are expressed as mean ± SD of three independent experiments. Statistical significance was determined using unpaired Student’s t-test and a P-value of less than 0.05 was considered statistically significant.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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