IAP antagonization promotes inflammatory destruction of vascular endothelium

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Abstract

In this study, we show for the first time that the therapeutic antagonization of inhibitor of apoptosis proteins (IAPs) inhibits B16 melanoma growth by disrupting tumor vasculature. Specifically, the treatment of mice bearing B16 melanoma with an IAP antagonist compound A (Comp A) inhibits tumor growth not by inducing direct cytotoxicity against B16 cells but rather by a hitherto unrecognized antiangiogenic activity against tumor vessels. Our detailed analysis showed that Comp A treatment induces NF- κ B activity in B16 tumor cells and facilitates the production of TNF. In the presence of Comp A, endothelial cells (ECs) become highly susceptible to TNF and undergo apoptotic cell death. Accordingly, the antiangiogenic and growth-attenuating effects of Comp A treatment were completely abolished in TNF-R knockout mice. This novel targeting approach could be of clinical value in controlling pathological neoangiogenesis under inflammatory condition while sparing blood vessels under normal condition.

Keywords IAPs; tumor; TNF; angiogenesis

Subject Categories Autophagy & Cell Death; Cancer; Cell Adhesion, Polarity & Cytoskeleton

DOI 10.15252/embr.201439616 | Received 24 September 2014 | Revised 9 March 2015 | Accepted 10 March 2015 | Published online 30 March 2015

EMBO Reports (2015) 16: 719–727

Introduction

The inhibitor of apoptosis protein (IAP) gene family encodes a group of structurally related proteins that were initially identified based on their ability to inhibit cell death. Although the precise mechanism is still debated, initial evidence showed that IAP proteins can act as direct inhibitors of pro-apoptotic caspases [1]. Given their cytoprotective properties and elevated expression level in many types of human cancer, great effort has been made to develop small

pharmacological inhibitors of IAPs [2,3]. The application of such inhibitors has shed new light on the regulatory function of IAPs. In particular, IAP antagonists killed cancer cells primarily by permitting the degradation of cIAP1 and 2 [4,5] rather than by de-repressing caspase activity. The rapid degradation of cIAP1 and 2 activates both the canonical and non-canonical NF- κ B signaling pathway, which in some tumor cells leads to secretion of TNF and thereby induces autocrine TNF-R1 signaling. In the absence of c-IAPs, TNF-R1 stimulation leads to RIP1-mediated formation of a death-inducing signaling complex consisting of RIP1, FADD, and caspase-8, resulting in cell death.

The tumor stroma is increasingly recognized as an integral part of cancer initiation, growth, and progression. TNF plays a critical role in this context, by acting as an amplifier of the inflammatory milieu. Thus, TNF production/secretion upon IAP antagonization may not only induce autocrine TNF-R signaling within the tumor cells but also engage paracrine TNF-R signaling in adjacent tissues. Here, we show that the treatment of mice bearing B16 melanoma tumors by an IAP antagonist compound A (Comp A) [5] inhibited tumor growth not by promoting direct cytotoxicity in tumor cells but by disrupting the tumor vasculature. Upon exposure to Comp A, tumor cells produced an increasing amount of TNF into the micro-environment. The increased local concentration of TNF together with Comp A induced endothelial cell death leading to attenuated B16 melanoma growth.

Results

IAP antagonization inhibits growth of B16 tumor *in vivo*

Compounds inhibiting IAPs have recently been shown to restore TNF-dependent apoptosis in melanoma cancer cells and xenografted NUDE mice [6–8]. Expanding on these findings, we first showed that the IAP antagonist Comp A inhibited tumor growth in immune-competent mice bearing B16 melanoma tumors. In these studies, matrigel containing Comp A or vehicle was mixed with B16 cells

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and subcutaneously injected into the flank region of C57/Bl6 mice [9]. In this syngeneic mouse melanoma model, tumor growth was significantly attenuated in the presence of Comp A compared to vehicle-treated controls (Figs 1A and EV1A). Surprisingly, histological analysis of B16 tumors exposed to Comp A showed no evidence of attenuated tumor cell proliferation (Ki-67) or apoptosis (active caspase-3) (Figs 1B and EV1B). Similarly, *in vitro* analyses showed that Comp A did not induce cytotoxicity or attenuate the proliferation rate of B16 tumor cells either alone or in conjunction with TNF (Figs 1C and EV1C). These data indicate that B16 melanoma cells belong to a cohort of tumor cells that resist the cytotoxic action of IAP antagonization as previously described [10]. Independently, IAP antagonization was shown to activate both the canonical and non-canonical NF- κ B signaling pathways as indicated by I κ B phosphorylation/degradation and NIK stabilization/NF- κ B2 p100 processing, respectively [4,5]. Accordingly, Comp A induced non-canonical NF- κ B activity in B16 cells demonstrated by the stabilization of NIK and NF- κ B2 processing of p100 yielding the active nuclear p52 (Fig 1D). Comp A-induced canonical NF- κ B activity in B16 cells was only slightly increased (Fig EV1D). Consistent with increased NF- κ B activity, TNF was transcriptionally upregulated (Fig 1E), and increasing amounts of TNF were secreted (Fig 1F) when B16 cells were exposed to Comp A. We therefore hypothesized that the discrepancy between attenuated B16 melanoma growth *in vivo* and B16 cell resistance *in vitro* could indicate that the tumor microenvironment rather than the tumor cells represents the primary target of Comp A.

IAP antagonization inhibits B16 tumor vascularization *in vivo*

The tumor microenvironment is made up of different components including blood vessels, tumor-infiltrating immune cells, and cytokines that affect tumor growth and progression [11]. In vehicle-treated B16 control tumor, immunofluorescence microscopy of histological sections revealed CD31-positive endothelial cells (ECs) 12 days after tumor cell implantation (Fig 2A and B) and establishment of tumor vasculature as demonstrated by multiphoton microscopy (Fig 2C). In contrast, B16 melanoma exposed to Comp A contained significantly fewer CD31-positive ECs and failed to establish a vascular network. The lack of CD31-positive cells on day 5 in vehicle-treated tumors (Fig 2A and B) indicated that the angiogenic switch had occurred between day 5 and 12 coinciding with exponential tumor growth (Fig 1A). Accordingly, the treatment of established B16 melanoma with Comp A 7 or 10 days post-implantation significantly attenuated tumor growth (Fig 2D) and reduced

tumor vascularization (Fig 2E and Movies EV1, EV2 and EV3). Importantly, Comp A treatment had no effect on lymphatic ECs or immune infiltration of B16 tumors for a period of up to 12 days after transplantation (Fig EV2). Together, these findings suggest that, rather than targeting the tumor cells *per se*, IAP antagonization attenuated growth of B16 melanoma *in vivo* by specifically targeting the tumor blood supply.

IAP antagonization promotes TNF-induced endothelial cell death

To better define the effects of IAP antagonization on vascular ECs, we next examined the cytotoxic activity of Comp A or an inactive control Comp B on cultured human umbilical vein ECs (HUVEC) and human dermal microvascular ECs (HDMEC). Unlike B16 tumor cells that were resistant to Comp A (Figs 1C and EV1C), both HUVEC and HDMEC were susceptible to Comp A, but only when co-treated with TNF (Figs 3A and EV3A). Indeed, treatment of ECs with Comp A in conjunction with TNF resulted in the depletion of cIAP1 and 2 and was associated with activation of executioner caspase-3 and PARP processing (Figs 3B and C, and EV3B). Previous data showed that genetic or pharmacological inhibition of cIAPs leads to RIP1-dependent apoptosis in response to TNF. By contrast, when caspase activity was inhibited, TNFR1 ligation resulted (in some cell types) in programmed necrosis [12]. Our data showed that EC death could predominantly be reduced by blocking caspase activity (zVAD treatment) but not by necrostatin-1 (Nec-1), an inhibitor of caspase-independent programmed necrosis (necroptosis) (Fig 3D) indicating that Comp A mainly promoted caspase-dependent TNF-induced apoptotic cell death in ECs. Accordingly, TNF-induced cell death was almost completely diminished when cells were treated with both zVAD and Nec-1 simultaneously (Fig 3D).

Although its precise function in angiogenesis remains controversial, TNF has been shown to modulate angiogenesis by inducing the expression of pro-angiogenic genes [13]. To investigate whether Comp A treatment interfered with the TNF-mediated transcriptional regulation of pro-angiogenic growth factors, we examined the expression levels of several angiogenic growth factors in ECs exposed to TNF in the presence or absence of Comp A. While our data confirmed the reported upregulation of VEGF-A, VEGF-C, and VCAM-1 in response to TNF (Fig EV3C), the expression levels were not significantly affected by Comp A co-treatment. Notably, Comp A alone failed to induce NF- κ B activation in ECs and only partially attenuated TNF-induced canonical NF- κ B activation (Fig 3E). These findings suggest that IAP antagonization in conjunction with

Figure 1. Comp A inhibits B16 melanoma growth *in vivo*.

- A Growth rates of B16 tumors mixed with matrigel and vehicle (control) or Comp A (2 μ M) and subcutaneously injected into the flank region of recipient mice. Tumor size was measured in 2 dimensions, and the calculated volume was recorded daily. Data points represent the mean $\pm$ SEM.
- B Histological tumor analysis on day 5 stained for Ki-67 or cleaved (cl.) caspase-3 antibody (red); nuclei were counterstained with DAPI (blue).
- C Cell viability of B16 cells treated with increasing amounts of Comp A alone or in combination with TNF (20 ng/ml) after 48 h. Error bars show SEM for $n \geq 4$.
- D Non-canonical NF- κ B activation in B16 cells was measured either by Western blotting using specific anti-NIK and anti-NF- κ B2 antibodies or by NF- κ B p52 ELISA after treatment with Comp A (2 μ M). Error bars show SEM for $n = 3$.
- E qRT-PCR analysis of TNF in B16 cells after treatment with Comp A (2 μ M) using specific primers for TNF and GAPDH. Error bars show SEM for $n \geq 3$.
- F Analysis of TNF secretion by TNF ELISA after treatment with Comp A (2 μ M). Data represent the mean $\pm$ SEM. Error bars show SEM for $n = 3$.

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-sided unpaired Student's *t*-test.

Source data are available online for this figure.

Figure 1.

Figure 2. Comp A inhibits tumor vasculature *in vivo*.

A B16 cells were mixed with matrigel and vehicle (control) or Comp A (2 μ M) and subcutaneously injected into the flank region of recipient mice. ECs were stained using a CD31 antibody (red) on day 5 or 12 post-implantation. Nuclei were counterstained with DAPI (blue).

B Intratumoral and peritumoral CD31-positive vascular structures of (A) were examined according to the intensity of CD31-specific staining.

C Intratumoral vasculature of tumors at day 12 (A) was analyzed by multiphoton microscopy using FITC-dextran staining of vessels.

D B16 cells were mixed with matrigel and subcutaneously injected into the flank region of recipient mice. After 7 or 10 days, tumors were treated with Comp A by subcutaneous injection next to the tumors. Tumor size was measured in 2 dimensions, and calculated volume was recorded daily. Data points represent the mean $\pm$ SEM.

E Intratumoral vasculature of tumors at day 12 (D) was analyzed by multiphoton microscopy using FITC-dextran staining of vessels.

Data information: * $P < 0.05$, ** $P < 0.01$, two-sided unpaired Student's *t*-test.

Figure 3.

Figure 3. Comp A promotes TNF-induced endothelial cell death.

- A HUVEC and HDMEC cells were treated with increasing amounts of Comp A or Comp B alone or in combination with TNF (20 ng/ml). Cell viability was measured after 48 h. Error bars show SEM for $n \geq 4$.
- B Whole-cell extracts of HUVEC cells were analyzed by Western blotting with primary antibodies for actin, cIAP1, cIAP2, and XIAP.
- C Caspase-3 processing and PARP cleavage were analyzed by Western blotting in HUVEC cell lysates with primary antibodies for actin, caspase-3, and PARP.
- D HUVEC cells were treated with TNF (20 ng/ml), Comp A (2 μ M), or both. Cell viability was measured after co-treatment with zVAD (30 μ M), Nec-1 (30 μ M), or both for 48 h. Error bars show SEM for $n = 3$.
- E Canonical NF- κ B activity was measured in HUVEC cells either by NF- κ B p65 ELISA or Western blotting using specific anti-phospho (ph) I κ B and anti-I κ B antibodies after treatment with Comp A (2 μ M) and TNF (20 ng/ml). Error bars show SEM for $n \geq 3$.
- F HUVEC cells were seeded on matrigel containing Comp A, TNF, or both, and capillary-like network was analyzed after 48 h with a bright-field microscope (developing network, upper panel). The established capillary-like network (lower panel) was exposed to Comp A, TNF, or both for 48 h and analyzed.
- G Staining of tubulin and cleaved (cl.) caspase-3 in EC capillary-like network (F) after treatments as indicated.
- H Analysis of the capillary-like network (F) with or without zVAD (30 μ M) treatment.

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-sided unpaired Student's *t*-test.

Source data are available online for this figure.

TNF-R signaling resulted in EC apoptosis rather than altering the TNF-induced transcriptional upregulation of pro-angiogenic growth factors.

To investigate whether Comp A in conjunction with TNF disturbed EC function, we conducted a tube formation assay. Both, controls and Comp A-treated HUVEC seeded on a layer of basement membrane (matrigel), formed extensive capillary networks within 48 h, indicating that normal EC function was not impaired by Comp A treatment alone (Fig 3F, upper panel). By contrast, network formation was inhibited on matrigel containing both, Comp A and TNF (Fig 3F, upper panel, developing network), but was unaffected in the presence of control Comp B (Fig EV3D). Similarly, when established capillary tubes were treated with TNF alone or with either Comp A or Comp B, only the combination of Comp A and TNF disrupted the endothelial network (Figs 3F and EV3D, lower panel, established network). Notably, disruption of the endothelial network by Comp A and TNF was associated with caspase-3 activation (Fig 3G), and inhibition of caspase activity by zVAD efficiently protected the capillary networks (Fig 3H). Together, these *in vitro* results corroborate the *in vivo* observations summarized in Figs 1 and 2 and point at a direct cytotoxic effect of Comp A toward ECs exposed to TNF.

IAP antagonization potentiates TNF-induced vascular disruption *in vivo*

To verify the *in vivo* TNF dependency of EC death in response to IAP antagonization, we implanted matrigel plugs supplemented with angiogenic growth factors and Comp A with or without TNF (but lacking tumor cells) into the flank of recipient mice. Perfused blood vessels growing into the matrigel plugs were then visualized by high molecular weight fluorescent FITC-dextran (2,000 kDa)-injected into the tail vein of these mice or by H&E staining (Fig 4A).

Strikingly, only matrigel plugs containing Comp A in combination with TNF showed a significant reduction in angiogenesis, whereas matrigel containing either Comp A or TNF was normally vascularized. This is in contrast to our initial observation that Comp A alone was sufficient to reduce tumor vascularization in matrigel plugs containing B16 tumor cells (Fig 2). These findings suggest that the tumor microenvironment including B16 tumor cells acts as a source of TNF (Fig 1) that is sufficient to induce EC death when combined with IAP antagonization.

To further confirm the critical role of TNF-R signaling during tumor growth and angiogenesis, we made use of TNF-receptor knockout mice lacking both TNF-R1 and R2 (TNF-R1/2^{-/-}) [14]. Strikingly, in contrast to wild-type mice, matrigel containing Comp A in combination with TNF showed no reduction in angiogenesis (Fig 4B) demonstrating that the anti-angiogenic effects observed upon Comp A treatment were dependent on TNF-R signaling.

Our data, summarized in Fig 1, demonstrate that B16 cells act as a source of TNF. In analogy to our initial experiment, matrigel plugs containing B16 tumor cells with or without Comp A were implanted into TNF-R1/2^{-/-} mice. In contrast to the growth-attenuating effect of Comp A in wild-type mice (Fig 1A), Comp A had no effect on B16 melanoma growth rates in the absence of TNF-R signaling (Fig 4C). Nor did Comp A adversely affect tumor vascularization (Fig EV4). These findings confirm that IAP antagonization has the capacity to promote TNF-dependent EC death in the B16 melanoma micro-environment.

Discussion

An increasing number of clinical trials are currently evaluating the use of different IAP antagonists as anticancer therapeutics [3]. Based on the evidence obtained over the last decade, IAP antagonists are

Figure 4. Comp A potentiates TNF-dependent vascular disruption *in vivo*.

- A, B Matrigel was mixed with vehicle (control), Comp A (2 μ M), or TNF (50 ng/ml) and subcutaneously injected into the flank region of recipient wild-type mice (A) or TNFR-1/2^{-/-} mice (B). Photography of plug after 12 days (first panel). Histological analysis was performed on day 12 by H&E staining (second panel). FITC-dextran is shown in green (Z-stacks, third and fourth panel).
- C B16 cells were mixed with matrigel and vehicle (control) or Comp A (2 μ M) and subcutaneously injected into the flank region of recipient TNFR-1/2^{-/-} mice. Tumor size was measured in 2 dimensions, and calculated volume was recorded daily (left panel). Data points represent the mean $\pm$ SEM. Histological analysis was performed on day 12 by H&E staining (right panel).

Figure 4.

thought to be more efficacious in patients whose tumors produce large quantities of inflammatory cytokines including TNF [15]. Accordingly, the cytotoxic activities of IAP antagonists were attenuated by TNF-blocking agents [16]. *In vitro* treatment of some cancer cells including melanoma cells could resist the cytotoxic activity of IAP antagonists even in the presence of exogenous TNF. However, *in vivo* tumor growth was efficiently inhibited by the IAP antagonist birinapant [6]. These findings indicated the critical involvement of the microenvironmental cues in shaping the specific response to IAP antagonization. Our results demonstrate for the first time that IAP antagonization induced the disruption of B16 tumor vasculature through TNF-mediated EC apoptosis. In line with these observations, the combined inactivation of *clap1* and *clap2* or *clap1* and *Xiap* is lethal as a result of cardiovascular defects, whereas single IAP knockout mice exhibited relatively subtle phenotype [17]. Also in zebrafish, knockout of the only c-IAP gene [18] leads to severe hemorrhage and vascular regression during development. Together, these data point to a central role of IAPs in EC homeostasis and function.

Two key components of tumor stroma, infiltrating immune cells and endothelial vasculature, were scrutinized in our studies. While our data conclusively identified ECs as the main target of Comp A within the tumor microenvironment, no alteration of immune cell infiltration of B16 tumors could be determined. However, our data do not formally exclude a role of IAP antagonists in immune cell function [19]. In particular, tumor-infiltrating macrophages may well be affected by IAP antagonization as previously demonstrated [20–22]. Indeed, loss of IAPs in the myeloid compartment results in up-regulation of many pro-inflammatory cytokines including TNF [23]. Thus, Comp A could stimulate the production of inflammatory cytokines by tumor-infiltrating immune cells and induce the cytotoxicity toward adjacent tissues including ECs.

In conclusion, we have provided evidence exposing a previously unrecognized antiangiogenic activity of a pan-IAP antagonist in the presence of elevated TNF levels under inflammatory conditions within the tumor microenvironment. Furthermore, compared to normal vessels, tumor vessels are considered to be more responsive to TNF based on their elevated TNF-R expression [24]. Thus, the elevated local concentration of TNF together with the improved TNF responsiveness of tumor vessels provides a favorable condition for the antiangiogenic activity of IAP antagonists to control tumor-associated pathological angiogenesis while minimizing collateral damage to normal blood vessels.

Materials and Methods

EC assays and IAP antagonists

Cell lines and culturing conditions were described previously [9]. *In vitro* matrigel tube formation assay was performed in 12-well plates and analyzed by an inverted-phase contrast microscope. The pan-IAP antagonists Comp A and B were obtained from Tetra-Logic Pharmaceuticals (Malvern, PA, USA).

Animal tumor models

For the tumor matrigel plug assay, 400 μ l of chilled matrigel was mixed with 1×10^6 B16-F1 tumor cells and Comp A (2 μ M final

concentration). Treatment of established tumors was performed by sequential subcutaneously injecting Comp A (2 μ M, 50 μ l) next to the tumors starting at day 7 ($\times 3$) or 10 ($\times 2$) post-implantation.

qRT-PCR

Total RNA was isolated using the standard phenol/chloroform method and analyzed using a 96-well plate Multicolor Real-Time PCR Detection System (CFX96 Touch™, Bio-Rad). Data were further evaluated using the standard Pfaffl method [25].

ELISA assays

NF- κ B ELISAs were performed using the TransAM NF- κ B p65 or p52 Kit (Active Motif) [26]. TNF ELISAs were performed using the Mouse TNF-alpha HIGH Sensitivity ELISA (eBioscience).

Further details are provided in the Appendix Supplementary Methods.

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Acknowledgements

We thank Prof. M. Uhlirova (Cologne, Germany) for providing Leica M 165 FC fluorescent stereomicroscope. We thank Diana Wagner-Stippich and Jan Zamek for technical assistance. This work was supported by grants from the DFG (KA 2853/4-1 Grant to HK and CM), SFB832, SFB670, and Köln Fortune Program 302/2014, University of Cologne (grant to HK).

Author contributions

AW carried out the majority of experiments. JMS, OC, PB, MA, SS, and KB performed the independent experimental replicates. PZ performed the IHC. CJ and ACS performed the multiphoton microscopy. DW, PK, MK, and CM provided essential tools. HK conceived the project.

Conflict of interest

The authors declare that they have no conflict of interest.

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