Regulation of Inflammation by the NF-κB Pathway in Ovarian Cancer Stem Cells

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Problem

The NFκB pathway is a major source of pro-inflammatory cytokines, which may contribute to cancer chemoresistance. We showed that constitutive NFκB activity is characteristic of the ovarian cancer stem cells (OCSCs). The aim of this study is to determine whether the inhibition of NFκB by Eriocalyxin B (EriB) in the OCSCs may induce cell death in otherwise chemoresistant cells.

Methods

OCSCs and mature ovarian cancer cells (mOCCs) were treated with increasing concentrations of EriB. Cell viability was measured using the Celltiter 96 assay, and caspase activity was quantified using Caspase-Glo™ assay. Cytokine levels were quantified using xMAP technology.

Results

EriB decreased the percent of viable cells in all cultures tested with GI_{50} of 0.5–1 μM after 48 hrs of treatment. The intracellular changes associated with EriB-induced cell death are: (i) inhibition of NF-κB activity; (ii) decreased cytokine production; (iii) activation of caspases; and (iv) down-regulation of XIAP. In addition, EriB is able to sensitize OCSCs to TNFα and FasL-mediated cell death.

Conclusion

Inhibition of the NFκB pathway induces cell death in the OCSCs. Because the OCSCs may represent the source of recurrence and chemoresistance, the use of NFκB inhibitors like EriB may prevent recurrence in patients with ovarian cancer.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal of all gynecologic malignancies. In 2009, it was estimated that 21,550 new cases were to be diagnosed and 14,600 deaths will result from this disease.[1] Newly diagnosed patients with ovarian cancer usually respond to surgery and chemotherapy but more than 80% of these responders eventually recur with chemo-resistant disease.[2,3] Thus, in EOC, the source of high mortality is disease recurrence. Unfortunately, the source of recurrence is unknown and therapies that can prevent recurrent disease are currently lacking.

Clinical and epidemiologic studies have suggested a strong association between chronic inflammation and cancer.[4] Chronic inflammation has been shown to play a critical role in initiating, sustaining, and advancing the growth of several cancers, including EOC.[5,6] A key molecular link between inflammation

and cancer is the NF-κB pathway. NF-κB controls many of the properties of cancer cells by regulating the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, and suppression of apoptosis. Therefore, specific inhibition of NF-κB has been suggested as a potential therapeutic target.

Growing number of scientific evidence suggests that the tumor represents a heterogeneous population of cells where a specific subgroup, the cancer stem cells (CSCs), has the potential to recreate the original tumor.[7] Our group recently reported the identification and characterization of the ovarian cancer stem cells (OCSCs) using the cell surface marker CD44.[8,9] These cells are chemoresistant and have the potential to recreate the original patient tumor in animal models. Thus, this cell population may have the capacity to survive treatment, rebuild the tumor, and initiate recurrence.

A major characteristic of the CD44^{+} OCSCs is the occurrence of a constitutive NF-κB pathway, which can be enhanced by ligation of Toll-like Receptor 4 (TLR4) and Tumor Necrosis Factor α (TNFα) receptor.[5,10,11] In this study, we tested the hypothesis that the inhibition of the NF-κB pathway may have a significant effect on the OCSCs. We used the compound Eriocalyxin B (EriB), which is an analog of oridonin, a natural ent-kaurene diterpene compound purified from *Isodon ericalyx var*. This natural product has been widely used in Chinese medicine as an anti-inflammatory and antibacterial agent.[12,13] Recent studies have shown that EriB has anti-tumoral effects in models of acute myeloid leukemia and has significant inhibitory effect on cell growth in several cancer cell lines.[12]

In our study, we demonstrate that EriB can inhibit both the constitutive and TNFα-induced NF-κB activation in the OCSCs. More importantly, we demonstrate that the inhibition of the NF-κB pathway promotes apoptosis in these cells. These findings suggest that inhibition of the NF-κB pathway may be an approach to prevent OCSC survival and therefore prevent ovarian cancer recurrence.

Materials and methods

Cell Lines and Culture Conditions

Ovarian cancer cells were isolated from malignant ovarian ascites or from ovarian tumors as previously described.[14,15] All patients signed consent forms, and the use of patient samples was approved under Yale University's Human Investigations Committee (HIC #10425). OCSCs and CD44^{−}/mature ovarian cancer cells (mOCCs) were isolated from these primary cultures and cultured as previously described.[16]

Growth Curves and Cell Viability Assay

Cells (4500 cells/well for OCSCs and 8000 cells/well for mOCCs) were plated in a 96-well plate. After 24 hrs, the medium was replaced with OptiMem (Gibco, Invitrogen, Carlsbad, CA, USA) for 4 hr followed by treatment. Growth curves were determined by placing treatment plates in the Incucyte system (Essen Instruments, Ann Arbor, MI, USA), where the growth curves are constructed from confluence measurements acquired during round-the-clock kinetic imaging. Cell viability was determined using CellTiter 96 Assay (Promega, Madison, WI, USA).

Protein Preparation and Cellular Fractionation

Protein was extracted and measured as previously described (ref). Cytoplasmic and nuclear fractions were separated using the NE-PER Nuclear and Cytoplasmic Extraction (Pierce Biotechnology, Inc., Rockford, IL, USA) according to manufacturer's instructions.

Caspase-3/7, -8, and -9 Activity Assay

The activities of caspases-3/7, -8, and -9 were measured using Caspase-Glo™ 3/7, 8, and 9 (Promega), respectively, as previously described.[17]

Mitochondrial Depolarization Assay

After treatment, cells were trypsinized and stained with JC-1 dye using the Mitocapture™ mitochondrial apoptosis detection kit (BioVision Research Products, Mountain View, CA, USA) according to the manufacturer's instructions. Data was gathered using the FACS Calibur System and analyzed using CellQuest software (BD Biosciences, Rockville, MD, USA).

SDS-PAGE and Western Blots

Gel electrophoresis and western blots were performed as previously described.[17]

Quantification of NF-kB Activity

NF-κB activity was measured using a luciferase reporter construct, pBII-LUC containing two κB sites before a Fos essential promoter (a gift from Dr S. Ghosh, Yale University). Cells were transfected, and luciferase activity measured as previously described.[10]

Quantification of Cytokines

Cytokine levels were measured from cell-free supernatants using Beadlyte® Human Multi-Cytokine Beadmaster™ kit according to the manufacturer's instructions. Data was acquired using the Bio-Rad 100 IS System (Bio-Rad, Hercules, CA, USA).

Immuohistochemistry

Cells were cultured in four wells chamber slides and fixed with 4% paraformaldehyde for 20 min at room temperature. Afterward, cells were stained with specific mouse monoclonal antibody for p65 as previously described.[18] Slides were then incubated with Alexa Fluor546 anti-mouse IgG and counterstained with Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA).

Results

EriB Induces Cell Death in EOC Cells

NF-κB has been shown to promote cell survival through the induction of target genes that can inhibit apoptosis.[19] Therefore, our first objective was to evaluate the effect of EriB on EOC cell survival. For this, we used a panel of both mOCCs and OCSCs isolated from either malignant ascites or ovarian cancer tumors.[15,16] The effect on cell viability was evaluated using the CellTiter 96 assay. EriB significantly reduced the number of viable cells in a dose- and time-dependent manner (Fig. 1a). Interestingly, in the OCSCs, the effect of EriB was density dependent, with increasing resistance as cell density increased (Fig. 1b).

EriB-induced Cell Death is Associated with Caspase Activation and Loss of Mitochondrial Membrane Potential

To determine whether the reduction in number of viable cells was caused by apoptosis, we evaluated cell morphology and caspase activity. As shown in Fig. 2a, cells treated with EriB showed typical morphology associated with apoptosis such as contracted cytoplasm, condensed chromatin, and the formation of apoptotic bodies. In addition, we found a significant increase in caspase 8, caspase 9, and caspase 3/7 activities in the treated cells in a dose-dependent manner (Fig. 2b).

As mentioned earlier, one of the most documented functions of NF-κB is its ability to promote cell survival through the induction of target genes, which inhibit the apoptotic machinery.[19] X-linked Inhibitor of Apoptosis Protein (XIAP) is an anti-apoptotic protein, which inhibits caspase 3 and caspase 9, and is highly expressed in EOC cells.[14,20] In addition, NF-κB has been shown to enhance the expression of XIAP.[21] Our next objective was to determine if EriB-induced cell death is associated with any changes in the levels of XIAP. We showed that EriB treatment significantly reduced the levels of XIAP in the EOC cells (Fig. 2c) and that the decrease in XIAP correlates with caspase activation.

Another well-characterized down-stream target of NF-κB is the anti-apoptotic protein Bcl$_2$, which functions to stabilize the mitochondria and therefore prevent the activation of caspases.[22,23] We next determined if EriB treatment has any effect on mitochondrial membrane integrity. We used the JC1 dye, which fluoresces bright red upon accumulation in intact mitochondria, but switches to green fluorescence once it is leaked into the cytoplasm upon the loss of mitochondrial membrane integrity.[24] Treatment with EriB for 12 hrs induced a significant shift in the JC1 dye fluorescence from red to green (Fig. 2d). This suggests that EriB-induced NF-κB inhibition alters the balance of proteins involved in the protection of the mitochondria leading to destabilization of the mitochondrial membrane and therefore triggering caspase-dependent cell death.

EriB Inhibits Constitutive NF-κB Activity and Constitutive Cytokine Production in the OCSCs

To further determine whether EriB's effect on OCSCs is associated with its capacity to inhibit NF-κB activity, we transfected OCSCs with a luciferase reporter linked to two NF-κB promoter elements.[10] Fig. 3a shows that EriB is able to inhibit baseline NF-κB activity in the OCSCs in a dose- and time-dependent manner. Low doses have a significant but transient effect in the first 2 hrs, but the higher
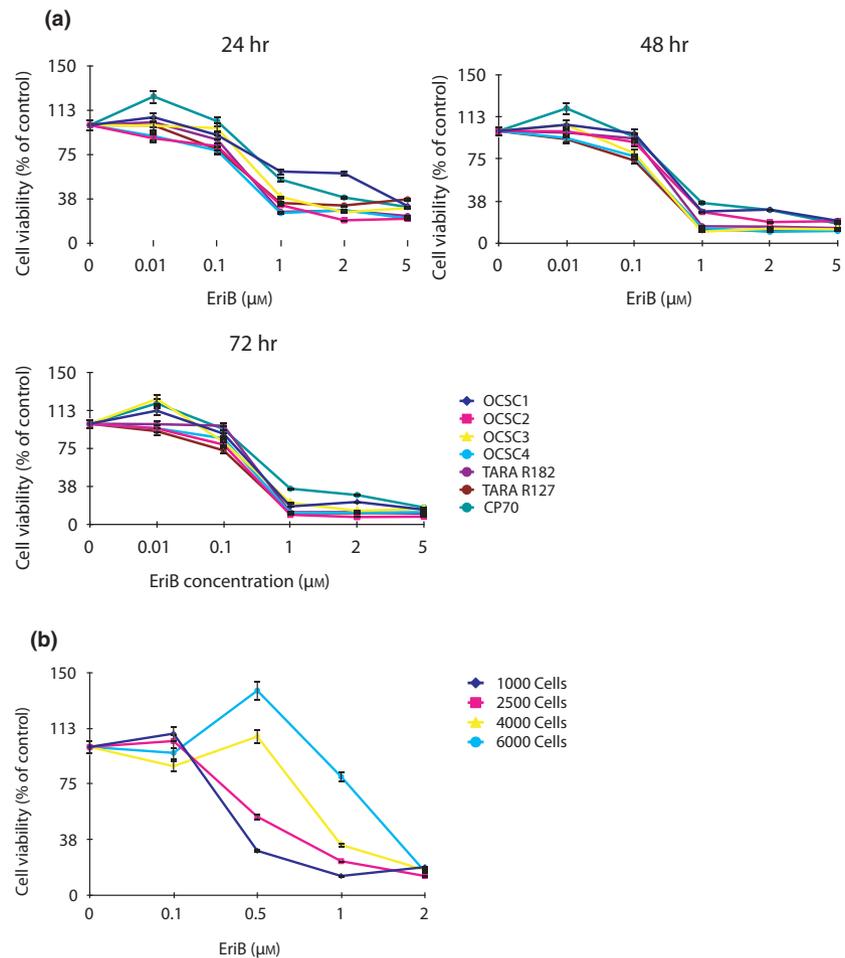
Fig. 1 Eriocalyxin B (EriB) induces cell death in epithelial ovarian cancer cells. A panel of ovarian cancer stem cells (OCSCs) and mOCCs were treated with increasing concentration of EriB. (a) Percentage of viable cells was quantified after 24, 48, and 72 hrs using Celltiter 96 assay; (b) OCSC1 was treated at different confluence and effect on viability determined after 48 hrs.

doses are able to maintain the inhibition for 12 hrs (Fig. 3a). These results confirm the specific effect of EriB on NF-κB. Moreover, this effect on NF-κB activity is reflected on the levels of secreted cytokines; when we evaluated the constitutive production of IL-6, MCP-1 and GRO-α in the OCSCs, we observed a significant decrease in these cytokines as early as 4 hrs after EriB treatment (Fig. 3b).

EriB Inhibits TNFα-induced NF-κB Activation and Cytokine Production in the OCSCs

So far we have shown that EriB can inhibit OCSCs baseline NF-κB activity. Our next objective was to determine if EriB could also inhibit induced NF-κB activity. TNFα is an endogenous stimuli known to induce NF-κB activity in numerous cell types.[25] We previously showed that instead of inducing cell death, TNFα is able to induce NF-κB activation and cytokine production in the OCSCs.[10] Thus, we pre-treated

OCSCs with increasing concentrations of EriB prior to TNFα treatment. As shown in Fig. 4a, TNFα alone (10 ng/mL) induced a significant increase in luciferase activity in a time-dependent manner, demonstrating NF-κB activation. This effect was blocked by pre-treatment with EriB even at the lower doses (1 μM). These results suggest that EriB can inhibit both constitutive and induced NF-κB activity.

To determine if the inhibition by EriB of TNFα-induced NF-κB activity translates to inhibition of cytokine production, the levels of IL-6, GRO-α, and MCP-1 were measured in OCSC supernatants. Similar to what was observed with the NF-κB luciferase assay, pre-treatment with EriB was able to down-regulate TNFα-induced increase in all these cytokines (Fig. 4b).

EriB Inhibits p65 Nuclear Translocation

To confirm the findings obtained with the luciferase reporter system and to determine where in the NF-κB
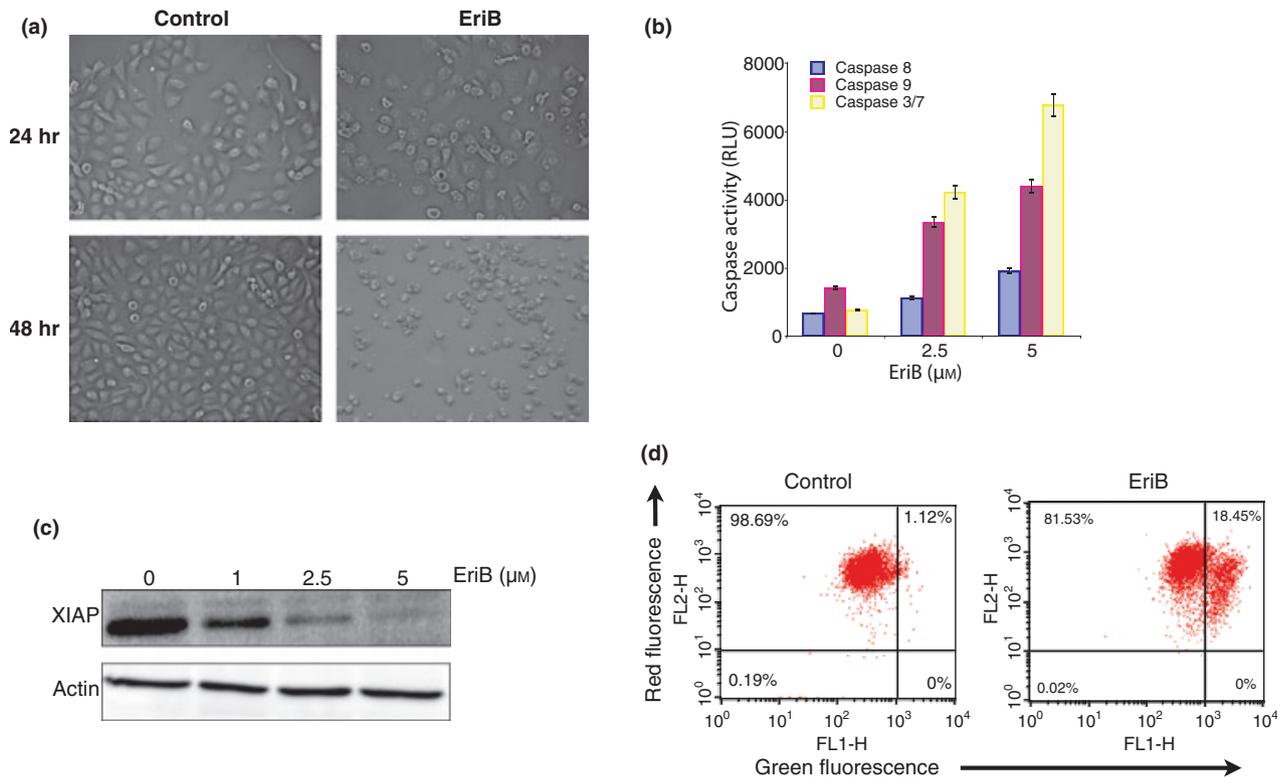
Fig. 2 Eriocalyxin B (EriB) induces apoptosis in epithelial ovarian cancer cells. A panel of ovarian cancer stem cells (OCSCs) and mOCCs were treated with increasing concentration of EriB for 24 hrs. (a) Effect on morphology was evaluated using light microscopy; (b) activity of caspases 8, 9, and 3/7 was quantified using Caspase Glo assay; (c) levels of XIAP was determined by western blot analysis; (d) effect on mitochondrial membrane stability was determined using the JC1 dye. Results shown are from OCSC1. Similar results were obtained with other cell cultures tested.

pathway EriB may exert its action, we evaluated the nuclear translocation of p65, which is a subunit of the NF-κB complex and a classical indicator of NF-κB activity. We evaluated both constitutive as well TNFα-induced p65 nuclear levels. We observed a decrease in the basal levels of nuclear p65 in the OCSCs after EriB treatment (Fig. 4c). In addition, EriB was also able to inhibit TNFα-induced increase in nuclear p65 (Fig. 4c). These results were further confirmed using immunocytochemistry where we observed a decrease in both constitutive and TNFα-induced nuclear p65 immuno-reactivity (Fig. 4d).

Our next objective was to determine whether the decrease in nuclear p65 is associated with changes in IκBα levels. It has been shown that following stimulation with a broad range of stimuli, such as TNFα, IκBα is phosphorylated by the IKK complex leading to the ubiquitination and proteosomal degradation of IκBα.[26,27] As shown in Fig. 4c, TNFα-induced p65 nuclear translocation corresponded with a significant

decrease on cytoplasmic IκBα. Interestingly, co-treatment with EriB prevented the TNFα-induced decrease in IκBα. This suggests that EriB may block p65 nuclear translocation by preventing the degradation of IκBα.

EriB is Able to Sensitize OCSCs to TNFα- and FasL-induced Cell Death

OCSCs are resistant to TNFα-induced cell death[14,28] and instead respond to TNFα treatment by upregulating NF-κB activity and enhancing cytokine production. Our next objective was to determine if the inhibition of NF-κB by EriB can sensitize OCSCs to TNFα-induced cell death. OCSCs were pre-treated with EriB prior to TNFα treatment, and the activation of the apoptotic pathway was determined by measuring caspase 3/7 activity and determining the effect on the mitochondrial membrane integrity. Our results show that TNFα alone was not able to induce
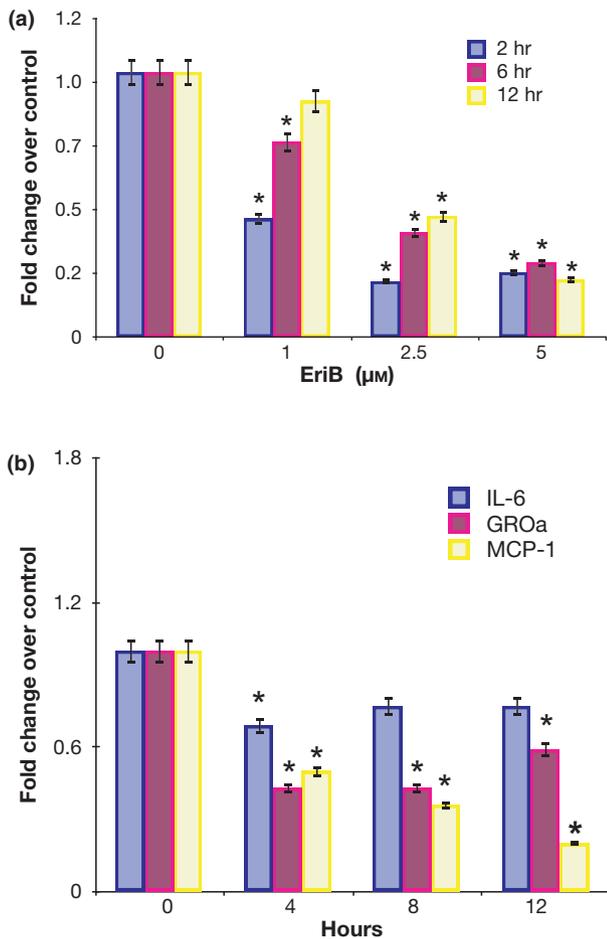
Fig. 3 Eriocalyxin B (EriB) inhibits constitutive NF-κB activity and constitutive cytokine production in the ovarian cancer stem cells (OCSCs). OCSCs were treated with increasing concentrations of EriB at the shown time points. (a) NF-κB activity was measured using a luciferase reporter system as described in the Methods section; (b) levels of IL-6, GRO-α, and MCP-1 were quantified using xMAP technology. Results shown are from OCSC1. Similar results were obtained with other OCSCs tested. *$P < 0.0001$.

the activation of caspase 3/7 in the OCSCs (Fig. 5a) nor was it able to destabilize the mitochondria (Fig. 5b). However, pre-treatment with EriB prior to TNFα significantly increased caspase 3/7 activity and also significantly induced a green shift in the JC1 dye (Fig. 5b) indicative of loss of mitochondrial membrane potential.

FasL is another pro-apoptotic receptor member of the TNFα family, and its function is regulated by the NF-κB pathway. We previously showed that EOCs in general are resistant to FasL-induced cell death.[14] To determine if EriB is also able to sensitize EOC cells to FasL-mediated apoptosis, OCSCs were pre-

treated with EriB followed by an anti-Fas monoclonal antibody which mimics FasL activity. Similar to what was observed with TNFα, anti-Fas alone was not able to induce caspase 3/7 activity in any of the cell cultures tested. However, pre-treatment with EriB was able to sensitize EOC cells to FasL-induced apoptosis (Fig. 5a). These results demonstrate that inhibition of NF-κB by EriB is able to reactivate both the TNFα and FasL apoptotic cascade in the OCSCs.

Discussion

In this study, we describe the effects of NF-κB inhibition by EriB on the OCSCs. We demonstrated that EriB, by blocking NF-κB activity, is able to suppress OCSC-mediated inflammation through the inhibition of cytokine production. More importantly, we show that it promotes OCSC death, therefore depleting the tumor of the possible source of tumor repair and recurrence.

CSCs or tumor-initiating cells represent a population of the tumor, which are chemoresistant and can undergo both processes of self-renewal and differentiation.[11,29,30] These cells can therefore survive chemotherapy and undergo self-renewal and differentiation to rebuild the tumor, thus leading to disease recurrence.[31] Targeting these cells is thus pivotal for the prevention of cancer recurrence and metastasis and the improvement of patient survival.

We previously showed chemoresistance and differentiation capacity to be some key features of the OCSCs.[16,24] Thus, it is possible that these cells are the source of EOC recurrence. Further studies showed that another remarkable characteristic of the OCSCs is constitutive NF-κB activity, which can be enhanced by TNFα ligation.[9,11] Moreover, these cells have a functional TLR4 pathway which can also contribute to enhanced NF-κB activity and cytokine production.[32] Therefore, in addition to being the possible source of recurrence, OCSCs may also be the source of a chronic inflammatory microenvironment.

Acute inflammation represents a critical signal to adult stem cells to initiate the process of tissue repair, which involves self-renewal (maintaining the pool of stem cells) and tissue renewal (differentiation on tissue specific cells).[7,33] In this setting, the source of the inflammatory signals has been associated with immune infiltrates, primarily macrophages, which respond to tissue damage by differentiating into the M2 type and secreting pro-inflammatory cytokines and chemokines.[34–36] Chronic inflammation, on the
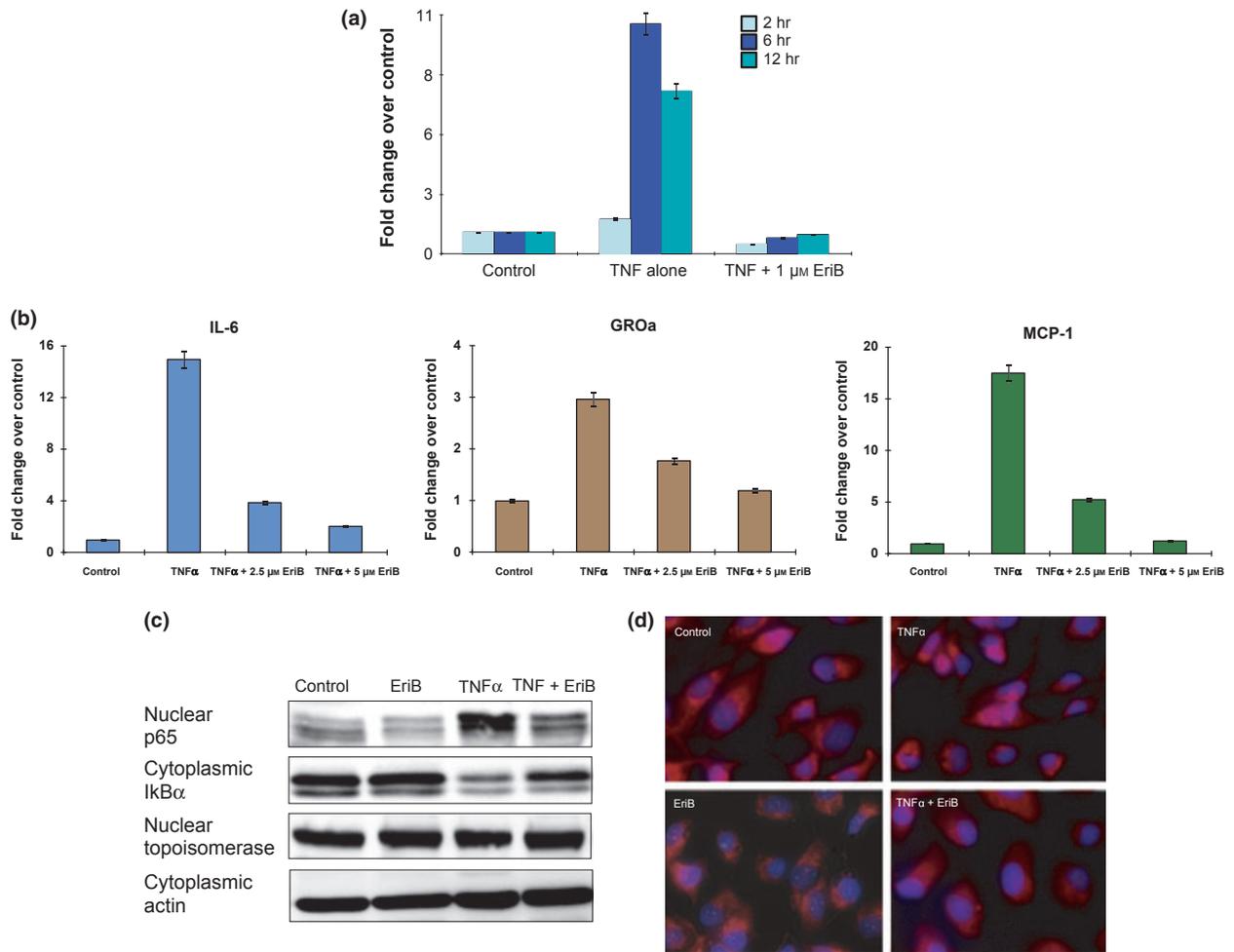
Fig. 4 Eriocalyxin B (EriB) inhibits Tumor Necrosis Factor α (TNFα)-induced NF-κB activation and cytokine production in the ovarian cancer stem cells (OCSCs). OCSCs were pre-treated with different doses EriB for 4 hrs prior to treatment with 10 ng/mL of TNFα for 2, 6, and 12 additional hours. (a) NF-κB activity was measured using the luciferase reporter system described earlier; (b) levels of IL-6, GROα, and MCP-1 were quantified after 12 hrs of EriB treatment using xMAP technology; (c) levels of nuclear p65 and total IκB were determined using western blot analysis; (d) co-localization of p65 and DAPI staining was determined using immunofluorescence. Results shown are from OCSC1. Similar results were obtained with other OCSCs tested.

other hand, may have detrimental effects by directly affecting abnormal progenitor cells and promoting tumorigenicity. Over two decades ago, Dvorak and co-authors described the tumor as an unhealed wound that produces a continuous source of inflammatory mediators (cytokines and chemokines).[37] This inflammatory environment promotes cell survival, enhances cell proliferation, and may even affect anti-tumoral immunologic response. Therefore, the demonstration that OCSCs can create a chronic inflammatory microenvironment through the NF-κB pathway provided us with a specific target for treatment.

We found that EriB is able to inhibit NF-κB activity, followed by suppression of cytokine production. This observation is relevant as cytokines are important for the process of tissue renewal and in the case of the tumor and tumor recurrence. Targeting this pathway may therefore allow prevention of recurrence and may also affect the pro-tumoral immunologic environment.

Interestingly, targeting the NF-κB pathway in the OCSCs induced apoptosis. These findings are in accordance with the role of NF-κB as a regulator of anti-apoptotic genes such as XIAP. Treatment with EriB induces a significant decrease in XIAP, which
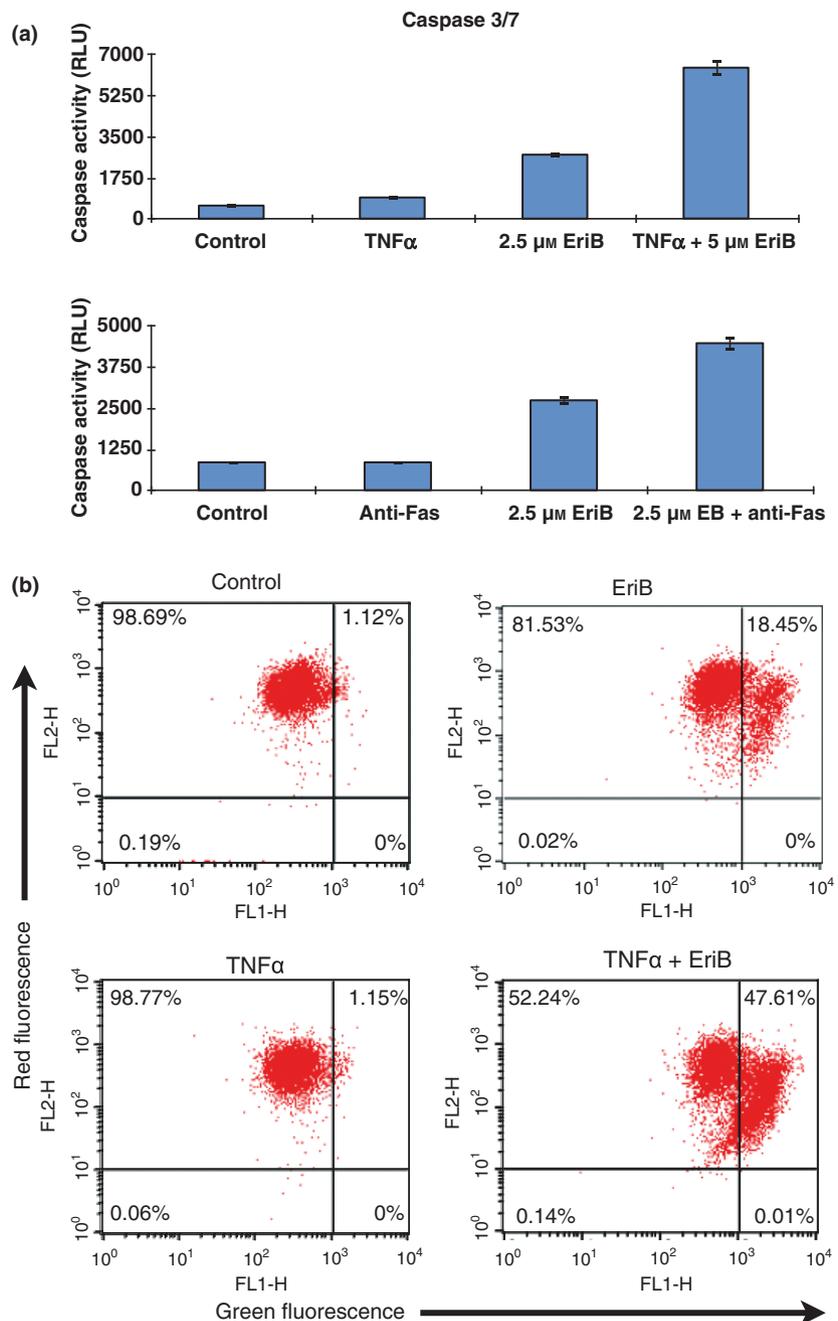
Fig. 5 Eriocalyxin B (EriB) sensitizes ovarian cancer stem cells (OCSCs) to Tumor Necrosis Factor α (TNFα)- and Fas-induced apoptosis. OCSCs were pre-treated with increasing concentrations of EriB for 4 hrs prior to treatment with 10 ng/mL TNFα or 500 ng/mL anti-Fas for additional 12 hrs, (a) caspase 3/7 activity was quantified using Caspase Glo assay; (b) effect on mitochondrial membrane stability was determined using the JC1 dye. Results shown are from OCSC1. Similar results were obtained with other OCSCs tested.

can then remove the inhibitory effects on the caspases. XIAP has been shown to be a central regulator of cell survival in EOC, and major attempts have been made, without much success, to develop XIAP inhibitors.[38–41]

We also found that inhibition of NF-κB can reverse the resistance of OCSCs to TNFα and FasL. TNFα has been shown to promote, in some types of tumors, cell growth instead of apoptosis.[42] We previously demonstrated that while TNFα can induce cell death in the mOCCs, it can enhance growth of the OCSCs.[10] This pro-apoptotic effect of EriB is not only associated with TNFα but also with FasL, another membranal receptor. Like TNFα, FasL is also ineffective in inducing cell death in the OCSCs. However, pre-treatment with EriB is able to sensitize OCSCs to FasL.

Finally, we evaluated how EriB inhibits NF-κB activity. Our data demonstrated that EriB decreased the levels of nuclear p65 in the OCSCs, both at the baseline level and after TNFα treatment. As NF-κB is a transcription factor, the inhibition of its nuclear translocation could explain the decrease in cytokines observed in the OCSCs after EriB treatment.

The nuclear translocation of p65 depends on its association with IκB.[43] Upon its phosphorylation of IκB, it gets degraded and this allows the dissociation and nuclear translocation of p65. As stated previously, OCSCs have constitutive NF-κB activity and the IκB levels in these cells cycles over a 24 -hr period. We found the inhibition of this cycle after the OCSCs were treated with EriB. This suggests that EriB is able to prevent IκB degradation.

In summary, we report a novel compound, which induces death in the OCSCs by inhibiting the NFκB pathway – a pathway that is highly activated in the OCSCs cells. Identifying and understanding the pathways in the OCSCs that can confer survival, resistance to apoptosis, and capacity for repair is relevant to improve survival of patients with ovarian cancer.

Acknowledgments

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