Reduced-gliotoxin induces ROS-mediated anoikis in human colorectal cancer cells

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Abstract. Reduced-gliotoxin is a small molecule derived from the secondary metabolites of marine fungi; compared to other gliotoxin analogues, it exhibits potent anticancer effects. However, the molecular basis of the death of colorectal cancer (CRC) cells induced by reduced-gliotoxin is unclear. Thus, the aim of this study was to investigate the potency of reduced-gliotoxin against CRC cells and to elucidate the underlying mechanisms. Cell morphology, flow cytometric analysis and western blot analysis were performed to examine the functions and mechanisms of cell death induced by reduced-gliotoxin. Our findings demonstrated that reduced-gliotoxin triggered rapid cell detachment and induced anoikis in CRC cells. Mechanistically, our data indicated that the anoikis induced by reduced-gliotoxin was associated with the disruption of integrin-associated cell detachment and multiple signaling pathways. Furthermore, reduced-gliotoxin induced the excessive production of reactive oxygen species (ROS) and the disruption of mitochondrial membrane potential (MMP), resulting in the activation of both endogenous and exogenous apoptotic pathways and eventually, in the apoptosis of CRC cells. The blockage of ROS generation with N-acetylcysteine (NAC) attenuated the anoikis induced by reduced-gliotoxin. Taken together, these results suggest that reduced-gliotoxin may prove to be a potential candidate in the treatment of CRC.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed types of cancer in both males and females worldwide (1,2). While the surgical resection of localized disease may be curative, mortality is primarily caused by metastatic progression, even with therapeutic regimens and the use of targeted therapies (3,4). Anoikis is an inherent cellular mechanism that 'cleans out' the detached epithelial cells in order to maintain tissue homeostasis and development (5-7). Cancer cells which survive during detachment from the extracellular matrix (ECM) and acquire metastatic potential have developed mechanisms with which resist anoikis (8,9). The breakdown of anoikis contributes to the development of many types of cancer, predominantly mammary and colon cancers (10,11). Therefore, enhancing the response of cancer cells to anoikis is considered to be an effective strategy for the treatment of metastatic colorectal cancer.

Marine fungi are an important source of structurally unique and biologically active natural products (12,13). In recent years, research has been conducted on the metabolites of marine fungi and obtained the optimized cultivation parameters with which to isolate and identify a series of novel and/or bioactive metabolites (14-17). Gliotoxin and its analogues, the diketopiperazines of various fungal species,
Rabbit monoclonal anti-glycogen synthase kinase (GSK)-3β (Cat. no. ab32391), rabbit monoclonal anti-phospho-GSK-3β (Ser9) (Cat. no. ab75814), rabbit monoclonal anti-β-catenin (Cat. no. ab32572), and rabbit monoclonal anti-cleaved caspase-9 (Cat. no. ab2324) antibodies were purchased from Abcam (Abcam, Cambridge, MA, USA). Rabbit polyclonal anti-GAPDH (Cat. no. 10494-1-AP), and mouse monoclonal anti- ACTB (Cat. no. 60008-1-Ig) antibodies were purchased from Proteintech Group (Proteintech Group, Chicago, IL, USA).

Colorectal cancer cell lines and cell culture. The colorectal cancer cell lines, HCT116 and HT-29, were purchased from the Culture Collection of Chinese Academy of Science (Chinese Academy of Science, Shanghai, China). The cells were cultured in RPMI-1640 medium (#11875500; Gibco/Thermo Fisher Scientific, Waltham, MA USA) containing 10% fetal bovine serum (#FBS-22A; Capricorn Scientific, Ebsdorfergrund, Germany) in a humidified atmosphere of 5% CO₂ at 37°C.

Apoptosis assay. The cell apoptotic rate was determined by flow cytometry using an Annexin V-FITC/PI dual staining kit (Nanjing KeyGen Biotech, Nanjing, Jiangsu, China), according to the manufacturer's instructions. Briefly, the CRC cells were seeded in 6-well tissue culture plates, and were then treated with various concentrations for the indicated periods of time. Cells present in the supernatant cells were collected by centrifugation at 626 x g for 10 min. The cells were counted (5x10⁴) and washed with PBS twice. The cells were then re-suspended in working solution (100 μl binding buffer with 5 μl Annexin V-FITC and 5 μl PI staining solution) for 10 min at room temperature in the dark. Subsequently, 400 μl binding buffer were added immediately prior to analysis using a flow cytometer (BD Biosciences, San Jose, CA, USA). The resulting data were analyzed using BD FACSDiva software, version 6.1.3 (BD Biosciences).

Western blot analysis. Western blot analysis was performed as previously described (24) with minor modifications. Briefly, total cell lysates were prepared in lysis buffer containing both protease and phosphatase inhibitors (KeyGEN Biotech). Protein concentrations were measured using a Bio-Rad assay kit (Bio-Rad, Hercules, CA, USA). Total proteins were separated on SDS-PAGE gel and transferred onto PVDF membranes (Bio-Rad), and the membranes were then blocked with 5% skimmed milk (BD Biosciences) in Tris-buffered saline with Tween-20 (TBS-T) and incubated with primary antibodies diluted in primary antibody solution I (Toyobo, Tokyo, Japan) overnight at 4°C. Anti-PARP, caspase-8, caspase-9, phosphor-Akt (Ser473), integrinβ4, PUMA, MCL1, caspase-3 phosphor-β-catenin (Thr41/Ser45) cleaved caspase-3, cleaved caspase-9 antibodies were diluted at 1:1,000. Anti-Akt, GSK-3β, phosphor-GSK-3β (Ser9) and β-catenin antibodies were diluted at 1:2,000. Anti-GAPDH and ACTB antibodies were diluted at 1:5,000. The following day, the membranes were washed and incubated with HRP-conjugated goat anti-rabbit IgG (H+L) (Cat. no. A16096; Invitrogen/Thermo Fisher Scientific) or goat anti-mouse IgG (H+L) (Cat. no. A16066; Invitrogen/Thermo Fisher Scientific) secondary antibody diluted at 1:5,000 in blocking buffer at room temperature for 2 h, followed by ECL (Bio-Rad) detection using a X-ray film or chemiluminescence equipment.
(Bio-Rad). After the detection of protein bands, the membranes were stripped and re-probed with anti-GAPDH or anti-ACTB antibodies to confirm equal loading of the samples.

**Mitochondrial membrane potential (MMP) assay.** MMP in the cells was detected using the MMP assay kit with JC-1 (Beyotime, Shanghai, China). According to the manufacturer's instructions, in brief, the cells were collected after treatment suspended with 5 µg/ml JC-1 in serum-free medium. The cells were then incubated at 37˚C for 20 min. MMP was detected by flow cytometry (green fluorescence for monomer form, Ex/Em: 490/530 nm; red fluorescence for aggregator form, Ex/Em: 525/590 nm).

**ROS assay.** ROS levels in the cells were monitored using the Reactive Oxygen Species assay kit (Beyotime). As instructed by the manual provided by the manufacturer, the cells were treated with reduced-gliotoxin for the indicated amounts of time, harvested and suspended with 10 µM of DCFH-DA in a serum-free medium, and then incubated with dye in 37˚C for 20 min. The treated cells were washed 3 times with serum-free medium and rinsed in PBS. ROS intensity was measured at an excitation/emission wavelength of 488/525 nm using a flow cytometer (BD Biosciences).

**Statistical analysis.** All experiments were performed at least 3 times, and the results are expressed as the means ± SD where applicable. All data were analyzed by a two-tailed unpaired Student's t-test for differences between 2 groups and by one-way ANOVA followed with Fisher's LSD test for multiple comparisons. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). A value of P<0.05 was considered to indicate a statistically significant difference.

**Results**

Reduced-gliotoxin triggers rapid cell detachment and induces the death of CRC cells. It has previously been reported that gliotoxin triggers rapid cell detachment in mouse embryonic fibroblasts and BEAS-2B human lung bronchial epithelial cells (25). Moreover, we have previously reported that gliotoxin induced the apoptosis of CRC cells (20). Therefore, in this study, we first examined whether reduced-gliotoxin, an analogue of gliotoxin, induces the death of CRC cells via the same mechanism. Indeed, we found that reduced-gliotoxin triggered rapid cell detachment in the HCT116 and HT-29 cells (Fig. 1A). As the detachment of cells can lead to cell death, we further examined the apoptosis-inducing effects of reduced-
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The anoikis induced by reduced-gliotoxin is associated with the activation of both endogenous and exogenous apoptotic pathways. The detachment of epithelial cells from the extracellular matrix induces cell apoptosis, a phenomenon known as anoikis (5). Furthermore, anoikis has been ascribed to the intrinsic and/or the extrinsic apoptotic pathway (26).

gliotoxin on CRC cells by flow cytometric analysis. The HCT116 and HT-29 cells were treated with escalating concentrations of reduced-gliotoxin, followed by Annexin V-FITC/propidium iodide (PI) staining coupled with flow cytometry. As shown in Fig. 1B, reduced-gliotoxin induced cell apoptosis in a dose-dependent manner.

Figure 2. The anoikis induced by reduced-gliotoxin is associated with the activation of both endogenous and exogenous apoptotic pathways. (A) Reduced-gliotoxin induced the cleavage of PARP and the activation of caspase-8 and caspase-9 in a dosage-dependent manner. The HCT116 and HT-29 cells were treated with reduced-gliotoxin at increasing concentrations for 24 h. The expression of PARP, caspase-8, caspase-9, cleaved caspase-9, PUMA and MCL1 was detected by western blot analysis. GAPDH was used as a loading control. (B) Reduced-gliotoxin induced the cleavage of PARP, and caspase-8, caspase-9 and caspase3 activation in a time-dependent manner. The HCT116 and HT-29 cells were treated with 4 µM reduced-gliotoxin for the indicated amounts of time, and the levels of PARP, caspase-8, caspase-9, caspase-3 and cleaved caspase-3 were detected by western blot analysis. GAPDH was used as a loading control. (C) Reduced-gliotoxin induced the loss of mitochondrial membrane potential (MMP) loss in the HCT116 and HT-29 cells. The cells were treated with increasing concentrations of reduced-gliotoxin for 24 h, and the loss of MMP was detected by JC-1 staining coupled with flow cytometry. All the values were expressed as the means ± SD (n=3). ***P<0.0001 vs. untreated cells, as shown by one-way ANOVA with Fisher's LSD test. re-G, reduced-gliotoxin.
Caspase-8 is a crucial adaptor in the extrinsic apoptotic pathway and caspase-9 is an important executor in the intrinsic apoptotic pathway (27). Therefore, in this study, we examined the activation of caspase-8 and caspase-9 in cells treated with reduced-gliotoxin. The HCT116 and HT-29 cells were treated with reduced-gliotoxin at the indicated concentrations for 24 h. The protein levels of integrinβ1 and integrinβ4 were detected by western blot analyses. ACTB was used as a loading control. The β-catenin degradation induced by reduced-gliotoxin was independent of the activation of GSK-3β in the HCT116 and HT-29 cells. The cells were treated with reduced-gliotoxin at the indicated concentrations for 24 h. The total and phosphorylated protein levels of β-catenin and GSK-3β were detected by western blot analyses. ACTB was used as a loading control. The inhibition of caspase-8 had no effect on the cell detachment and cell death induced by reduced-gliotoxin. The cells were treated with 5 µM reduced-gliotoxin for 24 h, in the absence and presence of the caspase-8 inhibitor, Z-IETD-FMK, at various concentrations. Western blot analyses were performed for the indicated protein. GAPDH was used as a loading control. 

The anoikis induced by reduced-gliotoxin is associated with integrinβ1, integrinβ4 and β-catenin degradation.

Figure 3. The anoikis induced by reduced-gliotoxin induced is associated with integrinβ1, integrinβ4 and β-catenin degradation, but not is dependent on caspase-8. (A) Reduced-gliotoxin induced integrinβ4 degradation in the HCT116 and HT-29 cells. The cells were treated with reduced-gliotoxin at the indicated concentrations for 24 h. The protein levels of integrinβ1 and integrinβ4 were detected by western blot analyses. ACTB was used as a loading control. (B) β-catenin degradation induced by reduced-gliotoxin was independent of the activation of GSK-3β in the HCT116 and HT-29 cells. The cells were treated with reduced-gliotoxin at the indicated concentrations for 24 h. The total and phosphorylated protein levels of β-catenin and GSK-3β were detected by western blot analyses. ACTB was used as a loading control. (C) The inhibition of caspase-8 had no effect on the cell detachment and cell death induced by reduced-gliotoxin. The cells were treated with 5 µM reduced-gliotoxin for 24 h, in the absence and presence of the caspase-8 inhibitor, Z-IETD-FMK, at various concentrations. Western blot analyses were performed for the indicated protein. GAPDH was used as a loading control. re-G, reduced-gliotoxin; CTNNB, β-catenin.
been implicated in triggering anoikis in colon carcinoma (30), we also examined whether reduced-gliotoxin decreases the protein level of β-catenin by western blot analysis. As shown in Fig. 3B, reduced-gliotoxin decreased β-catenin expression in a dose-dependent manner. The disengagement of integrin from the ECM leads to the activation of both the extrinsic and intrinsic apoptotic pathways (31). Thus, in order to verify the predominant pathway involved in the execution of the apoptosis of these cells, we used a caspase-8 inhibitor (Z-IETD-FMK) to block the extrinsic apoptotic pathway. As shown in Fig. 3C, the inhibition of caspase-8 activity did not reverse cell detachment and death induced by reduced-gliotoxin.

The anoikis induced by reduced-gliotoxin is independent of Akt activation or GSK-3β inactivation. As the phosphorylation of β-catenin by GSK-3β results in β-catenin degradation (32), we then examined whether the promotion of β-catenin degradation induced by reduced-gliotoxin required GSK-3β activity. Of note, reduced-gliotoxin treatment did not modify the expression of pan GSK-3β, but induced its phosphorylation on its Ser9 residue, which was shown to trigger its inactivation (Fig. 3B). These results indicate that the degradation of β-catenin by reduced-gliotoxin occurs independently of GSK-3β activity. During tumorigenesis, PI3K/Akt signaling has been shown to inhibit GSK-3β through phosphorylation at Ser9 (33). Thus, we then examined the phosphorylation of Akt at Ser473, which is strongly dependent upon PI3K activity. As shown in Fig. 4A, treatment of the CRC cells with reduced-gliotoxin increased the level of phosphorylated Akt at Ser473 in a concentration-dependent manner. Furthermore,
we used the PI3K-specific inhibitor, LY294002, and the Akt inhibitor, MK-2206, to examine the role of PI3K/Akt/GSK-3β in the anoikis induced by reduced-gliotoxin. As shown in Fig. 4B, Akt activity was inhibited by treatment with escalating concentrations of MK-2206, whereas the inhibition of GSK-3β induced by reduced-gliotoxin was not affected. Likewise, the activation of Akt induced by reduced-gliotoxin was also not affected by pre-treatment with the PI3K specific inhibitor, LY294002 (Fig. 4B). These findings thus suggest that reduced-gliotoxin triggers the phosphorylation of Akt and that this occurs independently of PI3K activity, while reduced-gliotoxin triggers the phosphorylation of GSK-3β and that this occurs independently of the PI3K/Akt pathway. We also examined the apoptosis-promoting effects of reduced-gliotoxin by flow cytometric analysis and western blot analysis in the presence or absence of PI3K/Akt inhibitors. As shown in Fig. 4C, PI3K/Akt inhibition did not affect the cell detachment and death induced by reduced-gliotoxin.

Excessive ROS production is the upstream regulator of the anoikis induced by reduced-gliotoxin. Gliotoxin has been found to increase ROS generation and induce oxidative stress in mouse fibroblasts (34), human hepatic stellate cells (35) and cultured macrophages (36). ROS generation is associated with the mitochondria, and as shown above, reduced-gliotoxin triggered the loss of MMP (Fig. 2B). Therefore, we examined the intracellular ROS levels by flow cytometry in the cells with or without reduced-gliotoxin treatment. As shown in Fig. 5A, reduced-gliotoxin increased ROS production in a dose-dependent manner. The two main sources of ROS are the...
Mitochondria and NADPH oxidase (NOX). In order to verify the main source of ROS generation induced by treatment with reduced-gliotoxin, we used DPI to block NOX activity. As it shown in Fig. 5B, DPI reduced most of the ROS generation. To identify the role of ROS in the anoikis induced by reduced-gliotoxin, NAC, as a ROS scavenger, was used. Of note, it was found that NAC almost completely abolished cell anoikis induced by reduced-gliotoxin (Fig. 5C). We further examined the effects of NAC on cell detachment, and multi-signaling pathway and caspase cascade activation induced by reduced-gliotoxin. As shown in Fig. 6A, the cell detachment induced by reduced-gliotoxin was attenuated by pre-treatment with NAC. In addition, β-catenin degradation, Akt activation and GSK-3β inhibition induced by reduced-gliotoxin-induced were all reversed (Fig. 6B). Additionally, the decrease in the levels of PARP, caspase-8 cleavage and pro-caspase-9 induced by reduced-gliotoxin were all reversed by NAC pre-treatment (Fig. 6C). These results suggest that ROS induction mediates the apoptotic pathways activated by reduced-gliotoxin and that ROS are a critical upstream regulator of the anoikis induced by reduced-gliotoxin.

Discussion

There is evidence to suggest that gliotoxin is a promising anticancer reagent that induces cytotoxic effects on cancer cells (21-23). Previously, we observed that reduced- exerted the most potent anticancer effects compared to other gliotoxin analogues (14). In this study, we examined the potency of reduced-gliotoxin against CRC cells and the underlying mechanisms. We demonstrated that reduced-gliotoxin triggered rapid cell detachment and induced the apoptosis of CRC cells. Mechanistically, our data indicated that the anoikis induced by reduced-gliotoxin induced is associated with the disruption of integrin-related cell detachment and multiple signaling pathways. Furthermore, reduced-gliotoxin induced ROS production, resulting in the activation of both endogenous and exogenous apoptotic pathways and eventually resulting in the apoptosis of CRC cells. The blockade of ROS generation by...
NAC completely reversed several reduced-gliotoxin-induced protein kinase signaling pathways and cell anoikis (Fig. 7). Taken together, these studies suggest that reduced-gliotoxin may be a potential candidate for the treatment of CRC.

Anoikis is defined as apoptosis that is induced by inadequate or inappropriate cell matrix interaction (11). Anoikis plays an important role in tissue homeostasis, disease development and cancer metastasis (5). Resistance to anoikis characterizes the ability of cancer cells to circumvent barriers to migration and invasion, thereby promoting metastatic progression (31). Therefore, a novel method that can enhance or induce anoikis to disrupt the settling of metastatic cancer cells at a distal site is an attractive strategy. In this study, we demonstrated that reduced-gliotoxin induced cell death by inducing anoikis (Fig. 1). Anoikis either disrupts mitochondrial homeostasis or triggers cell surface death receptors; hence, it is involved in both the intrinsic and extrinsic apoptotic pathways (11). In this study, the anoikis induced by reduced-gliotoxin was characterized by the activation of caspase-8, caspase-9 and PARP cleavage, suggesting that the intrinsic apoptotic pathway mainly mediates this event.

The induction of anoikis is caused by an absence of adhesive interaction between epithelial cells and their surrounding ECM. Integrins involved in the cell-ECM and cell-cell communication play an important role in regulating anoikis and metastasis (37). The overexpression of integrinβ1 and integrinβ4, which promote cell invasion and metastasis, are associated with a clinically aggressive phenotype of different types of cancer, including CRC (38-40). The inhibition of cell surface integrins is able to influence the first step of anoikis. It has been suggested this may prove to be an attractive target for CRC therapy (41). In this context, in this study, we demonstrated that the anoikis induced by reduced-gliotoxin might be associated with integrinβ1 and integrinβ4 degradation (Fig. 3). Previously, we reported that gliotoxin blocked the Wnt/β-catenin signaling pathway by inducing the degradation of β-catenin in CRC cells. A similar result was observed: Reduced-gliotoxin induced β-catenin phosphorylation and decreased the protein expression levels. β-catenin is a major oncprotein that is often activated in CRC and promotes tumor progression. Our data indicated that reduced-gliotoxin induced CRC cell anoikis and this was not only associated with the degradation of proteins interacting with ECM, but also with adherens junction proteins. Furthermore, we demonstrated that the degradation of β-catenin was accompanied by Akt activation and GSK-3β inactivation.

Reduced-gliotoxin can be oxidized to gliotoxin, thus releasing ROS, and it can also form mixed disulfides by thiol groups with proteins or antioxidants, such as glutathione (42). Therefore, it is not surprising to observe that ROS was produced by CRC cells treated with reduced-gliotoxin, whereas pre-treatment with NAC blocked ROS production and cell anoikis. In tumorigenesis, GSK-3β is the most important kinase for the abnormal phosphorylation of β-catenin, and the inactivation of GSK-3β promotes the accumulation of β-catenin. In this study, reduced-gliotoxin triggered the activation of Akt and the inactivation of GSK-3β. Moreover, the activation of Akt and the inactivation of GSK-3β were abrogated by pre-treatment with NAC. NAC inhibited the phosphorylation of Akt; it subsequently inactivated GSK-3β by phosphorylating at Ser9 as usual. However, inhibiting the activation of Akt with LY294002 or MK-2206 did not attenuate the inactivation of GSK-3β. Thus, ROS may act independently of Akt activation to mediate reduced-gliotoxin, which inactivates GSK-3β activity. Based on these findings, it is likely that ROS are crucial factors in the induction of anoikis and act as upstream signaling molecules in initiating cell anoikis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JC, QL, LH, CW, ML, ZZ, FW and LH performed the cell and molecular biology experiments; WL contributed to the marine natural productions; JC and QL wrote the manuscript; AI revised the manuscript and provided professional advice during the whole research process. HL and XY designed the experiments and analyzed the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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