ONCOLOGY LETTERS 12: 2632-2640, 2016

Novel recombinant protein FlaA N/C increases tumor radiosensitivity via NF-κB signaling in murine breast cancer cells

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Received April 14, 2015; Accepted July 20, 2016

DOI: 10.3892/ol.2016.4957

Abstract. The recombinant protein flagellin A (FlaA) N/C, derived from the flagellin protein of Legionella pneumophila, has been shown to increase the expression of cytoprotective cytokines, activate the nuclear factor-κB (NF-κB) signaling pathway, and increase the survival of mice following total body irradiation. Determining whether FlaA N/C has a sensitizing effect on tumor radiation or a direct tumoricidal effect is critical for its application as an effective radiation protection agent. The present study investigated the molecular mechanism underlying the tumor radiosensitivity of FlaA N/C. FlaA N/C was found to increase tumor apoptosis and autophagy, regulate the cell cycle and increase radiosensitivity in 4T1 tumor cells. Furthermore, FlaA N/C was found to promote radiosensitivity by activating NF-κB signaling. Finally, the present study analyzed FlaA N/C-enhanced radiosensitivity in animal models, and FlaA N/C was found to significantly prolong the survival period of mice after total body radiation. This indicates that FlaA N/C might be a novel radiation sensitizer in tumor radiation therapy.

Introduction

Breast cancer is the most common cancer among women worldwide (1,2). Although genetic, hormonal, lifestyle, and environmental risk factors have been established, the etiology and carcinogenesis of breast cancer warrant a more detailed understanding. The most common treatment for breast cancer includes breast-conserving surgery followed by a standard radiotherapy regimen (3). Radiotherapy, the use of high-energy ionizing radiation to eradicate tumor cells, is the mainstay of anticancer treatment. Radiotherapy is largely used for the local control of tumor progression and the preservation of organs; therefore, it is the major treatment course in the management of breast cancer. Radiation therapy can reduce the risk of locoregional recurrences following surgery by ~70%, and has been shown to improve overall survival for early-stage breast cancer, after breast-conserving surgery and for locally advanced disease following mastectomy (4–6). However, radiation hypersensitivity and the occurrence of radiotherapy-induced toxicity in normal tissue may affect treatment (7,8). Healthy cells located in the region that neighbors the tumor inevitably receive a considerable dose of radiation.

Radiation damage to biological systems is determined by the type of radiation, total dosage of exposure, dose rate and region of the body exposed (9). Three modes of cell death, necrosis, apoptosis, and autophagy, as well as accelerated senescence, have been demonstrated to occur in vitro and in vivo in response to radiation in cancerous and normal cells (10,11). Radiation damage may appear immediately after radiation therapy or can be delayed in surviving patients. The number of cancer survivors is steadily rising; thus, assessing how early- and late-radiation toxicity in normal tissues affects the quality of life for these patients is becoming increasingly important (12). The dose of radiation administered is restricted to avoid excess damage to healthy tissue, and this has limited its clinical application as part of modern treatment (13).

Cell death by apoptosis proceeds via 2 major pathways, the extrinsic (death receptor) and intrinsic (mitochondrial) pathways. The extrinsic and intrinsic pathways are initiated by specific ligands or various intracellular stimuli that activate effector caspases, ultimately fragmenting DNA and inducing cell death (14). Each pathway is involved in radiation-induced apoptosis (15). Inhibitor of apoptosis proteins (IAPs) are a family of endogenous anti-apoptotic proteins characterized by one or several baculoviral IAP repeat (BIR) domains. Certain IAPs contain a RING finger domain at the C-terminus, exhibiting E3 ubiquitin ligase activity, which is required for the ubiquitination and proteasomal degradation of various substrates (16). Cellular inhibitor of apoptosis (cIAP)1 and X-linked inhibitor of apoptosis protein (XIAP)2 tend to be involved in nuclear factor-κB (NF-κB) activity regulation and the suppression of caspase-8 activation (17). Aberrant IAP expression occurs in combination with cancer, particularly

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Key words: breast cancer, flagellin, nuclear factor-κB signaling, radiosensitivity, tumor
in hypoxic microenvironments (18). Previous studies on IAP amplification and overexpression demonstrated that IAPs, including cIAP1 and XIAP, may be novel predictive markers for radiotherapy resistance in individual cervical squamous cell carcinoma patients. Therefore, IAPs serve a critical function in interfering with the efficacy of radiotherapy and chemotherapy (19,20).

In contrast to apoptosis, the role of autophagy is paradoxical, as it can either induce autophagic cell death or provide a survival vehicle for the tumor against nutrition- and energy-deficient environments, as a result of chemotherapy, endocrine drugs and irradiation (21). During the process of radiotherapy and chemotherapy, multiple changes occur in the growth arrest and death pathways (22). Traditionally, irradiation is considered to induce cell death mainly via apoptosis. However, more recent studies have suggested that autophagy is also important in irradiation-induced cell death, which may aid to restore and improve radiosensitivity (23). For homeostasis, intracellular organelles and long-lived proteins are degraded by autophagy, a conserved cellular process (24). However, autophagy plays paradoxical roles in the initiation, development and metastasis of cancer. While autophagy exhibits an antitumor role, it also protects tumor cells against stress (25). Once autophagy is inhibited, the therapeutic effect is considered enhanced (26). XIAP and cIAP1 overexpression induces Beclin-1-dependent autophagy through NF-kB activation, suggesting that NF-kB signaling is important for radiation-induced cell apoptosis and autophagy (27).

The present study used a novel recombinant protein, namely flagellin A (FlaA) N/C, which was developed in an earlier study and is derived from the flagellin protein of Legionella pneumophila (28). FlaA N/C has been shown to increase the expression of several cytokracter cytokines, activate the NF-kB signaling pathway and significantly increase the survival of mice following total body irradiation, compared with flagellin or amifostine (28). However, in order to assess the effectiveness of FlaA N/C as a tumor radiation protection agent, determining whether FlaA N/C has a sensitizing effect on tumor radiation or has a direct tumoricidal effect is crucial.

The results of the present study found that pretreatment with FlaA N/C prior to radiation administration promoted apoptosis, autophagy and tumor radiosensitivity and regulated the cell cycle in mouse breast cancer 4T1 cells in vitro and in vivo. Furthermore, the results revealed that FlaA N/C regulated radiosensitivity by NF-kB signaling via toll-like receptor 5 (TLR5). Treatment with FlaA N/C clearly prolonged the survival of mice after total body irradiation. Thus, FlaA N/C may be a useful radiation sensitizer in breast tumor radiation therapy.

Materials and methods

Cells. Cells of the murine breast cancer 4T1 cell line (catalog no., CRL-2539; American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37°C and in 5% CO₂. All media and supplements were Invitrogen brand, purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Mice. Six-week-old female BALB/c mice (n=40) were purchased from Maccura Biotechnology Co., Ltd. (Chengdu, China). The animals were housed in groups of 5 or 6 in plastic mouse cages in a laminar flow housing cabinet, with an automatically controlled temperature of 22°C and 12 h of light. Specific pathogen free feed for mice was purchased from Chengdu Dossy Biological Technology Co., Ltd. (Chengdu, China) and the ddWater for mice was high pressure treated.

The research was conducted in accordance with the Declaration of Helsinki and the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, MD, USA). All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Chengdu Medical College (Chengdu, China).

Tumor model and radiation. Mice received a subcutaneous orthotopic injection of 5x10⁴ 4T1 tumor cells into the third thoracic mammary fat pad. After 7 days, purified FlaA N/C was subcutaneously injected into the BALB/c mice (10 mice per group), and recombinant protein tags (Beijing Protein Innovation Co., Ltd, Beijing, China) were used as a control. At 2 h subsequent to administration, the mice received 10-Gray (Gy) irradiation. Another 5 days later, 2 mice per group were anaesthetized, mice were sacrificed by cervical dislocation, and tumor tissue was preserved in liquid nitrogen for further analysis. The tumor volumes and body weights of the mice were measured every 2 days, and the mortality of mice was checked every day.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. 4T1 cells were irradiated with 10 Gy γ-rays following treatment with FlaA N/C for 2 h. Cells were cultured for 2 days (37°C; 5% CO₂). MTT reagent was added for 4 h and was then reduced using mitochondrial reductase (Beyotime Institute of Biotechnology, Hainen, China). The resulting purple crystals were dissolved in solubilization buffer and spectrophotometrically analyzed at 570 nm using a reference of 656 nm in a microplate reader (SpectraMax M5e; Molecular Devices, LLC, Sunnyvale, CA, USA). The inhibition index of proliferation was calculated as: 1 - absorbance of sample/absorbance of sample blank.

Clonogenic assay. Cells were seeded into 6-well plates and allowed to attach overnight. The cells were then treated with FlaA N/C for 2 h, irradiated with 10 Gy γ-rays, and cultured for 2 weeks. Plates were then washed with saline, fixed with 100% methanol, stained with crystal violet, washed with water, and air-dried. Colonies containing >50 cells were counted.

Western blot analysis. All protein samples for western blot analysis were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels and then transferred to nitrocellulose membranes, which were then blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% fat-free milk. All primary antibody [either mouse monoclonal; dilution, 1:2,000; catalog no.: β-actin, 60008-1-lg; or rabbit polyclonal; dilution, 1:2,000; catalog nos.: γ H2A histone family member X (γH2AX), 10856-1-AP; DNA-dependent protein kinases (DNA-PKs), 22129-1-AP;
B cell lymphoma-2 (Bcl-2), 12789-1-AP; Bcl-2-associated X (Bax), 50599-2-ig; BH3 interacting domain death agonist (Bid), 10988-1-AP; light chain 3 (LC3), 12135-1-AP; Beclin-1, 11306-1-AP; NF-xB, 14220-1-AP; RELA proto-oncogene, NF-kB subunit (P65), 10745-1-AP; cyclin dependent kinase inhibitor 1A (P21), 10355-1-AP; and TLR5, 19810-1-AP; ProteinTech Group, Inc., Chicago, IL, USA). Incubations were performed for 18 h at 4°C. Secondary antibody [either, for β-actin: horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (IgG), heavy and light chain (H+L); dilution, 1:5,000; catalog no., SA00001-1; or, for all other primary antibodies: HRP-conjugated goat anti-rabbit, IgG (H+L); dilution, 1:5,000; catalog no., SA00001-2; ProteinTech Group, Inc.] incubations were carried out at room temperature for 1 h. Secondary antibodies conjugated with HRP were used for detection by enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL, USA; or ECL Plus; GE Healthcare Life Sciences, Chalfont, UK) according to the manufacturers' instructions.

**Immunofluorescence.** Tissues were dewaxed and antigens retrieved using high pressure (0.12 MPa) for 3 min and then blocked with phosphate-buffered saline (PBS) containing 10% normal goat serum (Beyotime Institute of Biotechnology). Sections were stained with primary antibodies (rabbit polyclonal; dilution, 1:200; catalog nos.: γH2AX, 10856-1-AP; DNA-PKs, 22129-1-AP; LC3, 12135-1-AP; and TLR5, 19810-1-AP; ProteinTech Group, Inc.) for 30 min at 37°C, and then stained with Cy3-conjugated AffiniPure goat anti-rabbit IgG (H+L) secondary antibody (dilution, 1:500; catalog no., SA00009-2; ProteinTech Group, Inc.) for 30 min at 37°C. The samples were then re-stained with 4',6-diamidino-2-phenylindole for 10 min at 37°C. All immunofluorescence images were obtained using an Olympus BX51 microscope equipped with either a x20 or x40 objective lens and a DP50 camera (Olympus Corporation, Tokyo, Japan). Images were processed using Digital Production Control software (BX2 software; Olympus Corporation).

**Cell apoptosis assay.** Apoptotic cells were quantified using the Annexin V/propidium iodide (PI) double staining method and analysis of phosphatidylserine on the outer surface of apoptotic cell membranes (29). The treated 4T1 cells received 10-Gy irradiation, were harvested after 72 h, and washed twice with PBS. Subsequently, 10^4 cells were resuspended in 100 µl 1X binding buffer and stained with 5 µl fluorescein isothiocyanate (FITC)-conjugated Annexin V and 10 µl PI simultaneously. The cells were then incubated for 15 min in the dark and flow cytometry analysis (BD FACs Calibur flow cytometer with BD FACsComp™ software; BD Biosciences, Franklin Lakes, NJ, USA) was performed.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.** The TUNEL assay was performed using an Apoptosis Detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Apoptotic cells contained brown-yellow granules in the cytoplasm. The ratio of brown-yellow granules-prositive cells to normal cells in the microscopic field was used to determine the degree of apoptosis.

**Cell cycle assay.** The 4T1 cells were cultured for 24 h prior to FlaA N/C treatment for 2 h. The cells were exposed to 10-Gy irradiation and were cultured for another 72 h. After the cells were harvested, they were washed twice with PBS, treated with 50 µg/ml PI containing 20 µg/ml RNase, incubated at room temperature for 2 h, and analyzed by flow cytometry, using the aforementioned method.

**Statistical analysis.** Each experiment was performed at least 3 times independently. The effects of various treatments were compared by one-way analysis of variance and a two-tailed t-test using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences with a value of P<0.05 were considered to be statistically significant. The experimental results shown are presented as the means of multiple individual points from multiple separate experiments ± standard error of the mean.

**Results**

**FlaA N/C increased radiosensitivity in 4T1 cells.** A previous study demonstrated that, through the activation of NF-κB signaling, FlaA N/C regulates the expression of various cytokines, suppresses the inflammatory response and regulates multiple cellular functions important for radiation-induced intestinal injury (28). In addition, the effect of FlaA N/C on the radiosensitivity of tumor cells is currently unclear. In the present study, pre-treatment with FlaA N/C prior to radiation inhibited the proliferation of 4T1 cells (Fig. 1A). A clonogenic assay also showed that FlaA N/C inhibited the survival of 4T1 cells (Fig. 1B-C). The present study detected the expression of associated biomarkers of radiosensitivity and found that FlaA N/C clearly increased γH2AX and DNA-PKs in 4T1 cells (Fig. 1D).

FlaA N/C treatment prior to total body irradiation was studied in vivo. As shown in Fig. 1E, treatment with FlaA N/C increased the expression of γH2AX and DNA-PKs, suggesting that FlaA N/C has a marked capacity to increase tumor radio-sensitivity.

**FlaA N/C promoted apoptosis in 4T1 cells.** A large number of studies have shown that apoptosis is important in tumor radio-therapy (30,31). Therefore, the present study further tested whether FlaA N/C affects cell apoptosis after radiation. Treatment with FlaA N/C prior to radiation was found to increase apoptosis in 4T1 cells (Fig. 2A-B). When the expressions of Bcl-2, Bax and Bid were analyzed in 4T1 cells, FlaA N/C was found to increase Bax and Bid expression, while inhibited Bcl-2 expression (Fig. 2C).

In vivo experiments using the TUNEL assay in a 4T1-bearing animal model also proved that treatment with FlaA N/C increased tumor apoptosis. Compared with the control group, the tumor-bearing mice receiving FlaA N/C treatment prior to irradiation demonstrated significantly increased apoptosis in tumor cells (Fig. 2D).

**FlaA N/C promoted autophagy in 4T1 cells.** Cell death as a result of autophagy is important in radiation therapy for tumors. Therefore, the function of autophagy after treatment with FlaA N/C was analyzed. Following irradiation, FlaA N/C was found to increase the expression of LC3 and...
Figure 1. FlaA N/C increased radiosensitivity in 4T1 cells. (A) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that pre-treatment with FlaA N/C prior to radiation inhibited the proliferation of 4T1 cells; **P<0.01, ***P<0.001. (B) Bar graph of the clonogenic assay showed that pre-treatment with FlaA N/C prior to radiation inhibited cell survival of 4T1 cells; **P<0.01. (C) Clonogenic assay showed that pre-treatment with FlaA N/C prior to radiation inhibited cell survival of 4T1 cells. (D) Western blot analysis showed that FlaA N/C increased γH2AX and DNA-PK expression. (E) Immunofluorescence staining showed that FlaA N/C increased γH2AX and DNA-PK expression in vivo. Scale bars represent 50 μm. Con, control; Gy, Gray; FlaA, flagellin; γH2AX, γ H2A histone family member X; DAPI, 4',6-diamidino-2-phenylindole; DNA-PKs, DNA-dependent protein kinases.
Beclin-1 in 4T1 cells (Fig. 3A). Furthermore, the present study investigated tumor autophagy in tumor-bearing mice. As shown in Fig. 3B, FlaA N/C was found to increase LC3 expression in tumors, which indicated that FlaA N/C could promote cell death in the process of irradiation through cell autophagy.
**FlaA N/C regulated the cell cycle in 4T1 cells.** Changes in the cell cycle, particularly during the G2/M phase arrest, are critical in tumor radiation sensitization. The present study found that treatment with FlaA N/C promoted G2/M phase arrest (Fig. 4A-B). With regards to cell cycle biomarkers, FlaA N/C clearly increased the expression of P21 and inhibited the expression of CDK1 and Cyclin B1 in 4T1 cells. Con, control; Gy, Gray; FlaA, flagellin; P21, cyclin dependent kinase inhibitor 1A; CDK1, cyclin-dependent kinase.

**FlaA N/C increased radiosensitivity though NF-κB signaling.** Flagellin activates NF-κB signaling through interaction with TLR5 (32); therefore, TLR5 expression was first tested in 4T1 cells. As shown in Fig. 5A, 4T1 cells were positive for TLR5 expression. When the present study assessed the activation of NF-κB signaling following treatment with FlaA N/C, P65 expression was found to be upregulated. In addition, the subcellular localization of NF-κB was assessed by western blot analyses of the cytosolic and nuclear extracts. The analyses found that FlaA
N/C clearly increased NF-κB levels in the nuclear fraction while decreasing NK-κB levels in the cytosolic fraction, and the total NF-κB expression remained unchanged (Fig. 5B).

**FlaA N/C increases animal survival.** In the in vivo studies of FlaA N/C function, irradiation reduced the weight of mice, while the weight of mice in the FlaA N/C group remained unchanged (Fig. 6A). Treatment with FlaA N/C inhibited the 4T1 tumor growth prior to irradiation and further increased the overall survival of mice that received 10 Gy irradiation (Fig. 6B-C). As shown in Fig. 6D, hematoxylin-eosin staining further proved that FlaA N/C inhibited tumor growth, and is another indicator that FlaA N/C has a notable effect on tumor radiation sensitization.

### Discussion

Radiotherapy is a cornerstone of breast cancer treatment (33). Radiotherapy greatly reduces the risk of ductal carcinoma recurrence in situ and in lymph node-positive breast cancer (34). Even though radiotherapy shows promise in the treatment of all breast cancer subtypes, radiotherapy is associated with an increased risk of cardiovascular disease (35). In addition, breast tumors can acquire radioresistance (36). Finding agents that sensitize malignant cells to radiation may increase tumor response while minimizing toxicity to surrounding organs by lowering effective therapeutic doses.

Flagellin has been reported to increase the radiosensitization of cancer cells in several tumor types. Total flagellin activates the inflammatory reaction of normal tissues (37). In an earlier study, a recombinant protein, FlaA N/C, was constructed to reduce the side effects of flagellin. The study showed that FlaA N/C can reduce intestinal inflammation in total body irradiation (28). Thus far, whether FlaA N/C has an inhibitory effect on tumors that receive radiation therapy has remained unknown. The present study tested the irradiation effect of FlaA N/C in 4T1 cells in vitro and in vivo. Treatment with FlaA N/C was found to inhibit cell proliferation in 4T1 cells. The staining of radiosensitivity biomarkers also proved that FlaA N/C increased the radiosensitivity of 4T1 cells in vivo.

Apoptosis is crucial for cell death following radiotherapy, and autophagy is termed the ‘second apoptosis’ (38). The IAP family proteins, XIAP and cIAP1, induce autophagy by upregulating the transcription of Beclin-1, an essential autophagy gene. The E3 ubiquitin ligase activity of the two proteins activates NF-κB signaling, which results in the direct binding of P65 to the promoter of Beclin-1 and to its transcriptional activation (26). In cancer therapy, the role of autophagy is paradoxical; this cellular process may serve as a pro-survival or pro-death mechanism to counteract or mediate the cytotoxic effect of anticancer agents (39). The present study found that treatment with FlaA N/C prior to cell radiation increased cell apoptosis in 4T1 cells. In vivo experiments also proved that treatment with FlaA N/C 2 h after total body irradiation...

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**Figure 6.** FlaA N/C increased animal survival. (A) FlaA N/C prior to irradiation inhibited irradiation-induced weight loss. (B) Treatment with FlaA N/C prior to irradiation inhibited 4T1 tumor growth in vivo. (C) Pre-treatment with FlaA N/C prior to radiation prolonged the overall survival of mice. (D) Hematoxylin-eosin staining further proved that FlaA N/C inhibited tumor growth. Data are presented as the mean ± standard error of the mean. Scale bars represent 50 µm. Con, control; Gy, Gray; FlaA, flagellin.
clearly increased tumor apoptosis. FlaA N/C increased cell autophagy in 4T1 cells and in vivo experiments.

Shortening of the G2 checkpoint leads to decreased repair of radiation-induced damage, which can occur prior to cell division (39). For the radiosensitization of cancer cells, pharmacological agents that can inhibit specific checkpoint components, particularly the G2/M transition, have received attention recently. The checkpoint kinase (Chk) inhibitor, AZD7762, has demonstrated synergy with ionizing radiation to inhibit cancer growth by abrogating the G2/M checkpoint through selective inhibition of Chk1 (40). The present study found that FlaA N/C regulates the cell cycle and increases the G2/M phase arrest in 4T1 cells.

NF-κB can activate a great number of genes involved in stress responses, inflammation, apoptosis and autophagy. NF-κB subunit 1 (P50) homodimers, or P50/P65 or P50/c-Rel heterodimers, bind to the NF-κB DNA-binding sites in the promoter regions of numerous stress-response genes, suggesting a complex regulation network at gene and physiological levels, controlled by NF-κB in stress response (41). Accumulated evidence indicates that the transcription factor NF-κB is critical for cellular protection against a variety of genotoxic agents, including irradiation, and that the inhibition of NF-κB may result in radiosensitization in radioresistant cancer cells (42). In a previous study, human breast cancer cells treated with fractional γ-irradiation displayed enhanced NF-κB activation (43).

In conclusion, the present study indicates that, through activating NF-κB signaling, FlaA N/C regulates the function of apoptosis and autophagy and regulates cell cycle of radiation sensitization. The radiosensitization effects of FlaA N/C appear promising and indicate its clinical potential for use as a novel radiation sensitizer in tumor radiation therapy.

Acknowledgements

The present study was funded by the National Natural Science Foundation of China (grant no. 81001345), the Foundation of Sichuan Province Science and Technology Agency (grant no. 2014JY0039), the Foundation of Sichuan Province Education Office (grant no. 15ZA0248) and the Founding of Sichuan Province Academic and Technology Leaders in 2014.

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