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An Anti-apoptotic Role of NF-κB in TNFα-induced Apoptosis in an Ameloblastoma Cell Line

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Abstract: Nuclear factor-κB (NF-κB) is involved in the promotion of cell survival in a variety of cell types. The present study focused on the role of NF-κB in TNFα-induced apoptosis in an ameloblastoma. Immunohistochemical staining revealed p65 NF-κB protein to be expressed in ameloblastoma tissues. Furthermore, immunoblotting and immunocytochemistry analyses showed that the stimulation of TNFα in an ameloblastoma cell line (AM-1) induced p65 NF-κB translocation from the cytoplasm to the nucleus, indicating NF-κB activation. These findings were confirmed by an NF-κB luciferase reporter assay, which detected enhanced NF-κB transcription activity of AM-1 cells by TNFα stimulation. Moreover, pretreatment with SN50, a nuclear translocation inhibitor, prior to TNFα stimulation, effectively inhibited TNFα-induced NF-κB activation in AM-1 cells. In order to reveal the role of NF-κB activation during TNFα-induced apoptosis in AM-1 cells, an apoptosis assay was performed, and showed that the potential of TNFα in inducing apoptosis in AM-1 cells was significantly elevated by inhibiting the NF-κB activation. These results suggest that NF-κB plays an anti-apoptotic role in TNFα-induced apoptosis in AM-1 cells.

Key words: ameloblastoma, AM-1, TNFα, NF-κB, apoptosis

Introduction

NF-κB, one of the ubiquitously expressed transcription factors, is an activator of various inducible target genes that maintain the balance between pro- and anti-apoptotic cellular pathways, and also regulates other vital functions[1–3]. Furthermore, the activation of classical NF-κB, a heterodimer containing p50 and p65 subunits, provides a survival signal in the majority of cell types. NF-κB exists predominantly in cytoplasm as an inactive form by binding to the inhibitor of κBα (IκBα). However, upon activation, p50-p65 heterodimer translocates into the nucleus through

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phosphorylation of IκBα by IκB kinase (IKK). In the nucleus, p50-p65 heterodimer binds to specific DNA and transactivates transcription of specific genes[4,5]. Recent studies have demonstrated that the inappropriate regulation of NF-κB is closely associated with several diseases, such as autoimmune diseases and cancers[6].

Ameloblastoma, a neoplasm derived from the odontogenic epithelium, is usually considered to be benign and it also grows slowly. However, this tumor has the potential for local invasion with destructive behavior[7,8]. Very little is known about the molecular mechanisms that regulate its growth, cell differentiation, and invasion into bone. To elucidate this mechanism, it would be useful to establish an in vitro model. We previously reported that an immortalized ameloblastoma cell line, AM-1, was successfully established by transformation using HPV-16 DNA[9]. Recent studies have shown that TNFα induced survival activity in AM-1 cells through the serine-threonine kinase Akt and mitogen-activated protein kinase (MAPK) pathways, although prolonged treatment with TNFα effectively induced apoptosis of AM-1 cells. Furthermore, the potential of TNFα -induced apoptosis in AM-1 cells was elevated by inhibiting the Akt and MAPK survival pathways[10,11].

In various types of cells, NF-κB activation blocks TNFα-induced apoptosis[12,13]. However, the role of NF-κB activation during TNFα-induced apoptosis is still unknown in ameloblastoma. The present study shows some evidence that NF-κB plays an anti-apoptotic role in TNFα-induced apoptosis in ameloblastoma.

Materials and Methods

1. Tissue sample selection and cell culture

Specimens were surgically obtained from 24 patients with ameloblastoma at the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital. These tissues were fixed for 24–48 h in 4% formaldehyde (PFA) freshly prepared from paraformaldehyde in phosphate buffered saline (PBS) at 4℃. The tissue blocks were sliced and stained with hematoxylin and eosin for histological diagnosis according to the World Health Organization International histological typing of odontogenic tumors[14]. Ameloblastoma tissue sections were divided into 5 follicular types, 13 plexiform types, 3 basal cell types, and 3 desmoplastic ameloblastomas.

AM-1 cells were cultured in keratinocyte-SFM (GIBCO, Grand Island, New York, USA), penicillin-streptomycin (GIBCO), fungizone amphotericin B (GIBCO) and supplemented with bovine pituitary extract (Kyokuto, Tokyo, Japan). All cells were maintained in a humidified atmosphere of 5% CO_2 at 37℃. The medium was changed on alternate days.

2. Immunohistochemical staining

Ameloblastoma tissues specimens were sliced into 4 μm sections and then were mounted on 3-amino-propyltriethoxy-silane coated glass slides. The tissue sections were de-paraffinized and treated with 0.4% pepsin in 0.01 N HCl for 60 min at 37℃ for antigen retrieval. After rinsing in PBS, the sections were incubated with 3% hydrogen peroxide for 30 min to block endogenous activity. Next, 10% normal goat serum was applied for 1 h to reduce non-specific antibody binding. As a primary antibody, 1:50 diluted mouse monoclonal anti-p65 NF-κB (BD Pharmingen, San Diego, USA) antibody was applied overnight in a moist chamber at 4℃. After incubation with primary antibody, tissue sections were washed in PBS and treated with the Universal Immuno-enzyme Polymer (UIP) method using N-Histofine® Simple Stain MAX PO (M) (Nichirei Bioscience, Tokyo, Japan). The peroxidase activity was visualized by immersing the tissue sections in diaminobenzidine (Nichirei), resulting in a brown reaction product. Finally, the tissue sections were counterstained with methyl green and mounted. For a negative control, PBS was applied to substitute for the primary antibody. Samples were rinsed in PBS between each step.

3. Immunoblotting

To examine the effect of TNFα alone on AM-1 cells, serum starvation was performed for 4 h prior to stimulation of TNFα (Calbiochem, La

Jolla, CA, USA) and AM-1 cells were treated with/without SN50 (P-600, Biomol, USA). Next, the cells were lysed and homogenized using a buffer containing 50 mM Hepes/NaOH (pH 7.2), 0.5 M NaCl, 5 mM EDTA, 20 mM β-mercaptoethanol, 1% Triton X-100, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM p-amidinophenyl methane-sulfonyl fluoride hydrochloride, and protease inhibitor cocktails (SIGMA-Aldrich, San Louis, Missouri, USA).

The protein samples (15 μg) were separated using 10% or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, USA). After blocking in 5% dried milk solution, the sheets were incubated in 1:1000 mouse monoclonal anti-p65 NF-κB (BD Pharmingen), 1:1000 diluted rabbit polyclonal anti-IκB-α (sc-371, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or 1:1000 diluted rabbit anti-phospho-IκB-α (sc-8404, Santa Cruz) antibody. The secondary antibody was 1:2000 diluted horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham, Buckinghamshire, UK) or 1:1000 diluted horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham). The bound antibodies were visualized using the enhanced chemiluminescent (ECL) system (Amersham) according to the manufacturer's instructions.

4. Nuclear extract

Nuclear extracts were prepared according to the nuclear extraction kit protocol (Chemicon International, California, USA) as previously described[15]. Briefly, the cell pellet of 1×10^7 AM-1 cells was combined with ice-cold cytoplasmic lysis buffer containing 0.5 mM dithiothreitol (DTT) and 1:1000 dilution of protease inhibitor cocktail. Using a syringe with a 27-gauge needle, the cell suspension was disrupted, followed by centrifugation at 7000 rpm at 4℃ for 20 min. The supernatant was collected as the cytosol fraction. The remaining pellet was resuspended in ice-cold nuclear extraction buffer containing 0.5 mM DTT and 1:1000 dilution of protease inhibitor cocktail, disrupted with a syringe, and agitated in a low speed rotator at 4℃ for 60 min. After a final centrifugation of the cell suspension for 5 min at 10000 rpm, the supernatant was used as the nuclear extract.

5. Immunocytochemical staining

AM-1 cells were seeded onto chamber slides, stimulated, and fixed with 4% PFA for 15 min. After being rinsed in PBS, the fixed AM-1 cells were treated with 0.1% Triton X-100 for 10 min. The samples were incubated in PBS containing 0.1% bovine serum albumin (BSA) and 0.1% Tween-20 for 30 min to block nonspecific binding. Diluted anti-p65 NF-κB (1:100; BD Pharmingen) was applied as the first antibody and 1:100 diluted TRITC-Goat Anti-Mouse Ig-G (H + L) conjugate (Zymed Laboratories, San Francisco, CA, USA) was applied as the secondary antibody. The slides were then covered with cover slips and observed under a fluorescence microscope. After each step, the samples were rinsed in PBS.

6. NF-κB-luciferase-reporter assay

AM-1 cells cultured in a 6-well-dish were transiently co-transfected with 0.5 μg NF-κB luciferase reporter vector or empty control vector (LR0051, Panomics, Freemont, CA, USA) using FuGene 6 (Roche, Indianapolis, USA), and with 0.2 μg β-galactosidase control plasmid as an internal transfection efficiency standard and incubated overnight. A luciferase assay was performed using the luciferase assay system (Promega, Madison, WI, USA). Briefly, after washing with PBS, 40 μl of cell culture lysis buffer was added to the cells. The culture dish was rocked several times, scraped, and then the contents were transferred to the micro-centrifuge tube. The tube was vortexed for 15 seconds and centrifuged at 14000 rpm for 10 min. The resulting supernatant was collected and added to a luminometer tube containing luciferase assay reagent. The tube was then read for 10 s to measure luciferase activity using a Lumat LB5907 Luminometer (EG & Berthold Technology, Wildbad, Germany). The luciferase activity was normalized with the β-galactosidase activity.

7. Apoptosis assay

Apoptosis assay was carried out using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (APO-AF, Sigma). Annexin V can identify the externalization of phosphatidylserine during the early stage of apoptosis, whereas propidium iodide (PI) stains DNA of cells during the late stage of apoptosis[16]. Briefly, AM-1 cells were washed twice with ice-cold PBS and then resuspended in 250 μl of binding buffer (100 mM HEPES/NaOH, 1.4 M NaCl and 25 M $CaCl_2$, pH 7.5) containing Annexin V-FITC conjugate and PI solution. Cell suspensions were placed at room temperature for 10 min before flow cytometric analysis. The fluorescence of individual nuclei was measured using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ). Fifteen thousand events were acquired for each data point.

Results

1. Expression of p65 NF-κB proteins was detected in ameloblastoma tissues

Twenty of 24 ameloblastoma tissues (83.3%) were stained by anti-p65 NF-κB antibody with various immunoreactivities. Positive staining was observed in the cytoplasm and nuclei of tumor cells (Fig. 1). In the follicular and basal cell types, the expression of p65 NF-κB was stronger in the outer layer of the epithelial component than that in the inner stellate reticulum-like cells.

2. TNFα induced the nuclear translocation of p65 NF-κB in AM-1 cells

Treatment with 100 ng/ml TNFα for 10 min in AM-1 cells markedly increased the amount of p65 NF-κB proteins in the nucleus, then slightly decreased until 60 min (Fig. 2A). Conversely, in the cytoplasmic extracts, the amount of p65 NF-κB proteins decreased from 10 min and then slightly increased up to 60 min after TNFα stimulation (Fig. 2B). These results strongly suggest that TNFα stimulation induced the translocation of p65 NF-κB from the cytoplasm to the nucleus in AM-1 cells.

To confirm these results, immunocytochemical analysis was performed to examine the localization of p65 NF-κB in AM-1 cells. As shown in Figure 2C, the expression of p65 NF-κB was detected intensely in cytoplasm of untreated AM-1 cells, but very weakly in the nuclei. However, p65 NF-κB proteins were strongly expressed in the nuclei of AM-1 cells with TNFα stimulation.

The cell-permeable SN50 peptide carrying a functional domain, nuclear localization sequence (NLS), can inhibit nuclear translocation of NF-κB/Rel complexes in the intact cells[17]. In the present study, we used 100 μg/ml SN50 that has no effect on the apoptosis of AM-1 cells. The immunoblotting and immunocytochemical analyses showed that the pretreatment of AM-1 cells with SN50 prior to TNFα stimulation effectively inhibited TNFα-induced translocation of p65 NF-κB (Fig. 2).

3. TNFα induced the phosphorylation of IκB-α in AM-1 cells

An immunoblotting analysis was performed to determine whether IκBα is phosphorylated by TNFα stimulation during the process of p65 NF-κB translocation. Phosphorylated-IκBα (p-IκBα) was increased by 100 ng/ml TNFα stimulation in 10 min, and then gradually decreased until 60 min. Meanwhile, the amount of IκBα was decreased after 10 min and gradually recovered up to 60 min (Fig. 3).

4. NF-κB transcriptional activity was enhanced by TNFα stimulation in AM-1 cells

An NF-κB-luciferase-reporter assay was performed in AM-1 cells in order to measure the NF-κB transcriptional activity by TNFα stimulation. The stimulation by TNFα significantly enhanced the transcriptional activity of NF-κB in AM-1 cells ($p < 0.01$). Meanwhile, the pretreatment of SN50 prior to the TNFα stimulation extensively inhibited TNFα-induced transcriptional activity of NF-κB in AM-1 cells ($p < 0.01$; Fig. 4).

5. SN50 elevated the potential of TNF in inducing apoptosis in AM-1 cells

To examine whether apoptotic cells were affected by blocking the NF-κB signaling path-

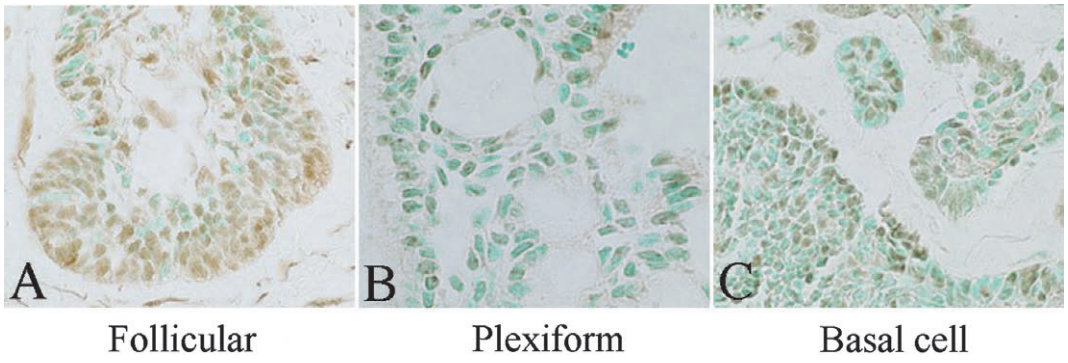

Fig. 1 The expression of p65 NF-κB in ameloblastoma tissues. Ameloblastoma tissues were immunohistochemically stained with anti-p65 NFκB antibody (A-C). (A) Follicular ameloblastoma, (B) plexiform ameloblastoma, and (C) basal cell ameloblastoma.

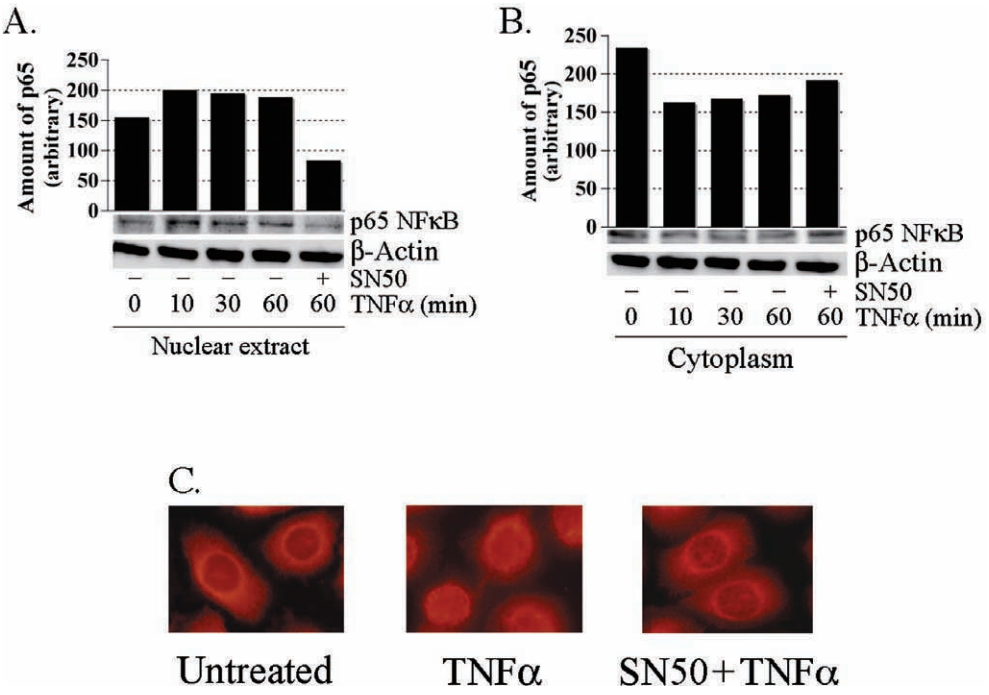

Fig. 2 TNFα induced p65 NF-κB translocation in AM-1 cells. AM-1 cells were starved for 12 h, treated with/without 100 μg/ml SN50 for 1 h and stimulated with 100 ng/ml TNFα for 10, 30, and 60 min. The nucleus and the cytoplasm of the cell extracts were separated and analyzed using an immunoblotting assay. (A) Nuclear extracts and (B) cytoplasm of TNFα-induced p65 NF-κB translocation in AM-1 cells. (C) Immunocytochemical analysis of TNFα-induced p65 NF-κB translocation in AM-1 cells. AM-1 cells were starved for 12 h, treated with/without 50 μg/ml SN50 for 1 h and stimulated with 100 ng/ml TNFα for 10 min. Next, the cells were stained with anti-p65 NF-κB antibody.

way, an apoptosis assay was performed in AM-1 cells by using FACScalibur[17]. As shown in Figure 5, treatment with TNFα significantly increased the number of apoptotic cells with Annexin V and PI positive. Furthermore, by applying SN50 prior to the TNFα stimulation, the number of apoptotic cells was extensively increased, with 19.3% additional cells in comparison to the treatment with TNFα alone.

Discussion

NF-κB exerts its anti-apoptotic effects in a wide variety of cells to protect them from various apoptotic agents. This transcription factor promotes

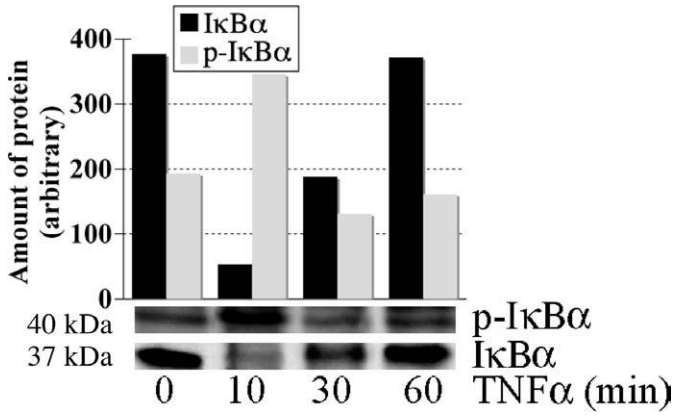

Fig. 3 TNFα induced the phosphorylation of IκB in AM-1 cells. AM-1 cells were starved for 12 h and stimulated with 100 ng/ml TNFα for 10, 30, and 60 min. Cell extracts were analyzed by immunoblotting assay using anti-IκB or anti-phospho-IκB antibody.

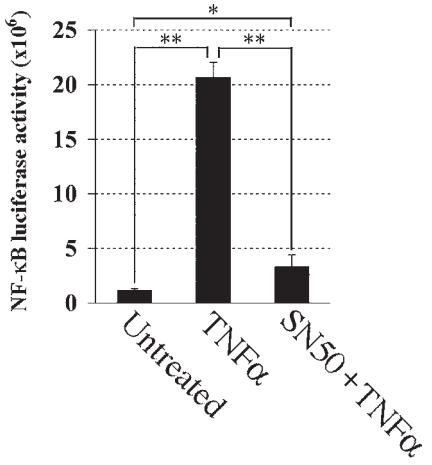

Fig. 4 TNFα elevated NF-κB transcriptional activity in AM-1 cells. AM-1 cells were transiently transfected as described in Materials and Methods, treated with/without 100 μg/ml SN50 for 3 h, and stimulated with 100 ng/ml TNFα for 6 h. Values are the mean of three different experiments ($^{*}p < 0.05$, $^{**}p < 0.01$).

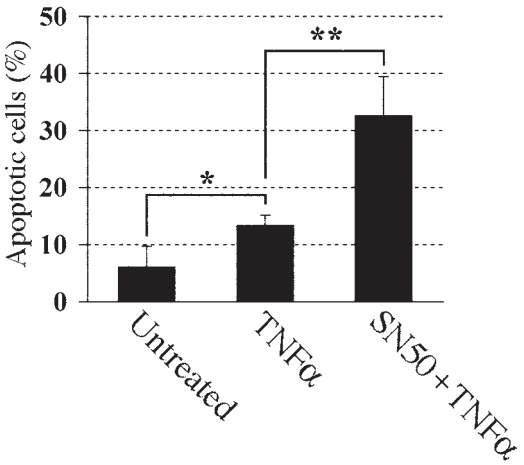

Fig. 5 SN50 significantly elevated the potential of TNFα in inducing apoptosis in AM-1 cells. AM-1 cells were treated with/without 100 μg/ml SN50 for 3 h before stimulation with 100 ng/ml TNFα for 24 h. The cells were then incubated with FITC-conjugated Annexin V and PI solution and analyzed by flow cytometry as described in Materials and Methods. Values are the mean of three different experiments ($^{*}p < 0.05$, $^{**}p < 0.01$).

cell survival through the expression of genes encoding anti-apoptotic proteins which directly block caspase activation, such as survivin and X chromosome-linked inhibitor of apoptosis protein (XIAP), members of IAP family proteins, or some acting at the mitochondrial level, like bcl-2 family proteins[18]. Furthermore, NF-κB can directly activate pro-oncogenes such as cyclin D1 and c-Myc, and also has been implicated in the development or progression of human tumors[19,20]. A previous study showed that two members of bcl-2 family proteins, bcl-2 and bcl-X, were observed more predominantly in the outer layer cells than in inner layer cells of an ameloblastoma[21,22]. Similar patterns were also reported for the expression of survivin in ameloblastoma[23]. Consistent with these studies, p65 NF-κB reactivity was much higher in the outer layer cells than in inner layer cells of ameloblastoma specimens in the current study. These results suggest that the outer layer cells of

ameloblastoma have higher anti-apoptotic activities in comparison to the inner layer cells. Moreover, these members of the bcl-2 and IAP families might be involved in mediating the anti-apoptotic effects of NF-κB in ameloblastoma.

Many types of tumors show the constitutive activation of NF-κB to enable tumor cells to escape apoptosis and to acquire drug resistance[24–26]. This constitutive activation of NF-κB can be caused by the following mechanisms: genetic alterations affecting the genes encoding NF-κB and IκB, constitutive activation of IKK that leads to acceleration of IκB phosphorylation, or mutations inactivating IκBs[24]. However, in the present study, p65 NF-κB proteins were detected in the nuclear extract and p-IκBα were detected in the cytoplasmic extract of untreated AM-1 cells (Fig. 2-A, B). Furthermore, the expression of p65 NF-κB was not only observed in the cytoplasm, but also slightly in the nucleus of untreated AM-1 cells by immunocytochemical analysis (Fig. 2-C). These findings suggest that the constitutive activation of NF-κB might occur in AM-1 cells. Further study is needed to reveal this mechanism.

Treatment with TNFα induced the phosphorylation of Akt and MAPK in AM-1 cells, which might be involved in survival and/or proliferation of ameloblastoma[10]. However, when the treatment with TNFα was prolonged until 24 h, apoptosis was induced in AM-1 cells[11]. The present study showed that treatment with TNFα induced NF-κB activation in AM-1 cells. Furthermore, SN50 inhibited TNFα-induced NF-κB activation. To understand the role of NF-κB in TNFα-induced apoptosis in AM-1 cells, an apoptosis assay was performed and revealed that the potential of TNFα in inducing apoptosis in AM-1 cells was significantly elevated by inhibiting the NF-κB activation. These results are direct evidence that NF-κB plays an anti-apoptotic role in TNFα-induced apoptosis in AM-1 cells. Therefore, given the established anti-apoptotic role of NF-κB in TNF-induced apoptosis in AM-1 cells, the inhibition of NF-κB activation might be a promising strategy to enhance the apoptotic effect of TNFα in ameloblastoma.

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